MECHANISMS OF ACTION OF AND CELLULAR RESISTANCE TO CHEMOTHERAPEUTIC AGENTS

MECHANISMS OF ACTION OF AND CELLULAR RESISTANCE TO CHEMOTHERAPEUTIC AGENTS IN HUMAN CELLS: POSSIBLE APPLICATIONS TO QUANTITATIVE MUTAGENESIS

By

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ABSTRACT

The aim of this study was to investigate the mechanisms of action of and development of cellular resistance to various anticancer agents in human (HeLa) cells using a combined genetic and biochemical approach. The agents employed for this purpose included: the purine nucleoside analogues, toyocamycin, tubercidin, and 6-methylmercaptopurine riboside (6-MeMPR); the protein synthesis inhibitor, puromycin; and the microtubule stabilizer, taxol.

To investigate the mechanisms of action and cellular resistance to the purine nucleoside analogues, stable first-step toyocamycin, tubercidin and 6-MeMPR resistant HeLa mutants were isolated. These mutants exhibited high degrees of resistance and cross-resistance to various adenosine kinase-activated nucleoside analogues, possessed <2% of the adenosine kinase activity of parental HeLa cell extracts and exhibited severely reduced cellular uptake and macromolecular incorporation of adenosine *in vivo*. These results indicate that in human cells the cytotoxic effects of toyocamycin, tubercidin and 6-MeMPR are dependent upon adenosine kinase-catalyzed phosphorylation of these drugs to their respective monophosphates and that resistance to these agents results from a deficiency in adenosine kinase activity *in vivo*.

Further insight into the nature of the genetic and biochemical alteration(s) affecting adenosine kinase in these mutants was achieved using SDS-polyacryamide gel electrophoretic and immunoblot analysis. Immunoblots revealed that each toyocamycin, tubercidin and 6-MeMPR resistant mutant contained similar amounts of cross-reacting material that had the same electrophoretic mobility as adenosine kinase in parental HeLa cells. Therefore, the lesion in these mutants must be a missense type of alteration in the structural gene for adenosine kinase.

The utility of the 6-MeMPR resistant mutant selection system for quantitative mutagenesis studies was also investigated. Numerous favourable attributes appropriate to mutagenesis studies were found using this selection system. These included: the obtainment of highly resistant mutants which were stable in the absence of drug, the absence of cell density or cross-feeding effects in the selection system, maximum phenotypic expression required a relatively short time period and mutagen treatment increased the mutant frequency in a linear dose-dependent manner. Thus, selection for genetic alterations at the adenosine kinase locus appears to provide a valuable system for quantitative mutagenesis studies in human cells.

The combined genetic and biochemical approach was also used to investigate the development of resistance to

puromycin and taxol. Therefore, first- and second-step mutants resistant to each of these drugs were selected and characterized. Cross-resistance and uptake studies with the puromycin resistant mutants suggest that the most common mechanism for the development of cellular resistance to puromycin in human (HeLa) cells involves an alteration in membrane permeability that reduces drug uptake/transport. Similar studies with the taxol resistant mutants suggest the existence of two possible mechanisms for the development of resistant to this agent in human (HeLa) cells. One mechanism involves a biochemical lesion that specifically affects a microtubule-related cellular component. The second mechanism, however, nonspecifically affects cellular membrane permeability and results in reduced drug uptake/transport.

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LIST OF ABBREVIATIONS

AA^r	8-azaadenine resistant genetic marker
ACS	aqueous counting scintillant
ADP	adenosine-5'-diphosphate
AK	adenosine kinase
AK^+	adenosine kinase activity present
AK^-	adenosine kinase activity absent
Ama^r	α-amanatin resistant genetic marker
AMP	adenosine-5'-monophosphate
APRT	adenine phosphoribosyltransferase
ara-c	1-β-D-arabinofuranosylcytosine
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
$BUdR^r$	5-bromodeoxyuridine resistant genetic marker
CHO/WT	Chinese hamster ovary/wild type
cis-Platin	cis-diamminedichloroplatinum (II)
CRM	cross-reacting material
CTP	cytosine-5'-triphosphate
D_{10}	dose of a compound that reduces the plating efficiency of a cell line to 10% of that observed in the absence of the drug
dCTP	deoxycytosine-5'-triphosphate
DFCS	dialyzed fetal calf serum
2-D gels	two-dimensional gel electrophoresis
Dip^R	diphtheria toxin resistant genetic marker

DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRB	5,6-dichloro-1-β-D-ribofuranosyl benzimidazole
Drb^R	5,6-dichloro-1-β-D-ribofuranosyl benzimidazole resistant genetic marker
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
EMS	ethylmethanesulfonate
FCS	fetal calf serum
HGPRT	hypoxanthine-guanine phosphoribosyl-transferase
IEF	isoelectric focusing
$LaCl_3$	lanthanum chloride
mA	milliamperes
α-MEM	alpha-modified minimal essential medium
6-MeMPR	6-methylmercaptopurine riboside
6-MeMPR^r	6-methylmercaptopurine riboside resistant genetic marker
MGBG	methylglyoxal(bis)guanylhydrazone
$MgCl_2$	magnesium chloride
mmf proteins	modulators of membrane fluidity proteins
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
6-MPR	6-mercaptopurine riboside
M_r	relative molecular mass
mRNA	messenger ribonucleic acid

2-MSH	2-mercaptoethanol
NaOH	sodium hydroxide
Oua^R	ouabain resistant genetic marker
PBS	phosphate buffered saline
P-100 dish	100-mm-diameter dish
POPOP	1,4-bis-2-(5-phenyloxazolyl)benzene
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Thg^r	6-thioguanine resistant genetic marker
TK	thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
Tub^r	tubercidin resistant genetic marker
UMP	uridine-5'-monophosphate
UV	ultraviolet
VM26	teniposide (4'-demethylepipodophyllotoxin thenylidene-β-D-glucoside)
VP16-213	etoposide (4'-demethylepipodophyllotoxin ethylidine-β-D-glucoside)

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1. INTRODUCTION

In 1953, with the discovery of the three-dimensional structure of DNA, the era of molecular biology was born (Watson and Crick, 1953). This development represented the fusion of the two formerly independent fields of genetics and biochemistry and was first used to study the simplest living cells. Further attempts to investigate the "molecular biology" of man, however, were met with overwhelming problems. Although the study of human biochemistry could parallel biochemical studies in other organisms, possible parallels between human genetics and genetic studies in lower organisms were greatly hindered for several reasons. First, in contrast to the generation time of 20 minutes for typical prokaryotes (eg. Escherichia coli), that of man approximates 25 years. In addition, specific matings that would provide the answers to particular genetic questions were not possible in man. Lastly, both the human genome and the regulation of gene expression and differentiation in higher eukaryotes appeared to be far more complex than in prokaryotic organisms.

In 1955, a solution that in part solved these problems was offered by Puck and Marcus (1955, 1956). They postulated that, even though the human body contains approximately 10^{13} nucleated somatic cells, it should be

possible to treat these as though they were equivalent to Escherichia coli cells and consequently apply the methods of microbial genetics to such cell populations. In this way, the study of the genetics of somatic cells could substitute for that of the germ cells utilized in classical eukaryotic genetics.

With this, came the realization that certain techniques were required to achieve the genetic analysis of somatic cells. These included: (a) reliable methods for sampling somatic cells from experimental animals or humans and for their establishment in long term cultures, (b) methods for propagating these cultures into large and reasonably stable populations and (c) simple, rapid and reliable means for growing single cells into clonal populations thereby allowing the selection, recognition and isolation of mutant colonies. Furthermore, during the development of these techniques, it was necessary to: (a) design incubators that precisely controlled the temperature, relative humidity and CO_2 concentration in order to simulate in vivo growth conditions, (b) formulate media and media supplements conducive to highly efficient growth of single cells into colonies and (c) design methods for isolating colonies, growing stocks and ensuring their clonal nature.

By 1958, most of these requirements had been met (Puck and Marcus, 1955; Puck et al., 1956, 1958) and it was

possible to culture mammalian cells for extended periods of time outside the body. As a result, somatic cell genetics was established as a new approach to studying the genetics of man and other higher eukaryotes. Since this time, the field of somatic cell genetics has grown enormously. It has provided a means to understand not only gene and chromosome structure, but the complex genetic organization and regulation of multicellular eukaryotic organisms in general and man in particular (Wright et al., 1980; Ruddle, 1981; Puck and Kao, 1982).

Somatic cell genetics has contributed greatly to our knowledge and understanding of the mechanisms of drug action (Thompson and Baker, 1973; Wright et al., 1980; Puck and Kao, 1982), as well as the processes of mutagenesis (Siminovitch, 1976), genetic complementation (Kao et al., 1969; Puck and Kao, 1982) and gene regulation (Davidson, 1974), and to the development of the methodologies required for gene transfer and gene mapping (Shows and Sakaguchi, 1980; Ruddle, 1981; Puck and Kao, 1982). Since the scope of this thesis deals with the mechanisms of action of and development of resistance toward chemotherapeutic agents, as well as possible applications to quantitative mutagenesis studies, only these areas of somatic cell genetics will be discussed in greater detail.

Somatic cell genetics has contributed to understanding the mechanisms of action of many different drugs affecting cell function (Lewin, 1980; Wright et al., 1980). The isolation, from established cell lines, of mutants resistant to these drugs, combined with genetic and biochemical characterization of these mutants, has made this understanding possible. Drug resistant mutants have provided a powerful approach to understanding drug action because they allow comparison of an abnormal (resistant) state with a normal state within an isogenic background (Thompson and Baker, 1973). By allowing the examination of the metabolic consequences of altering one particular component of a cell, a drug resistant mutant serves as a tool for probing functional relationships between cellular components. In this way, somatic cell mutants have:

(a) provided insight into the mode of action of numerous drugs, many of which are clinically important in chemotherapy or antibiotic therapy (Thompson and Baker, 1973; Gupta, 1983a,b,c,d, 1985; Lewin, 1980; Wright et al., 1980),

(b) provided insight into the genetic origin of resistance to chemotherapeutic agents and thereby allowed development of more rational and effective chemotherapeutic drug combinations (Curt et al., 1984; Schimke, 1984; Goldie and Coldman, 1984),

(c) provided in vitro models for the study of human genetic diseases (Martin and Gelfand, 1981; Osborne, 1981),

(d) created an opportunity to study the structure, role and regulation of the affected function in the drug resistant mutants and thereby helped understand the functioning and physiology of a normal cell (Thompson and Baker, 1973; Lewin, 1980; Curt et al., 1984),

(e) provided well characterized genetic markers which serve as reliable and accurate end points for quantitative mutagenesis studies which assess human risk due to mutagenic and/or carcinogenic agents (Hollstein et al., 1979; Kilbey et al., 1984).

1.1 Somatic Cell Lines

Somatic cell genetics relies heavily on established somatic cell lines in culture. Since the first demonstration that single cells of an animal could grow outside the body (Harrison, 1907), interest in using cell culture techniques to study the various aspects of mammalian genetics has steadily increased. Analysis of established somatic cells in vitro offers numerous advantages:

(a) Compared to the whole organism (ie. man), the generation time of a single cell is approximately 10^4-fold shorter. Thus, the possible rate of experimentation is greatly accelerated (Puck and Kao, 1982; Wright et al., 1980).

(b) Individual cells undergo mitotic division and form discrete colonies. This allows one to obtain genetically uniform cell populations (Wright et al., 1980).

(c) The composition of the culture medium in which cells are maintained can be readily manipulated. Thus, a particular cellular phenotype can be observed under numerous conditions (Thompson, 1979).

(d) The genetic properties of a cell line and the response of that same cell line to environmental influences are not masked by the presence of different types of somatic cells as is the situation with a whole organism (Wright et al., 1980).

(e) The use of somatic cells in culture readily lends itself to the experimental approaches and techniques of molecular biology (Puck and Kao, 1982).

A major restriction imposed on the use of somatic cells in culture has been the limited number of cell lines that have been established (Lewin, 1980). Primary cultures of fibroblasts (derived from man and other animals) which show uniform diploid karyotypes have been readily obtained. However, the utility of these cell strains for the purposes of somatic cell genetics has been somewhat restricted due to: (a) the fact that these cells have a finite life span in culture (Hayflick, 1965) and (b) they generally cannot be handled with the ease of "permanent" cell lines (ie. they

exhibit low plating efficiencies and cannot be grown in suspension culture, Thompson and Baker, 1973). To circumvent these problems, investigators have turned to the use of "permanent" (established) cell lines; cultures which appear to have acquired immortality, generally at the expense of changes in karyotype. A partial list of some established cell lines and their characteristics is presented in Table 1.

The first established human cell line, HeLa, originated from the biopsy of a cervical carcinoma of a black patient, Henrietta Lacks. Since their establishment by Gey et al. (1952), HeLa cells have been the focus of numerous research interests, including those of the geneticist (Nelson-Rees et al., 1974; Bengtsson et al., 1975; Hsu et al., 1976). The continued use of HeLa cells in vitro stems from a number of favourable characteristics that they offer in culture. These include: (a) their ease of growth (minimal growth requirements) in suspension culture or on solid surfaces, (b) their high plating efficiency (80 to 100%), (c) their relatively rapid growth rate (doubling time = 18 to 20 hours), (d) their transformed phenotype ("infinite" life span in culture) and (e) their human origin (Lewin, 1980).

Table 1. Established Somatic Cell Lines

Name	Organism and tissue of origin	Chromosome complement of species (2n)	Current mode and range of chromosome number in cell line
HeLa	human cervical carcinoma (fibroblast)	46	heteroploid 71 (38-106)
L	mouse connective tissue (fibroblast)	40	heteroploid 70 (40-115)
3T3	mouse embryo (fibroblast)	40	heteroploid 76 (70-80)
S49	mouse lymphoma	40	pseudo diploid (39-41)
Friend	mouse erythroleukemia	40	pseudo diploid 39 (35-43)
CHO	Chinese hamster ovary (fibroblast)	22	pseudo diploid 21 (20-22)
V79	Chinese hamster lung (fibroblast)	22	pseudo tetraploid
Don DonC	Chinese hamster lung (fibroblast)	22	pseudo diploid 23
BHK 21	Syrian hamster kidney (fibroblast)	44	pseudo diploid 44 (42-45)
BSC 1	African green monkey kidney (epithelioid; SV40 infected)	60	pseudo tetraploid 115
RAG	Mouse renal adenocarcinoma (epithelioid)	40	heteroploid 74
-	Rana pipiens embryo	26	haploid
S2	D. melanogaster embryo	4	diploid

Table 1 was taken from Lewin (1980).

1.2 Somatic Cell Mutants

Since the first isolation of somatic cell mutants (Puck and Fisher, 1956), numerous efforts have been made toward elucidating the genetic mechanisms that allow altered phenotypes to be selected in culture (Thompson and Baker, 1973; DeMars, 1974; Siminovitch, 1976; Caskey and Kruh, 1979). Understanding the mechanisms of origin of altered phenotypes is necessary if variant cells isolated in culture are to be useful models for *in vivo* genetics. Thus, numerous attempts have been made to determine whether altered phenotypes are due to changes in DNA (ie. mutations) or the result of stable directed shifts in genetic expression (epigenetic events). Early studies which postulated that epigenetic mechanisms were the basis of most of the selected variant phenotypes (Harris,1967, 1971; Chu and Malling, 1968; Mezger-Freed, 1972) have been met with more convincing documentation (DeMars, 1974; Chasin and Urlaub, 1975; Siminovitch, 1976; Thompson, 1979) which illustrates that changes in DNA are generally responsible for altered phenotypes isolated in culture.

Several criteria have been used to assess the mutational origin of variant phenotypes isolated from cultured somatic cells (Thompson and Baker, 1973; Siminovitch, 1976; Lewin, 1980; Puck and Kao,1982). These are:

(a) the altered phenotype should be stably transmitted through consecutive generations in the absence of selective conditions,

(b) the spontaneous frequency of occurrence and reversion of the altered phenotype should be low,

(c) the frequency of occurrence of the mutation should be enhanced by mutagen treatment,

(d) the altered phenotype should be associated with both an altered gene product (ie. a protein) and an altered gene (observable at the DNA level),

(e) it should be possible to map the mutation to a specific region of the genome (ie. a particular genetic locus).

Presently, it is believed that most procedures select altered cells with genetic properties satisfying most of these criteria (DeMars, 1974; Siminovitch, 1976; Wright et al., 1980). However, it must be realized that non-mutational mechanisms of "gene inactivation or silence" in certain genetic systems may play a role in the isolation of variant phenotypes (Milman et al., 1976; Morrow, 1977; Bradley, 1979; Turker et al., 1984).

1.2.1 Drug Resistant Mutants

The development of selection procedures for isolating a variety of stable phenotypic variants has been extremely rapid in the last two decades. Numerous procedures for selecting temperature sensitive, auxotropic, lectin

resistant and drug resistant mutants exist (Thompson and Baker, 1973; DeMars, 1974; Clements, 1975; Siminovitch, 1976; Caskey and Kruh, 1979; Thompson, 1979; Lewin, 1980; Wright et al., 1980; Puck and Kao, 1982). Of these mutant classes, the intrinsic nature of drug resistant mutants has been most often explored because of the availability of numerous selective agents, the variety of specific mutants that can be isolated and the relative ease with which these mutants can be obtained. Since this thesis deals with the selection and characterization of drug resistant mutants, only this class of mutants will be discussed in greater detail.

Theoretically, any compound that is cytotoxic to cells in culture can be used to select drug resistant mutants. In practice, the selection of these mutants involves exposure of wild-type (sensitive) cells to a cytotoxic agent, followed by the isolation of surviving clones exhibiting the drug resistant phenotype. Appropriately, a drug resistant mutant is defined as any cell line that retains normal plating efficiency, but not necessarily normal growth rate, in the presence of a drug concentration that significantly reduces the plating efficiency of wild-type (sensitive) cells (Thompson and Baker, 1973).

Generally, mutants are obtained by two different procedures: (a) a single-step protocol or (b) a multi-step protocol. In single-step protocols, normally sensitive cells acquire resistance following single exposure to one given concentration of a drug (Thompson and Baker, 1973; Thompson, 1979; Lewin, 1980). Depending upon the nature of the affected function, a number of resistant mutants, each exhibiting different levels of resistance, may result. When different concentrations of a drug are used in separate selections, the resistant mutants may characteristically display ranges of resistance appropriate to only their particular selective concentration. However, mutants "completely" resistant to a drug are usually obtained (if this is at all possible) by using concentrations which exceed those necessary to reach the 10^{-4} survival level of parental cells (ie. plating efficiency = 0.01%).

In order to obtain mutants resistant to high concentrations of a drug, extension of the single-step protocol to the multi-step (or continuous selection) protocol may be required (Thompson and Baker, 1973; Lewin, 1980). Using this protocol, successive increments in resistance may be obtained by submitting the parental cells to consecutive single-step selections with graded increases in drug concentration. Assuming that the basis of resistance in these variants lies in mutation, the necessity of their

selection using the multi-step protocol implies that several mutations are required to achieve high resistance (Lewin, 1980).

This suggests that in a single-step protocol the occurrence of multiple events in a single cell is rare, but by isolating mutant cells after each event in a multi-step protocol, it is possible to obtain cells that have gained all the necessary mutations. It must be realized, however, that variant phenotypes obtained using the multi-step protocol often revert to the parental (sensitive) phenotype in the absence of selective pressure and, due to their phenotypic instability, may not be mutants in the true sense (Schimke, 1982, 1984). During selection, attempts to minimize the number of steps required to achieve a high degree of resistance are made since the occurrence of multiple events may complicate identification of the genetic basis of the alterations (Thompson and Baker, 1973; Lewin, 1980).

1.2.1.1 Application of Selection Procedures for Drug Resistant Mutants to Quantitative Mutagenesis Studies

Humans are exposed to an extremely large number of mutagens and/or carcinogens from a variety of sources: cooking food, natural chemicals in our diet, man-made chemicals, industrial pollutants, etc. In order to assess potential human risk from exposure to these agents, it is

important to screen large numbers of these compounds for their mutagenic and/or carcinogenic activity. However, it is impractical for both technical and monetary reasons to do the bulk of the mutagen/carcinogen screening using experimental animals. Consequently, there is a need to develop reliable, short term in vitro tests that have the ability to detect the mutagenicity and/or carcinogenicity of chemicals or their ability to interact with DNA.

Measurement of the frequency of mutants in cell culture under various conditions is a critical aspect of somatic cell genetics (Thompson and Baker, 1973; Lewin, 1980). Observations concerning the frequency and distribution of spontaneous mutants are necessary to characterize the variability of a cell culture and to substantiate the origin (random vs. adaptive) of any mutants. As well, evidence indicating whether a particular phenotype is increased by mutagen treatment is one criterion used to assess the probability that the variant phenotype results from an alteration in DNA. Lastly, if the frequency of occurrence of a mutant phenotype is a reliable indicator of genetic variation in a cell culture, assay of the mutant phenotype may be used as a test system to evaluate the potential mutagenicity and/or carcinogenicity of various chemicals. The determination of mutant frequencies in cell culture has allowed certain selection procedures for various

drug resistant mutants to be developed into accurate and reliable short term tests necessary for studies in quantitative mutagenesis (Hollstein et al., 1979; Kilbey et al., 1984).

1.3 Mechanisms of Drug Resistance Exhibited by Mammalian Cell Lines

Study of the mechanisms involved in the development of drug resistance by mammalian cells is an area of active research (Hill, 1982; Ling, 1982; Curt et al., 1984; Hutchison, 1984). Practically speaking, this research is of importance to the field of cancer chemotherapy since the appearance of a single drug resistant mutation could eventually lead to treatment failure. Understanding the mechanisms of drug resistance *in vitro* and in animal models has provided information concerning possible resistance mechanisms in man, has suggested modifications in the design of new drugs and dosing schedules and has led to the development of combination chemotherapy as an effective means for cancer treatment (Goldie and Coleman, 1982).

Determination of the mechanisms by which mammalian cells become resistant to different drugs has centered around comparative analysis of the "normal" biochemistry of the parental cells and the "altered" biochemistry of the drug resistant mutants. There are several well-defined examples indicative of the mechanisms of action of specific

drugs and acquired resistance to them (Table 2). In general, drug resistance exhibited by mammalian mutant cell lines is associated with one or more of the following mechanisms (Hill, 1982; Curt et al., 1984):

(a) defective cellular uptake or transport of the drug

(b) altered metabolism of the drug to the active (lethal) species

(c) increased drug metabolism or inactivation

(d) increased levels of the cellular target site (ie. gene amplification)

(e) an altered target site

(f) altered intracellular nucleotide pools

(g) increased DNA repair mechanisms

As can be seen from the partial list of cytotoxic agents presented in Table 2, resistance to a given drug (adriamycin or cytarabine) can occur via a number of independent mechanisms. An equally important "indirect" mechanism for the development of drug resistance to certain agents occurs when resistance to one specific agent simultaneously confers cross-resistance to structurally dissimilar drugs with different mechanisms of action (Curt et al., 1984). The pathway by which cells acquire such pleiotropic drug resistance appears to be at the level of membrane transport of these drugs (Bech-Hanson et al., 1976; Wilkoff and Dulmadge, 1978; Kaye and Bodin, 1980; Curt et al., 1984).

Table 2. Mechanisms of Resistance to Various Cytotoxic Agents

General mechanism	Drug	Specific alteration
Defective uptake/ transport	Adenosine Daunomycin Puromycin Taxol	Altered cell membrane permeability (Inaba and Johnson, 1978; Cohen et al., 1979; Fallon and Stollar, 1982; Gupta, 1983d)
	Cytarabine (Ara-C)	Decreased membrane nucleoside binding sites (Wiley et al., 1982)
	Daunomycin VM26	Increased efflux (Skovsgaard, 1978; Lee and Roberts,1984)
Defective metabolism to the active species	Adenosine	Alteration in adenosine kinase (McBurney and Whitmore, 1974)
	8-Azaadenine	Decreased adenine phosphoribosyl-transferase activity (Jones and Sargent, 1974)
	Cytarabine	Decreased deoxy-cytidine kinase activity (Richardson et al., 1982)
	5-Fluorouracil	Decreased uridine kinase activity (Reyes and Hall, 1969)

General mechanism	Drug	Specific alteration
	6-Mercaptopurine 6-Thioguanine	Decreased hypoxanthine-guanine phosphoribosyltransferase (Brockman 1963; Caskey and Kruh, 1979)
	6-Methylmercaptopurine Riboside Pyrazofurin Toyocamycin Tubercidin	Lack of adenosine kinase activity (Gupta and Siminovitch, 1978b, Dix et al., 1979; Gupta and Singh, 1983)
Increased drug inactivation	Cytarabine	Increased deoxycytidine kinase activity (Stewart and Burke, 1971)
	6-Mercaptopurine 6-Thioguanine	Increased membrane alkaline phosphatase (Scholar and Calabresi, 1979)
Increased cellular target site	Adriamycin Colchicine	Increased membrane glycoproteins (Ling, 1975; Baskin et al., 1981)
	Methotrexate	Increased dihydrofolate reductase gene copy number (Schimke, 1980)
	Pyrazofurin	Increase in UMP synthetase (Suttle, 1983)
Altered target site	Colcemid Taxol Vincristine	Altered tubulin (Cabral et al., 1980, 1981; Keates, 1981)

General mechanism	Drug	Specific alteration
	Emetine	Altered 40S ribosomal subunit (Boersma, 1979; Reichenbacher and Caskey, 1979)
	5-Fluorouracil	Altered thymidylate synthetase (Hall, 1977)
	Methotrexate	Altered dihydrofolate reductase (Flintoff and Essani, 1980)
	Puromycin	Altered protein synthetic apparatus (Fallon and Stollar, 1982)
Altered intracellular nucleotide pools	Cytarabine	Increased intracellular CTP and dCTP pools (DeSaint Vincent and Butten, 1979)
Increased DNA repair	Adriamycin Alkylators cis-Platin	Increased efficiency of excision of damaged bases and/or ligation of excised segment (Erickson et al., 1978; Carr and Fox, 1981; Erickson et al., 1981; Bedford and Fox, 1982)

Table 2 was constructed from Hill (1982) and Curt et al. (1984).

Those mechanisms of acquired resistance that are relevant to the anticancer agents employed in this work will be discussed in greater detail in the following sections of this thesis.

1.4 Cytotoxic Agents Involved in the Present Work

1.4.1 Purine Nucleoside Analogues

Antimetabolites are defined as structural analogues of physiological substances that have the ability to produce a deficiency in the natural metabolites of a biological system (Krakoff, 1984). Although numerous antimetabolites exist, they can generally be divided into two main groups according to their mechanism of action: (a) those antimetabolites which are incorporated into DNA, RNA and/or protein (ie. the "informational" biopolymers) in place of the natural monomers and (b) those antimetabolites which inhibit the formation of essential metabolites (Timmis and Williams, 1967).

Among the various antimetabolites belonging to group (a), the purine nucleoside analogues represent a diverse group of compounds which are structurally related to the normal purine nucleosides. Purine nucleoside analogues can be broadly divided into two groups: (a) N-nucleosides and (b) C-nucleosides (Suhadolnik, 1981). The base of N-nucleosides is joined to the ribose sugar moiety by a nitrogen-carbon bond whereas in C-nucleosides a carbon-

carbon bond joins the base and ribose sugar. Of the purine nucleoside analogues involved in this work, the pyrrolopyrimidine nucleosides toyocamycin and tubercidin and the adenosine analogue 6-methylmercaptopurine riboside (6-MeMPR) represent the group of N-nucleosides.

1.4.1.1 Pyrrolopyrimidine Nucleosides

The pyrrolopyrimidine ribonucleosides, toyocamycin and tubercidin belong to an important class of nucleoside antibiotics which exhibit a wide spectrum of biological activities. In addition to possessing potent antibacterial, antifungal, antiparasitic and antiviral activities (Ritch and Glazer, 1984), these compounds are highly cytotoxic to mammalian cells in culture and have been effective against numerous experimental tumors including Sarcoma 180, Ehrlich ascites tumor, Jensen carcinoma and nodular basal cell carcinomas (Owen and Smith, 1964; Klein et al., 1975; Suhadolnik, 1981).

Toyocamycin was first isolated from Streptomyces toyocaensis by Nishimura et al., (1956). Its chemical structure was later determined by Ohkuma (1961) to be 4-amino-5-cyano-7-β-D-ribofuranosyl-pyrrolo-(2,3-d)pyrimidine (Figure 1). In contrast to adenosine which possesses a nitrogen atom at position 7, toyocamycin possesses a C-CN function at this same position. As a consequence of its close structural relationship to adenosine, toyocamycin is

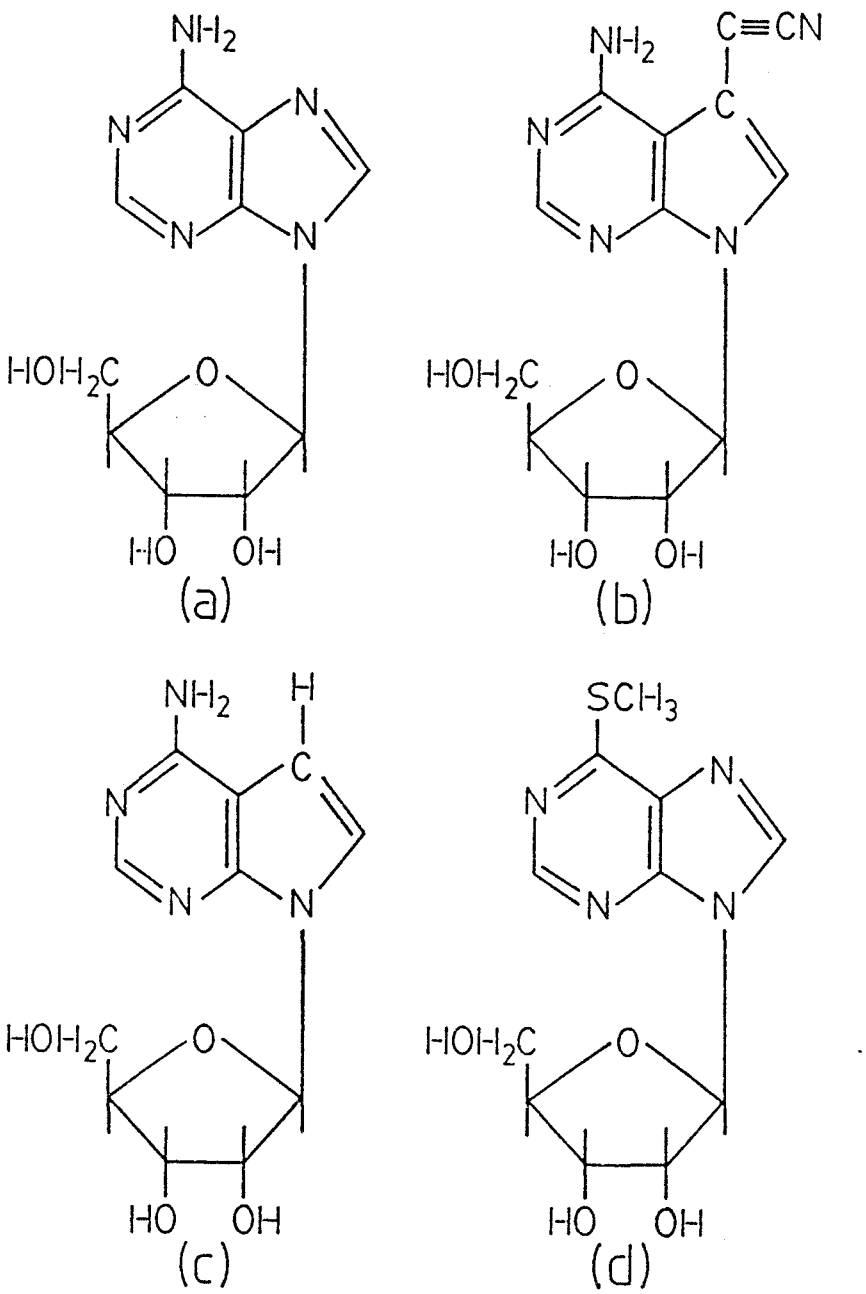


Figure 1. Structural formulae of the purine nucleoside, adenosine (a), the pyrrolopyrimidine nucleosides, toyocamycin (b) and tubercidin (c), and the adenosine analogue, 6-methylmercaptopurine riboside (d).

an excellent substrate for adenosine kinase but is not subject to phosphorolysis or deamination (Lindberg et al., 1967).

Toyocamycin is phosphorylated by Ehrlich ascites tumor cells to the mono-, di- and triphosphate derivatives and is subsequently incorporated into RNA in these cells (Suhadolnik et al., 1967). In mouse L cells, toyocamycin incorporation into RNA selectively inhibits rRNA synthesis (Tavatian et al., 1968, 1969). Studies concerned with RNA maturation in cultured Novikoff hepatoma cells and HeLa cells show that while processing of 45S RNA to 38S RNA continues, formation of mature 28S and 18S RNA is markedly inhibited by toyocamycin (Hamelin et al., 1973; Weiss and Pitot, 1974). Further support of this mechanism of action was obtained in Friend erythroleukemia cells where toyocamycin was shown to allow the processing of 45S pre-rRNA but inhibit the maturation of nucleolar 28S and 18S rRNA and the appearance of new ribosomes in the cytoplasm (Hadjiolova et al., 1981). Theories concerning the mechanism by which toyocamycin inhibits rRNA processing include: (a) an inability of the initial 45S transcript to associate properly with required proteins and (b) changes in rRNA conformation induced by analogue incorporation such that rRNA processing enzymes do not function properly (Tavitian et al., 1968). Toyocamycin has been shown to interfere with

RNA metabolism by preventing polyadenylation and/or methylation of adenosine moieties (Swart and Hodge, 1978).

The antibiotic tubercidin was isolated from culture filtrates of Streptomyces tubercidicus (Anzai et al., 1957). Its chemical structure (Figure 1) has been established (Suzuki and Marumo, 1960) as 7-deazaadenosine or 4-amino-7β-D-ribofuranosyl-pyrrolo-(2,3-d)pyrimidine (simply, tubercidin results from the substitution of the N-7 of adenosine with a methylene group). Tubercidin, like toyocamycin, has been shown to be an excellent substrate for adenosine kinase (Lindberg et al., 1967), undergoes intracellular phosphorylation to the mono-, di- and triphosphate forms and is incorporated into DNA and RNA of bacterial and mammalian cells (Acs et al., 1964; Bloch et al., 1967; Ross and Jaffe, 1972). It has been reported to inhibit rRNA processing (Weiss and Pitot, 1974; Cohen and Glazer, 1984), methylation of nuclear RNA and tRNA (Stern and Glazer, 1980; Chang and Coward, 1975), protein and nucleic acid biosynthesis (Aca et al., 1964; Uretsky et al., 1968) and polyamine biosynthesis (Coward et al., 1977), as well as cause visible nuclear damage (Bassleer et al., 1976). In Ehrlich-Lettre tumor cells, tubercidin has also been shown to inhibit mitochondrial respiration (Miko and Drobnica, 1975). Numerous other effects of tubercidin have been reported in studies where tubercidin is known to

substitute for the adenosine residue of nicotinamide adenine dinucleotide (Bloch et al., 1967), cyclic AMP (Walter, 1976), S-adenosylmethionine (Wainfan and Landsberg, 1973) and S-adenosylhomocysteine (Chang and Coward, 1975). Thus, the general mechanism(s) of action of tubercidin is (are) related to its incorporation into nucleic acids and cofactors, and to its ability to produce rapid and profound inhibition of protein synthesis.

1.4.1.2 6-Methylmercaptopurine Riboside (6-MeMPR)

6-MeMPR (9-β-D-ribofuranosyl-6-methylthiopurine) is an easily obtainable synthetic derivative of 6-mercaptopurine (Timmis and Williams, 1967). In contrast to adenosine, which has an NH_2 group at position 6 of the adenine moiety, 6-MeMPR has a methylthio (SCH_3) function at that same position (Figure 1). Initial interest in the mechanism of action of 6-MeMPR arose because it was extremely cytotoxic toward numerous cell lines in culture including human cancer cells resistant to 6-mercaptopurine and 6-mercaptopurine riboside (Bennett et al., 1965, 1966). 6-MeMPR has also been reported to be an effective inhibitor of the growth of several mouse tumors (Paterson, 1961) and experimental neoplasms (Ehrlich ascites carcinoma) in vivo (Paterson and Wang, 1968).

At present, the mechanism by which 6-MeMPR inhibits growth is not completely understood. It has been clearly

demonstrated that the effects of 6-MeMPR on cell growth require that it be metabolized to the nucleoside monophosphate, a reaction that is catalyzed by adenosine kinase (Bennett et al., 1965, 1966; Schnebli et al., 1966). The nucleoside monophosphate has been shown to inhibit phosphoribosylaminotransferase, thereby blocking de novo purine biosynthesis (Elion, 1967; Hill and Bennett, 1969). This inhibition is reflected by a decrease in ATP and GTP pools observed following 6-MeMPR exposure (Nelson and Parks, 1972). However, the relevance of this decrease in endogenous purine nucleotides to the mechanism of 6-MeMPR cytotoxicity has been questioned (Tidd and Paterson, 1974).

Intracellular 6-MeMPR di- and triphosphates have also been reported but their contribution to 6-MeMPR-mediated cytotoxicity remains to be elucidated (Zimmerman et al., 1974). Hypotheses concerning the possible action(s) of these di- and triphosphates include the inhibition of ribonucleotide reductase and nucleic acid polymerases, as well incorporation into nucleic acid.

6-MeMPR has also been reported to stimulate tyrosine aminotransferase degradation in Reuber H35 hepatoma cells (Koontz and Wicks, 1984). The precise mechanism of this degradation and whether generalized protein degradation results from 6-MeMPR exposure is not known.

1.4.2 The Protein Synthesis Inhibitor, Puromycin

Puromycin (6-dimethylamino-9-[3-deoxy-3-(p-methoxy-L-phenylananylamino)-β-D-ribofuranosyl]-β-purine), an antibiotic produced by the mold Streptomyces alboniger (Perez-Gonzalez et al., 1983), possesses a structure (Figure 2) which resembles the terminal aminoacyl adenosine moiety of tRNA (Fryth et al., 1958). Since its discovery in 1952 (Porter et al., 1952), puromycin has been found to inhibit the growth of a broad spectrum of microorganisms, tissues (Takeda et al., 1960; Gorski et al., 1961; Hulton, 1961; Ferguson, 1962; Nemeth and de la Haba, 1962) and experimental tumors in mice (Troy et al., 1953; Baker et al., 1955a,b). These effects appear to be the result of puromycin-induced inhibition of protein synthesis (Yarmolinsky and de la Haba, 1959; Nathans and Lipmann, 1961).

The structural similarity between puromycin and the terminal aminoacyl adenosine moiety of tRNA, first noted by Yarmolinsky and de la Haba (1959), provided the basis for understanding the mechanism of puromycin action. These investigators hypothesized that the antibiotic inhibits protein synthesis by acting as an analogue of esterified tRNA. Continued efforts revealed that puromycin, like aminoacyl tRNA, can serve as an acceptor of the nascent peptide chain of ribosome-bound peptidyl tRNA (Morris and Schweet, 1961; Rabinovitz and Fisher, 1962). The entire

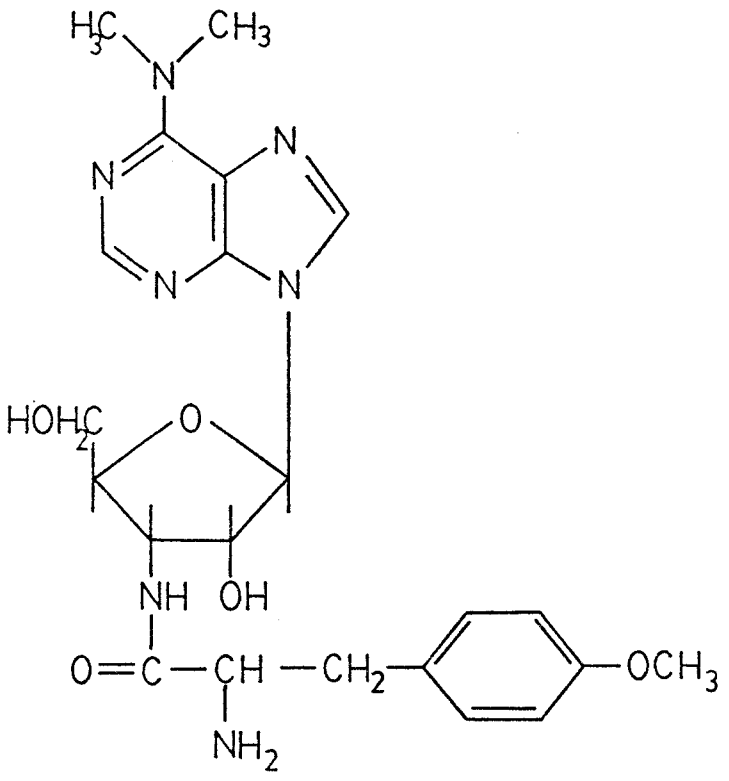


Figure 2. The structural formula of the aminoacyl-tRNA analogue, puromycin.

puromycin molecule becomes linked to the nascent chain by a peptide bond between the amino group of the p-methoxy-phenylalanine moiety and the carboxyl-terminal end of the polypeptide (in a reaction similar to the transfer of peptide from peptidyl-tRNA to the next aminoacyl-tRNA) and the tRNA is released from the ribosome. No further peptide bond formation can occur because of the chemical stability of the C-N bond which links the p-methoxyphenylalanine moiety of puromycin to the nucleoside residue (Franklin and Snow, 1971). Consequently, a prematurely terminated polypeptide chain that has incorporated one molecule of puromycin is released from the ribosome (Allen and Zamecnik, 1962; Nathans, 1964). In short, puromycin inhibits protein synthesis by competing with aminoacyl-tRNA for a site at the peptidyl-transferase centre on the large ribosomal subunit. In place of aminoacyl-tRNA, it accepts nascent peptides, thereby interfering with the elongation cycle of protein synthesis and causing premature release of incomplete polypeptide chains (Pestka, 1971).

1.4.3 The Microtubule Stabilizing Agent, Taxol

In addition to being an important component of the cytoskeleton, microtubules are an integral part of the mitotic spindle, cilia, flagella and cytoplasm of interphase cells. Consequently, they have been implicated in a variety of cellular functions including mitosis, saltatory motion,

secretion, morphology and growth control (Dustin, 1978, 1984; Roberts and Hyams, 1979). Advances that have increased our understanding of the biochemistry, assembly and function of microtubules in each of these cellular processes are: (a) the development of procedures for the *in vitro* polymerization of microtubules (Weisenberg et al., 1972; Shelanski et al., 1973; Borisy et al., 1974), (b) the development of fluorescent antibody techniques for studying the organization of microtubules in cultured cells (Brinkley et al., 1975; Weber et al., 1975), (c) detailed morphological studies (Dustin 1978, 1984) and (d) the use of various microtubule inhibitors (Dustin, 1978, 1984). As a direct consequence of their highly specific mechanism of action, these latter agents have not only played a central role in characterizing microtubule structure and function but they have shown great promise in the field of cancer chemotherapy (Bender, 1981; Bender and Chabner, 1982).

Many different compounds (for example, colchicine, colcemid, griseofulvin, maytansine, nocodazole, podophyllotoxin) which interfere with microtubule functions are currently known. These mitotic poisons inhibit the polymerization of tubulin *in vitro* and cause the disassembly of microtubules when added to cells in culture by binding to specific microtubule structural component(s) (Dustin, 1978, 1984). Among the antimitotic agents that interact with microtubules, one compound, taxol, is quite unique.

Taxol (a low molecular weight alkaloid isolated from the stem bark of the western yew Taxus brevifolia, Wani et al., 1971) has a complex structural formula (Figure 3.). The drug is an ester that has been shown to be a taxane derivative containing a rare oxetan ring and is the first compound of this type to have antileukemic and tumor inhibitory properties (Wani et al., 1971). Since its discovery, taxol has been shown to be a potent growth inhibitory agent towards numerous cell types (Schiff et al., 1979; Manfredi et al., 1982; Lataste et al., 1984). In addition, this microtubule poison exhibits antitumor activity toward P388 leukemia, B16 melanoma and human Mx-1 mammary tumor xenografts insulated under the subrenal capsule of nude mice (Kisner et al., 1983).

Studies in vitro have shown that in contrast to other antimitotic alkaloids that inhibit microtubule formation, taxol enhances both the rate and yield of microtubule assembly (Schiff et al., 1979). In the presence of taxol, the critical concentration of microtubule protein required for assembly is reduced and the microtubules formed are resistant to depolymerization by low temperatures (Schiff et al., 1979; Thompson et al., 1981). Interestingly, taxol possesses the ability to assemble tubulin under other conditions in which polymerization would not normally occur. These include the absence of microtubule-associated proteins

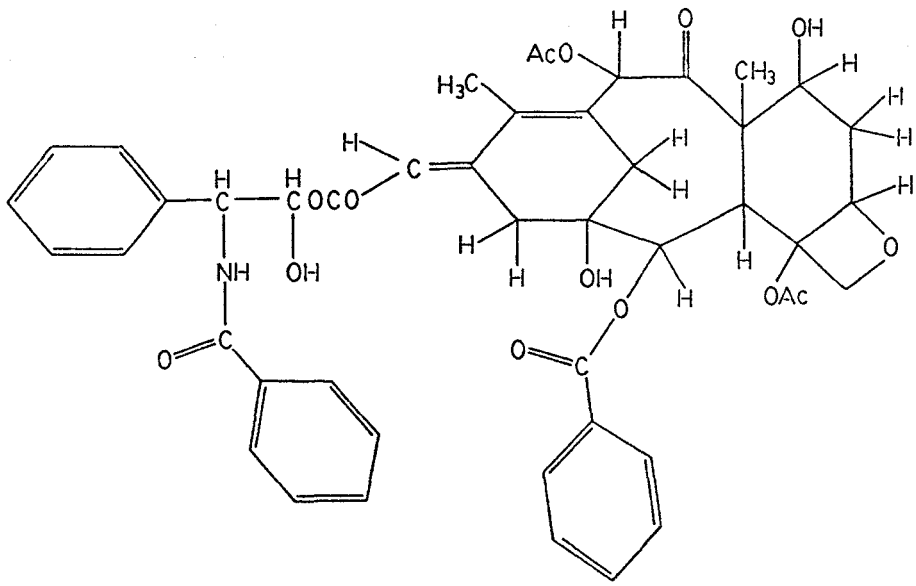


Figure 3. The structural formula of the antimitotic agent, taxol.

or exogenous guanosine-5'-triphosphate (Kumar, 1981; Schiff and Horwitz, 1981). Assembled microtubules incubated in the presence of taxol become resistant to depolymerization by calcium which suggests that there is a taxol binding site on microtubules (Schiff and Horwitz, 1981). Further work with (^{3}H)-taxol has shown that *in vitro* this drug does bind to microtubules and that binding to polymerized tubulin occurs with a stoichiometry approaching one (Parness and Horwitz, 1981). Similar to the situation *in vitro*, taxol has been shown to promote microtubule assembly and stability to various depolymerizing agents *in vivo* (Schiff and Horwitz, 1980; Masurovsky et al., 1980).

Although the effects of taxol have been studied in numerous cell systems (Manfredi et al., 1982; Lataste et al., 1982) and appear to be related to the tubulin-microtubule system in each cell line, the precise mechanism of action of taxol *in vivo* or *in vitro* is not totally understood.

1.5 Rationale and Objectives of the Current Study

As mentioned earlier, the purine nucleoside analogues, toyocamycin, tubercidin and 6-MeMPR, as well as the protein synthesis inhibitor, puromycin and the microtubule stabilizing agent, taxol have shown potential for applications to the field of cancer chemotherapy. Consequently, extensive efforts have been made in an attempt

to understand their intracellular activities. As yet, however, the precise details concerning the mechanisms of action, cellular toxicity and development of resistance to these anticancer agents are not well established.

One approach that should provide insight into these areas is the combined genetic and biochemical approach to mutant characterization. The introduction of a specific lesion into cells, followed by careful genetic and biochemical characterization of the nature of this lesion and its physiological consequence (drug resistance), is a powerful means with which to study the role of cellular constituents in determining the phenotypic character of a mutant cell. In an attempt to use this approach, several investigators have isolated mutants exhibiting varying degrees of resistance to each of the aforementioned anticancer drugs. However, in most cases, the mutants were isolated in cell systems other than those of human origin. Since any future clinical applications of these agents would be to the treatment or therapy of different types of human cancer, it is important to understand clearly the mechanisms of action and development of resistance to these agents in human cells. Therefore, in the present work, the combined genetic and biochemical approach to mutant characterization was undertaken in an attempt to gain further insight into the mechanism of action and development of resistance to

chemotherapeutic agents in human cells. For this purpose, stable mutants exhibiting varying degrees of resistance to toyocamycin, tubercidin, 6-MeMPR, puromycin and taxol were isolated in HeLa cells. Subsequent studies performed with these mutants have not only provided valuable information concerning the mechanism of action and development of resistance to these anticancer agents in human cells, but have shown that selection for purine nucleoside analogue resistant mutants in culture may provide a valuable system for quantitative mutagenesis studies involving cells originally established from humans.

2. MATERIALS AND METHODS

2.1 Materials

Material	Abbreviation	Source
Aclacinomycin A	(NSC-208734)	DSCB/NCI*
Actinomycin D	-	Sigma Chemical Co.
Acrylamide	-	Bio Rad Laboratories
Adenosine	-	Aldrich Chemical Co.
Adenosine-5'-triphosphate	(ATP)	Sigma Chemical Co.
Adriamycin	-	Sigma Chemical Co.
Alpha-modified minimal essential medium	(α-MEM)	Grand Island Biol. Co.
Ammonium persulfate	-	Fisher Scientific Co.
Aqueous counting scintillant	(ASC)	Amersham Corporation
1-β-D-Arabinofuranosyl-cytosine	(ara-C)	Sigma Chemical Co.
Bis acrylamide	-	Bio Rad Laboratories
Bisantrene	(NSC-337766)	DSCB/NCI*
Bovine serum albumin	(BSA)	Sigma Chemical Co.
Bromophenol blue	-	Sigma Chemical Co.
Chlorambucil	-	Sigma Chemical Co.

Material	Abbreviation	Source
Chromatography paper (#1 and #3)	-	Whatman Paper Limited
Cisplatin	-	Verlacoid Chemical Co.
Colchicine	-	Sigma Chemical Co.
Coomassie brilliant blue R	-	Sigma Chemical Co.
Cupric sulphate	-	Sigma Chemical Co.
Daunomycin	-	Sigma Chemical Co.
Dialyzed fetal calf serum	(DFCS)	Grand Island Biol. Co.
Ellipticine	(NSC-71795)	DSCB/NCI*
Erythro-9-(2-hydroxy-3-nonyl) adenine	(EHNA)	Burroughs Wellcome Co.
Ethylmethanesulfonate	(EMS)	Eastman Kodak Co.
Fetal calf serum	(FCS)	Grand Island Biol. Co.
Filtration units (0.22μm)	-	Millipore Corporation
5-Fluorouracil	-	Sigma Chemical Co.
Glass fiber filters	(GF/C)	Whatman Paper Limited
Glycerol	-	BDH Chemicals Limited
Glycine	-	Bio Rad Laboratories
Harringtonine	(NSC-124147)	DSCB/NCI*

Material	Abbreviation	Source
ICR-170	-	Calbiochem-Behring Corp
Lanthanum chloride	-	Sigma Chemical Co.
Magnesium chloride	-	Sigma Chemical Co.
Maytansine	(NSC-153858)	DSCB/NCI*
Membrane tubing (Spectrapor #1)	-	Spectrum Medical Ind.
2-Mercaptoethanol	(2-MSH)	Sigma Chemical Co.
Methotrexate	-	Sigma Chemical Co.
Methylene blue	-	Sigma Chemical Co.
Methylglyoxal(bis)-guanylhydrazone	(MGBG)	Aldrich Chemical Co.
6-Methylmercaptopurine riboside	(6-MeMPR)	Sigma Chemical Co.
N-Methyl-N'-nitro-N-nitrosoguanidine	(MNNG)	Aldrich Chemical Co.
Mithramycin	-	Sigma Chemical Co.
Mitomycin C	-	Sigma Chemical Co.
Mitoxantrone	(NSC-301739)	DSCB/NCI*
Nitrocellulose membranes	-	Mandell Scientific Co.
Phenol (Lowry) reagent	-	Fisher Scientific Co.

Material	Abbreviation	Source
Podophyllotoxin	-	Polysciences Inc.
Potassium phosphate (mono- and dibasic)	-	Fisher Scientific Co.
PPO/POPOP toluene concentrate	-	New England Nuclear
Puromycin	-	Sigma Chemical Co.
Pyrazofurin	-	Dr. R.L. Hamil of Lilly Research Labs
Sodium carbonate	-	BDH Chemicals Limited
Sodium deoxycholate	-	Sigma Chemical Co.
Sodium dodecyl sulfate	(SDS)	BDH Chemicals Limited
Sodium tartrate	-	Sigma Chemical Co.
Taxol	(NSC-125973)	DSCB/NCI*
N,N,N',N'-Tetramethyl-ethylenediamine	(TEMED)	Bio Rad Laboratories
Tissue culture dishes (60-mm-diameter/plastic)	-	Corning Lab. Sci. Co.
Tissue culture dishes (100-mm-diameter/plastic)	-	Nunclon
Tissue culture dishes (24-well/plastic)	-	Flow Laboratories

Material	Abbreviation	Source
Toluene	-	Fisher Scientific Co.
Toyocamycin	(NSC-63701)	DSCB/NCI*
Trichloroacetic acid	(TCA)	Fisher Scientific Co.
Tris [tris(hydroxymethyl)-aminomethane]-base	-	Sigma Chemical Co.
Tris-HCl	-	Sigma Chemical Co.
Tubercidin	-	Sigma Chemical Co.
Uridine	-	Sigma Chemical Co.
Vinblastine sulphate	-	Sigma Chemical Co.
VM26	(NSC-122819)	DSCB/NCI*
VP16-213	-	Dr. A. von Wartburg of Sandoz Limited
X-Omat XAR-5 film	-	Eastman Kodak Co.

* Drug Synthesis and Chemistry Branch of the National Cancer Institute, National Institute of Health, Silver Springs, MD.

All of the nucleoside analogues and drugs were freshly dissolved in their respective solvents (water, ethanol or DMSO) and filter sterilized before each experiment. The final concentration of ethanol or DMSO did not exceed 0.5% when the drug was added to the cells. At this concentration, there was no observed effect on the growth of HeLa cells.

All the other chemicals were of the highest analytical reagent grade available.

(b) Enzymes

Trypsin (EC 3.4.4.4) was purchased from Difco Laboratories.

(c) Radiochemicals

(^{125}I)-Donkey anti-rabbit IgG F(ab')$_2$ fragment (18 μCi/μg) was purchased from the Amersham Corporation. (^{3}H)-Adenosine (42 Ci/mmole) and (^{3}H)-daunomycin (1.5 Ci/mmole) were obtained from the New England Nuclear Corporation.

(d) Antiserum

Antibody specific to adenosine kinase (AK) was provided by K. Mehta, Department of Biochemistry, McMaster University.

(e) Cell Culture and Cell Lines

The phenotype and origin of the different HeLa cell lines employed in these studies are presented in Table 3. All the drug-resistant mutants investigated are direct descendents from the parental, HeLa cell line (clone S_3). The various cell lines were routinely grown in monolayer cultures at 37^{o}C in alpha-modified minimal essential medium (α-MEM ; Stanners et al., 1971) in humidified incubators containing a fixed 95% air - 5% CO_2 atmosphere. This medium contains all four ribonucleosides and deoxyribonucleosides (10 μg/mL each) and was supplemented with 10% fetal calf

Table 3. Characteristics of the HeLa Cell Lines Used in the Present Study

Cell Line	Phenotype and Origin
HeLa (clone S_3)	Human, epithelial cells established from a cervical carcinoma by Gey et al.(1952) and cloned by Puck et al. (1956).
TOY^{rI}-2	Toyocamycin-resistant mutants of HeLa cells selected in the presence of 50 ng/mL of toyocamycin (Section 3.1).
TUB^{rI}-2, TUB^{rI}-4 and TUB^{rI}-5	Mutants obtained for resistance to tubercidin by plating HeLa cells in the presence of 500 ng/mL of tubercidin (Section 3.1).
6-$MeMPR^{rI}$-1, 6-$MeMPR^{rI}$-2 6-$MeMPR^{rI}$-3 and 6-$MeMPR^{rI}$-4	Mutants resistant to 6-methylmercaptopurine riboside obtained by plating HeLa cells in the presence of 5 µg/mL of 6-methylmercaptopurine riboside (Section 3.1).
PUR^{rI}-27	Puromycin-resistant mutants of HeLa cells selected in the presence of 250 ng/mL of puromycin (Section 3.4).
PUR^{rII}-26D, PUR^{rII}-27C and PUR^{rII}-28B	Second-step mutants obtained for resistance to puromycin by plating PUR^{rI}-27 cells in the presence of 10 µg/mL of puromycin (Section 3.4).

Cell Line	Phenotype and Origin
TAX^{RI}-24A	Mutants resistant to taxol obtained by plating HeLa cells in the presence of 5 ng/mL of taxol (Section 3.5).
TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G	Second-step mutants obtained for resistance to taxol by plating TAX^{RI}-24A cells in the presence of 100 ng/mL of taxol (Section 3.5).
CHO/WT (Pro^{-})	Proline-requiring Chinese hamster cells established from ovarian tissue (Tijo and Puck, 1958).

serum (FCS) (referred to as regular medium). However, for the experiments involving various nucleoside analogues, a deficient medium, α-MEM minus nucleosides supplemented with 10% dialyzed fetal calf serum (DFCS), was employed (referred to as minus nucleosides or special medium). The doubling time of HeLa cells in both types of media was approximately 18-20 hours.

Since the stability of the drug-resistant phenotype of the second-step mutants was uncertain, these cell lines were cultured under continuous selective pressure. This was achieved by growing these cells in the presence of the drug at the same concentration against which they were initially selected. Phosphate buffered saline (PBS), used for washing the cells, contained 0.8% sodium chloride, 0.02% potassium chloride, 0.115% disodium hydrogen phosphate and 0.008% potassium dihydrogen phosphate. Trypsinization of all cell lines was accomplished by adding a minimal volume of 0.125% trypsin to each plate for approximately 5 minutes. Cell counts were made with a Coulter Electonic Counter - Model Z_f (Coulter Electronics, Inc.)

2.2 Methods

2.2.1 Selection of Mutants

The selection of various types of drug-resistant mutants was carried out using procedures which paralleled those reported elsewhere (Gupta, 1983d; Gupta and Singh,

1983). Initially, three 100-mm-diameter dishes (P-100 dishes) were set-up such that they contained 10^7 exponentially growing cells in 10 mL of regular medium (α-MEM + nucleosides + 10% FCS). After 6 or 7 hours, which was sufficient time to allow the cells to adhere to the plates and resume growth, the cells in two dishes were mutagenized by adding 300 to 400 μg of ethylmethanesulfonate (EMS) per mL for 20 hours (the other dish, which was not treated with the mutagen, served as the control population and was cultured in the same manner as the mutagenized cells). After treatment, the mutagen-containing medium was removed, the cells were washed twice with 5 mL of phosphate buffered saline (PBS) and 1 mL of 0.125% trypsin was added for about 5 minutes.

Post trypsinization, the mutagenized cells from all dishes were resuspended in regular medium to yield a single suspension. Duplicate aliquots of 0.3 mL of this suspension were counted with the use of a Coulter electronic counter (Coulter Electronics, Inc.) to determine the cell density. Appropriate dilutions were then made in regular medium such that triplicate 60-mm-diameter dishes containing 250 mutagenized cells per dish were set-up in 5 mL of nonselective regular medium. These plates were incubated at $37^{o}C$ in a 5% CO_2 atmosphere for 11 to 12 days, after which time the dishes were stained with 0.5% (w/v) methylene blue

in 50% methanol (v/v) and aggregates of 40 or more cells were counted as surviving colonies. These counts were used to determine the percent cell survival after mutagen treatment.

The remainder of the cell suspension was divided between five or six 100-mm-diameter dishes and cultured for the appropriate expression period (Section 3.) in nonselective regular medium to allow time for mutation fixation. If the required expression period was of sufficient duration to allow the cells to approach confluency, the mutagenized cells were trypsinized, resuspended in medium, combined and at least 50% of the total cell population replated in fresh, nonselective regular medium. After allowing an appropriate time for expression, the mutagenized cells were trypsinized, resuspended in medium and combined to yield a single cell suspension.

The selection of mutants in both the control and mutagenized cell populations was achieved by plating $1x10^6$ cells per 100-mm-diameter dish in medium containing the appropriate selective concentration of the drug (Section 3.). The appropriate selective drug concentration is one which will select for mutants which are at least two- to three-fold resistant to the drug and will decrease the background growth of normal cells to a point where virtually

none is observable. Typically, 1.0 mL of drug solution (twelve times the desired final concentration) and 1.0 mL of cell suspension containing the desired number of cells were added, in this order, to 100-mm-diameter dishes containing 10.0 mL of growth medium. For the selection of mutants resistant to taxol and puromycin, regular medium was used but for the selection of mutants resistant to the nucleoside analogs, special medium (α-MEM minus nucleosides + 10% DFCS) was used.

In addition to those plates used for the selection, triplicate 60-mm-diameter dishes, each containing 250 cells in 5 mL of nonselective regular medium, were set-up in order to determine the plating efficiency of the cells. Both mutant selection and plating efficiency dishes were incubated for 11 to 12 days at 37^{o} C in a 5% CO_2 environment during which time no change of medium or fresh addition of selective drugs was carried out. Subsequently, the dishes used for mutant selection were checked for the growth of any surviving colonies. Selected drug-resistant colonies were picked, plated and grown in separate 60-mm-diameter dishes in regular nonselective medium and subsequently tested for their degree of resistance to the selective agent. All dishes used for mutant selection and determining plating efficiencies were stained with methylene blue and aggregates of 40 or more cells were counted as colonies. From the

number of cells plated, the number of mutant colonies observed and the plating efficiency of the cells, the mutation frequency was calculated for both the control and mutagenized cell populations.

2.2.2 Measurement of the Degree of Resistance

The degree of resistance of the mutants toward the drug against which they were selected and the degree of cross-resistance of the mutants toward drugs other than those against which they were selected were determined. Where no prior information regarding the toxicity of a particular drug toward these HeLa cell mutants existed, a preliminary drug resistance test was performed to determine the approximate range over which the drug was toxic to the cells. This was accomplished by plating the cells in the presence of increasing concentrations of the drug ranging from less than 1.0 ng/mL to 1.0 mg/mL. Subsequent to this preliminary test, a more precise determination of the degree of resistance was achieved by repeating the drug resistance test using drug concentrations which were chosen such that the majority of them lay within the 10 to 90% range of cell survival.

Experimentally, the degree of resistance or cross-resistance of the mutant cells was determined in the following manner; initially, 250 and 1000 cells (in a 0.5 mL volume of growth medium) were seeded in duplicate into the

wells of 24-well tissue culture dishes. Each of these wells contained 0.5 mL of the various dilutions of the drugs (eleven dilutions were done such that duplicate concentrations would be present in addition to two control, drug-free wells) at twice the final concentrations desired in the growth medium. Therefore, each 24-well dish represented a drug resistance test which contrasted the normal growth of mutant cells in the absence of any drug with the growth of those same cells in the presence of increasing concentrations of the drug. These dishes were incubated for 11 to 12 days at 37°C in a 5% CO_2 atmosphere, after which time they were stained with 0.5% (w/v) methylene blue in 50% methanol (v/v). From the number of colonies surviving at the different drug concentrations, relative plating efficiencies (the ratio of the number of colonies surviving at a given drug concentration to that obtained in the absence of drug) were recorded. From these relative plating efficiencies, dose-response curves were constructed for each cell line in the presence of a variety of drugs and the relative effect of each drug on the mutant established by comparing the D_{10} values. The D_{10} value of a drug towards a cell line refers to the dose of the drug which reduces the relative plating efficiency of the cell line to 10% of that obtained in the absence of any drug.

2.2.3 Cellular Uptake and Incorporation of (^{3}H)-Adenosine and (^{3}H)-Daunomycin

The cellular uptake and incorporation of (^{3}H)-adenosine and the cellular uptake of (^{3}H)-daunomycin were performed by procedures similar to those described earlier (Gupta, 1983d; Mehta and Gupta, 1983). Initially, approximately $5x10^5$ cells of each cell line were seeded (in duplicate for each time period) into the wells of 24-well tissue culture dishes in regular medium. The following day, when the cells were nearly confluent, the medium was carefully aspirated from each well and the uptake experiment started with the addition of 0.25 mL of the appropriate medium. When determining the cellular uptake of (^{3}H)-adenosine, the medium used was α-MEM minus nucleosides + 10% DFCS, supplemented with 1.0 µCi/mL of (^{3}H)-adenosine (specific activity, 42 Ci/mmol), 10 µg/mL of the adenosine deaminase inhibitor erythro-9-(2-hydroxyl-3-nonyl) adenine (to prevent deamination of adenosine) and $5x10^{-5}$ M uridine (to prevent the cellular toxicity caused by addition of exogenous adenosine). When determining the cellular uptake of (^{3}H)-daunomycin, the medium used was α-MEM + nucleosides + 10% FCS, supplemented with $2x10^{-7}$ M (^{3}H)-daunomycin (specific activity, 1.5 Ci/mmol). At different intervals, the labelled medium was carefully aspirated and the cells washed three times with 0.5 mL of PBS. After the final wash,

the cells were dissolved in 0.5 mL of lysis buffer (0.4% deoxycholic acid in 0.1 N NaOH).

To quantitate the total cellular uptake of radioactive adenosine, one-half of the cell lysate was counted after addition to 4 mL of aqueous counting scintillant (ACS). To determine the amount of radioactive adenosine which had been incorporated into cellular macromolecules, the other half of the cell lysate was added to 1.0 mL of ice-cold 10% trichloroacetic acid (TCA). After 30 minutes, the precipitate was collected on Whatman glass microfibre filters (GF/C) and washed five or six times with ice-cold 5% TCA (6 to 7 mL each time). The filters were dried and counted in 4 mL of a toluene-based scintillation fluid containing PPO and POPOP.

Alternatively, to quantitate the total cellular uptake of radioactive daunomycin, the total cell lysate was counted directly after its addition to 4 mL of aqueous counting scintillant.

Parallel control experiments were run in which duplicate wells for each time point were set-up containing regular medium and no cells. When the uptake was to be started, the regular medium was aspirated and 0.25 mL of the appropriate uptake medium added. After the appropriate uptake interval, the labelled medium was removed, the wells were washed three times with 0.5 mL of PBS and 0.5 mL of the

lysis buffer was added. When assaying total cellular uptake of (^{3}H)-adenosine or (^{3}H)-daunomycin, the resulting solution was added directly to aqueous counting scintillant. Alternatively, for (^{3}H)-adenosine uptake and incorporation into macromolecules, the resultant solution was added to 1.0 mL of 10% TCA for 30 minutes, filtered on Whatman glass microfibre filters (GF/C), washed with 5% TCA, dried and counted in the toluene-based scintillation fluid. The background radioactivity present in these control wells was subtracted from the total radioactivity assayed at each interval. At the same time, the total number of cells in each of two additional wells per cell line was determined by trypsinization and counting of duplicate aliquots in a Coulter counter. The cellular uptake of (^{3}H)-adenosine and (^{3}H)-daunomycin, as well as the uptake of (^{3}H)-adenosine into cellular macromolecules, in different cell lines was normalized to a constant number of cells. The cellular uptake and incorporation of radioactive adenosine or daunomycin in duplicate samples in these experiments usually differed by less than 10% and their averages were taken in all cases. Assuming the average amount of (^{3}H)-adenosine or (^{3}H)-daunomycin taken up by the sensitive HeLa cells to be 100, the relative uptake in the mutant cells was calculated.

2.2.4 Measurement of Protein

Protein concentrations were determined spectrophotometrically according to the method of Lowry et al. (1951) using ovalbumin as the standard.

2.2.5 Assay of Adenosine Kinase (EC 2.7.1.20)

The cell extracts required for the measurement of AK activity were prepared in the following manner. Initially, three or four 100-mm-diameter dishes for each cell line to be assayed were seeded with $5x10^6$ cells in regular medium. After 2 days, when the cells were not yet confluent, the medium from the dishes was aspirated, the cells trypsinized, resuspended in 10 mL of regular medium and counted. Equal numbers of cells (approximately $4x10^7$ cells) from each cell line were aliquoted and centrifuged in an IEC Centra-7R refrigerated centrifuge (International Equipment Company) at 1500 rpm for 10 minutes at 4^oC. The supernatant was discarded and the cell pellet resuspended in 3 mL of ice-cold PBS. This suspension was again centrifuged at 1500 rpm for 10 minutes at 4^oC, the supernatant disgarded and the PBS wash repeated. After the second wash, the cell pellet was resuspended at a concentration of $2x10^7$ cells per mL in ice-cold 0.2 M potassium phosphate buffer, pH 7.0, and the cells disrupted by sonication using a Bronson sonicator (Model W140D-Bronson Sonic Power Company) for two 30 second bursts interspersed by a 1 minute interval. At all times during the sonication, the cells/extracts were kept on ice. The

resulting crude cell extract was centrifuged at 30,000 x g in a Sorvall SS34 rotor for 30 minutes at 4°C. The supernatant was placed in a Spectrapor #1 dialysis tubing bag (cutoff, 6000-8000; Spectrum Medical Industries) and dialyzed for 16 hours at 4°C against 200 volumes of 0.02 M potassium phosphate buffer (pH 7.0) with one change of buffer during the dialysis period. The dialysate was then used immediately for the assay of AK activity.

The assay of AK activity in the cell extracts was carried out essentially as described by Chan et al. (1973) and Gupta and Siminovitch (1978b). The assay mixture (a final volume of 250 µL) contained 50 mM potassium phosphate buffer, pH 7.0, 2.5 mM ATP, 0.25 mM $MgCl_2$ and $4x10^{-5}$ M (^3H)-adenosine (specific activity, 42 Ci/mmole). The reaction, which was carried out at 37°C in a constant temperature water bath (Canlab Laboratory Equipment), was initiated by the addition of between 25 and 50 µL of the cell dialysate. At various intervals, 50 µL aliquots of the reaction mixture were removed and added to 1 mL of ice-cold 0.1 M $LaCl_3$, which precipitates AMP. After 30 minutes, the AMP was collected on Whatman glass microfibre filters (GF/C), which were washed five or six times with 6 to 7 mL of ice-cold H_2O. The filters were dried and counted in 4 mL of a toluene-based scintillation fluid containing PPO and POPOP. A parallel control experiment was run in which a reaction

mixture, minus any cell extract, was incubated at 37°C and after the appropriate time intervals, 50 µL aliquots were removed, precipitated, filtered, washed, dried and counted. The background radioactivity bound to these filter papers was subtracted from the total radioactivity assayed at each interval. Under the conditions employed, the duplicate samples showed less than 10% variation and their averages were taken. In each case, the amount of (^{3}H)-AMP produced by each cell extract was converted from cpm into nanomoles of (^{3}H)-AMP. From this, the specific activity of AK in the cell extracts was calculated by dividing the observed AK activity (ie. nmoles of nucleotide formed) by the protein concentration in each of the cell extracts.

2.2.6 Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Slab-Gel Electrophoresis

Samples were prepared for electrophoresis in the following manner. Initially, a 100-mm-diameter dish was set-up for each cell line in regular medium. When the cells were one-half to two-thirds confluent, the medium was removed, the cells were washed twice with 5 mL of PBS, trypsinized, counted and equal numbers of cells ($1x10^7$) resuspended in regular medium. The cell suspensions were spun at 1500 rpm for 10 minutes at room temperature, after which time the supernatant was discarded and each pellet resuspended individually in 1 mL of regular medium and transferred to

1.5 mL Eppendorf centrifuge tubes. The tubes were spun for 2 minutes in a microcentrifuge (Fisher Scientific Company), the supernatant discarded and each pellet resuspended in 1.0 mL of sample buffer [0.125 M Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 2.5% (v/v) 2-MSH, 0.002% (w/v) bromophenol blue tracking dye]. All samples were heated to 100^{o}C for 5 minutes to denature the proteins and subsequently spun in a microfuge for 10 minutes. From each of these concentrated samples, one-fifth dilutions were made by taking 20 µL of each concentrated sample and adding 80 µL of sample buffer (this corresponded to a crude extract made from $2x10^5$ cells). These diluted samples were loaded on the gel (total volume loaded was 100 µL).

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) using slab gels with a discontinuous buffer system. Two glass plates per gel, of the same width but different lengths (dimensions are 20 cm x 22 cm and 20 cm x 24 cm), were washed with detergent, rinsed with distilled H_2O, left to air dry and further cleaned by wiping with a 50% methanol solution. Plastic spacers (1.5 mm thick) were covered with vaseline and placed along the longer sides of the larger plate, the shorter plate placed on top and the two plates clamped onto a Bio-Rad two gel electrophoresis apparatus (Bio-Rad Laboratories) with the shorter plate facing the apparatus. Subsequently, the

separating gels were made by mixing the following reagents so that their final concentrations were 10% acrylamide (30% : 0.8% acrylamide : bis acrylamide, w/w), 0.375 M Tris-HCl (pH 8.8), 0.1 % (w/v) glycerol, 0.025% TEMED, 0.075% (w/v) ammonium persulfate and 0.1% SDS. The solution was degassed prior to the addition of freshly prepared ammonium persulfate and SDS. After the addition of ammonium persulfate and SDS, the solution was mixed and poured between the two sets of glass plates. Each separating gel was overlaid with 1 mL of distilled H_2O and allowed to polymerize at room temperature for approximately 30 minutes. During this time, a stacking gel solution (sufficient for two gels) was made by mixing the following reagents so that their final concentrations were 5% acrylamide (30% : 0.8% acrylamide : bis acrylamide, w/w), 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 0.05% glycerol and 0.075% TEMED. After the separating gel had polymerized, the overlaid distilled H_2O was completely removed before the stacking gel was poured. The stacking gel solution was mixed, 0.064% ammonium persulfate added and the solution poured on top of the separating gel. Ten-well combs were inserted between the plates and pushed down until approximately 2 cm of stacking gel lay between the bottom of the combs and the beginning of the separating gel. After 20 minutes at room temperature (when the polymerization was complete), the combs were

removed and the excess fluid drained from the wells. The previously prepared samples were loaded (100 µL) into individual wells and carefully overlaid with electrophoresis running buffer [0.30% (w/v) Tris-base, 1.44% (w/v) glycine and 0.1% (w/v) SDS]. Electrophoresis running buffer was added to both the upper reservoir (a sufficient volume to cover the gels) and lower reservoir and any bubbles trapped under the gels were removed. An initial voltage of 150 volts was applied until the samples had run from their respective wells and migrated through one-half of the stacking gel. At this point the voltage was decreased to 80 volts and electrophoresis allowed to continue overnight.

When the dye front had travelled 14 cm into the separating gel, the gels were removed from the plates and one gel placed into a tray of staining solution (the other gel was used for the immunoblot-Section 2.2.7). The staining of proteins was accomplished by leaving the gel in a solution of 0.25% Coomassie blue, 50% methanol and 10% glacial acetic acid (v/v) for 2 to 3 hours at room temperature with gentle shaking. The staining solution was then removed, the gel was washed with distilled H_2O to remove excess stain, placed in destaining solution (20% ethanol, 10% glacial acetic acid, v/v) and the shaking continued. Destaining was allowed to occur overnight with at least three changes of the destaining solution until the

protein bands in the gel were clearly visible. The destained gel was placed onto a piece of Whatman #1 chromatography paper and dried using a Bio-Rad slab-gel dryer (Bio-Rad Laboratories).

2.2.7 Immunoblots

Immunoblotting of proteins onto nitrocellulose was performed in a manner similar to that described by Towbin et al. (1979). The proteins were first subjected to electrophoresis in 10% SDS-polyacrylamide one-dimensional slab gels (Section 2.2.6). After electrophoresis, the gel was removed, soaked in transfer buffer (25mM Tris-base, 192 mM glycine, pH 8.3, 20% methanol) for approximately 10 minutes and then placed on a nitrocellulose membrane (0.45 µm pore size, 14 cm x 14 cm, Schleicher and Schuell, Inc.) which had also been previously soaked in the transfer buffer. The gel and adhering nitrocellulose were placed between two 14 cm x 14 cm sheets of Whatman #3 chromatography paper and then sandwiched between plastic grids. The whole assembly was placed (keeping the nitrocellulose sheet toward the positive electrode) into an electrophoretic Trans-Blot chamber (Bio Rad Laboratories), the chamber filled with transfer buffer and the proteins transferred to nitrocellulose by overnight electrophoresis at 80 mA. Following transfer, the nitrocellulose sheet was removed from the apparatus and placed in a heat-sealed

plastic bag containing 15 mL of 3% BSA in saline (0.9% sodium chloride, in 10 mM Tris-HCl, pH 7.4). After rocking (using a Speci-Mix Aliquot Mixer, Sybron/Thermolyne Corporation) for 3 hours at room temperature to saturate the remaining protein binding sites, the blot was removed from the plastic bag and either used immediately for antibody application or air-dried and stored at $4^{o}C$.

For the first antibody application, the blot was rinsed with saline and then rocked for 3 hours at room temperature in the presence of antiserum diluted 1:30 into 15 mL of saline containing 3% BSA. The blot was then washed with five or six changes of saline (200 mL per wash) over a period of 1 to 2 hours to remove any unbound antibody and subsequently rocked overnight at room temperature in the presence of the second (indicator) antibody directed against the immunoglobulins of the first antiserum. (^{125}I)-Labelled donkey anti-rabbit IgG $F(ab')_2$ fragment (18 µCi/µg), diluted to $5x10^5$ cpm per mL in saline containing 3% BSA, was used as indicator antibody. On the following day, the blot was extensively washed with saline (seven or eight changes over 2 hours, 200 mL per wash), air dried on Whatman #3 chromatography paper and covered with Saran wrap. The radioactivity was detected by overnight autoradiography at room temperature using Kodak X-Omat XAR-5 film. The film was developed using an automatic x-ray film processor.

2.2.8 Preparation of the Mutagens and Mutagen Treatment of HeLa Cells

2.2.8.1 Ethylmethanesulfonate (EMS)

Prior to mutagenesis, a fresh solution (20 mg/mL) of EMS was prepared by adding 50 µL of EMS stock solution (1220 g/L) to 3.0 mL of regular medium. This solution was filter sterilized by passage through a 0.22 µm filter unit (Millipore Corporation). Appropriate volumes (such that the range of final concentrations tested was between 0 and 400 µg/mL) of EMS solution were added directly to 100-mm-diameter dishes containing 10.0 mL of regular medium and the actively growing HeLa cells. The cells were exposed to the mutagen for 16 to 20 hours at 37°C in a 5% CO_2 atmosphere after which time the mutagen-containing medium was removed, the cells were washed twice with 5 mL of PBS and appropriate platings carried out.

2.2.8.2 N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

Prior to mutagenesis, a fresh stock solution of MNNG was prepared by dissolving 3.0 mg of crystalline MNNG per mL of absolute ethanol. From this, an appropriate dilution was made into 10.0 mL of regular medium such that the desired final concentration of MNNG was obtained (2.0 µg/mL). This MNNG solution was added to 100-mm-diameter dishes containing actively growing HeLa cells and exposure allowed for a period of 4 hours at 37°C in a 5% CO_2 atmosphere.

Subsequently, the mutagen-containing medium was removed, the cells were washed twice with 5 mL of PBS and appropriate platings performed.

2.2.8.3 ICR-170

A fresh stock solution of ICR-170 was prepared by dissolving 2.0 mg of crystalline ICR-170 per mL of absolute ethanol. From this, appropriate dilutions were made to obtain the desired final concentrations of ICR-170 in 10.0 mL of regular medium (the range of final concentrations tested was between 0 and 3.0 µg/mL). These ICR-170 solutions were added individually to 100-mm-diameter dishes containing actively growing HeLa cells and exposure allowed for a period of 16 hours at 37°C in a 5% CO_2 atmosphere. After this time, the mutagen-containing medium was removed, the cells were washed twice with 5 mL of PBS and appropriate platings carried out.

2.2.8.4 Ultraviolet (UV)-light

Actively growing HeLa cells were exposed to UV-light at room temperature. The UV-irradiation of the cells was carried out in 100-mm-diameter dishes (without covers) that had the regular medium removed and replaced with 1 mL of PBS. The UV-dose administered was varied by increasing the duration (seconds) of the exposure and this time of exposure was subsequently converted to energy (ergs/mm^2) of UV-exposure. The UV-source was a 254 nm Mineralight^R Lamp

(Ultra-Violet Products, Inc.) and the incident dose of 23.5 ergs/mm^2/sec was calibrated using a Black Ray^R ultraviolet intensity meter (Ultra-Violet Products, Inc.).

The control cells in each case were treated with an equivalent amount of the solvent.

2.2.9 Determination of the Mutagen Sensitivity of HeLa Cells

HeLa cells were treated with various concentrations of the mutagens to determine their toxic effect. For this purpose, approximately 1x10^6 HeLa cells were seeded into several replicate 100-mm-diameter dishes in 10 mL of regular medium 1 day prior to mutagenesis. The following day, when the cells were growing actively, the medium was removed from the dishes and replaced with 10 mL of fresh regular medium to which the desired concentrations of the mutagens had been added. The control cells were treated with an equivalent amount of solvent. For those mutagens where very limited prior information regarding their toxicity toward HeLa cells existed, numerous mutagen concentrations were employed to create a wide range of lethality (between 0 and 100%). However, where some prior information regarding toxicity existed, the mutagen concentrations were chosen such that most points would lie within the 10 to 90% range of cell survival. After the appropriate treatment period, which differs for the various mutagens (Section 2.2.8),

cells were washed twice with 5 mL of PBS, trypsinized, resuspended in regular medium and duplicate aliquots counted. Two hundred and fifty cells for each mutagen concentration used were plated in triplicate in 60-mm-diameter dishes in 5 mL of regular medium. These plates were incubated for 11 to 12 days at 37°C in a 5% CO_2 environment and then stained with 0.5% methylene blue (w/v) in 50% methanol (v/v). From the average number of colonies observed at different concentrations of the mutagen, the percentage survival of the cells was obtained as the ratio of the number of colonies observed at any particular mutagen concentration to that observed for the control (nonmutagenized) cells. Based on these studies, 3 or 4 concentrations of each mutagen, which were expected to cause between 10 and 90% lethality, were chosen for the mutagenesis experiments.

2.2.10 Procedures for Determining the Optimal Conditions for Studying the Mutagenic Response of the Adenosine Kinase (AK) Locus

2.2.10.1 Effect of the Cell Density of AK^+ Cells on the Recovery of AK^- Cells

To determine whether the recovery of AK^- mutants was affected by cell density, a reconstruction experiment was carried out in which a constant known number of AK^- (resistant) HeLa cells were plated in the presence of

increasing numbers of AK^+ (sensitive) HeLa cells. Initially, 100-mm-diameter dishes were set-up containing 10.0 mL of special medium supplemented with 6-MeMPR at twelve times the desired final concentration (5 μg/mL). Next, one of the AK^- mutant cell lines (TOY^{rI}-2, TUB^{rI}-4 or 6-$MeMPR^{rI}$-3) was trypsinized, resuspended in special medium, counted and 200 cells (present in 1.0 mL of special medium) added to each plate. The control plates contained only AK^+ cells. Similarly, the appropriate number of AK^+ cells (ranging from $1x10^5$ to $2x10^6$ cells/plate) were added to each plate (excluding one set which contained only AK^- cells) in 1.0 mL volumes. After this final addition, the total volume of medium (α-MEM minus nucleosides + 10% DFCS + 5 μg/mL 6-MeMPR) present in each dish was 12.0 mL. All dishes were incubated at 37°C in a 5% CO_2 atmosphere for 11 to 12 days with no change of medium or fresh addition of drug. After this time the plates were stained with methylene blue and aggregates of 40 or more cells were counted as colonies.

2.2.10.2 Expression Time Requirement for the AK^- Mutant Phenotype

Before any genetic marker can be used for quantitative mutagenesis, it is necessary to determine the time required for the expression of the mutant phenotype. To obtain this information, $1x10^7$ HeLa cells were plated into each of six 100-mm-diameter dishes containing regular

medium. After 6 or 7 hours, when the cells had attached to the plates and were growing actively, five dishes were mutagenized with either EMS at a concentration of 350 µg/mL or MNNG at a concentration of 2.0 µg/mL (the remaining dish of HeLa cells served as the control). These concentrations of the mutagens caused 40 to 60% cell killing. After treatment, the mutagen-containing medium was removed, the cells were washed twice with 5 mL of PBS, trypsinized and resuspended in special medium (the same was done for the control culture). Duplicate aliquots of the suspension were counted and 250 cells, from both mutagenized and nonmutagenized cultures, were plated in triplicate in 60-mm-diameter dishes in 5 mL of regular medium to determine the survival after mutagen treatment. In addition, ten to twelve 100-mm-diameter dishes (for both cultures) containing 10.0 mL of special medium were supplemented with 1.0 mL of 6-MeMPR at 12 times the desired final concentration (5 µg/mL). To these dishes, 1.0 mL volumes containing $1x10^6$ mutagenized or control cells were added to allow selection for the 6-$MeMPR^r$ phenotype. The remainder of each cell suspension was replated in several 100-diameter dishes in regular medium to allow further time for the expression of the mutant phenotype. On days 3, 6, 9, and 12 post-mutagenesis, platings for the selection of the 6-$MeMPR^r$ mutants, plating efficiency and continued expression of the mutant phenotype

(excluding day 12) were repeated. All plates were incubated at 37°C in 5% CO_2 atmosphere for 11 to 12 days with no medium change or fresh addition of drug. Subsequently, the plates were stained with 0.5% methylene blue (w/v) in 50% methanol (v/v) and aggregates of 40 or more cells counted as colonies. The mutant frequency was calculated for both the mutagen-treated and control cultures on each day and the profile of the expression period for the 6-MeMPR^r phenotype determined.

2.2.11 Determination of the Concentration Response of the Adenosine Kinase (AK) Locus to Various Mutagenic Agents

A typical mutagenesis experiment began by seeding approximately $4x10^6$ HeLa cells into each of seven or eight 100-mm-diameter dishes in regular medium. After 2 days, the medium was removed, the cells were washed twice with 5 mL of PBS, trypsinized and resuspended to yield a single cell suspension. Duplicate aliquots were counted and $1x10^7$ cells were seeded into each of twelve 100-mm-diameter dishes containing 10 mL of regular medium. When the cells had attached to the plates and were growing actively (6 to 7 hours), they were treated with various concentrations of the mutagenic agents that were expected to result in 10 to 90% cell killing. The experiment was set-up such that there were two 100-mm-dishes used for each concentration of mutagen and

for the control cells. Subsequent to mutagen treatment, the mutagen-containing medium was removed and cells treated with different mutagen concentrations were independently washed twice with 5 mL of PBS, trypsinized and pooled. Two hundred and fifty cells from each mutagen-treated and the control culture were plated in triplicate, in 60-mm-diameter dishes containing 5 mL of regular medium, to determine the cell survival. All of the remaining cells were plated in several 100-mm-diameter dishes in 10 mL of regular medium at a density of $3x10^6$ to $5x10^6$ cells per dish and incubated at 37^oC in a 5% CO_2 environment. When the cells approached confluency (after 2 to 3 days), the medium was removed, the cells were washed twice with 5 mL of PBS, trypsinized, resuspended in regular medium and replated into several 100-mm-diameter dishes containing regular medium such that at least 50% of the initial population was retained. This was repeated as required, to allow further time for the expression of the AK^- phenotype, until the selection for 6-$MeMPR^r$ mutants was carried out.

The procedure employed for the selection of 6-$MeMPR^r$ mutants was similar to that used for other mutant selections. However, the conditions used here were optimized with respect to the concentration of the selective drug, the number of cells plated per dish and the minimum expression time necessary for obtaining the maximum mutagenic response

after mutagen treatment. In general, the selective drug concentrations were chosen such that virtually no background growth of sensitive cells was observed and cell concentrations (ie. the number of cells per dish) were chosen such that they allowed analysis of a maximum number of cells in a dish without adversely affecting the recovery or scoring of the mutant cells. In addition, the expression period represented the time subsequent to mutagen treatment when new plateaus of increased mutation frequency were reached. To select the 6-MeMPR^r mutants, 1.0 mL volumes of both the 6-MeMPR solution (twelve times the desired final concentration) and each different mutagen-treated cell suspension containing the desired number of cells ($1x10^6$ cells/ml), were added, in that order, to 100-mm-diameter dishes containing 10.0 mL of special medium. For the selection, ten to twelve dishes were set-up for each mutagen-treated culture and control non-mutagenized cells. Two hundred and fifty cells from each culture were also plated in triplicate in 60-mm-diameter dishes in 5 mL of regular medium without drug to determine the plating efficiency of the cells at the time of selection. All dishes were incubated at 37^oC in a 5% CO_2 atmosphere for 11 to 12 days during which time no change of medium or fresh addition of drug was carried out. Subsequently, the dishes were stained and aggregates of 40 or more cells were counted as

colonies. From the number of cells plated, the average number of mutant colonies observed per dish and the plating efficiency of the cells, the mutant frequency in each mutagenized and control population was calculated as follows:

$$\text{mutant frequency} = \frac{\text{mean number of mutant colonies per dish}}{\text{number of cells plated per dish} \times \text{plating efficiency of the cells}}$$

3. RESULTS AND DISCUSSION

3.1 Drug Resistance and Biochemical Studies of First-Step Hela Mutants Resistant to Toyocamycin, Tubercidin and 6-Methylmercaptopurine Riboside

As discussed in the Introduction, the pyrrolopyrimidine nucleoside antibiotics, toyocamycin and tubercidin, are cytotoxic to mammalian cells in culture and growth inhibitory to a wide variety of bacteria, fungi, parasites, DNA viruses and double and single-stranded RNA viruses (Ritch and Glazer, 1984). In addition, they have been shown to be effective against a wide variety of experimental tumors (Owen and Smith, 1964; Klein et. al., 1975). Similarly, the purine nucleoside analogue, 6-methylmercaptopurine riboside, is highly cytotoxic to a number of cultured cell lines (Koontz and Wicks, 1984) including cancer cells originating from different tumor-types (Bennett et al., 1965; Nelson and Parks, 1972).

Phosphorylation of natural nucleosides or nucleoside analogues is of critical importance in various biochemical, pharmacological and pathological situations. In this respect, because of the structural similarity between toyocamycin, tubercidin, 6-MeMPR and adenosine, these three nucleoside analogues are readily phosphorylated to their mono-, di- and triphosphates in cells (Acs et al., 1964;

Suhadolnik et al., 1967; Ross and Jaffe, 1972; Zimmerman et al., 1974). These phosphorylated derivatives are structurally similar to the adenine nucleotides (AMP, ADP and ATP) and take their place in a wide variety of cellular reactions which leads to cell death. At present, however, it is not clear whether the cytotoxicity caused by the toyocamycin, tubercidin and 6-MeMPR families of nucleotides results from the inhibition of, or interference with, one distinct cellular reaction or a cumulative effect on several cellular reactions.

Several studies in various systems have shown that it is possible to isolate first-step mutants highly resistant to toyocamycin, tubercidin and 6-MeMPR (Bennett et al., 1966; Gupta and Siminovitch, 1978b; Rabin and Gottesman, 1979; Thacker, 1980; Gupta and Singh, 1983; Gupta and Mehta, 1984; Yamanaka et al., 1984). Such mutants exhibit a high degree of cross-resistance to numerous other adenosine analogues activated by enzymic phosphorylation but differing in their mechanism of cellular toxicity. Further, it was found that these mutants possess greatly reduced (<1% relative to parental cells) levels of the activity of the purine salvage pathway enzyme, adenosine kinase (AK), which must phosphorylate these adenosine analogues before they become cytotoxic to cells. This implied that the mechanism of acquired resistance of these mutants was the result of an

alteration in the initial step required for the biosynthesis of lethal metabolites (that is, adenosine kinase catalyzed phosphorylation of the drug). Lastly, it was discovered that these mutants, which were in effect AK^-, still possessed the ability to phosphorylate adenosine at a rate that was between 5% and 15% of that exhibited by the parental sensitive cells (Gupta and Mehta, 1984). It was not clear whether the observed phosphorylation of adenosine by these mutants was the result of "residual" AK activity or some other cellular phosphorylating activity present in CHO cells.

Since most of the aforementioned work has been performed in cell systems other than those of human origin and since we eventually wanted to test the potential of the adenosine kinase locus serving as a genetic endpoint for quantitative mutagenesis studies in human cells (Section 3.3), the purpose of this work was to isolate HeLa mutants resistant to toyocamycin, tubercidin and 6-MeMPR and determine the mechanism of action of these nucleoside analogues in a human cell system. As such, efforts were made: a) to determine whether it was possible to select first-step HeLa mutants which were resistant to toyocamycin, tubercidin and 6-MeMPR, b) once mutants were isolated, to determine whether they possessed a high degree of resistance to their selective adenosine analogue as well as cross-

resistance toward other adenosine analogues, c) to determine the level(s) of AK activity exhibited by these mutants relative to that demonstrated by the parental cell line and d) to determine the degree of adenosine uptake shown by these mutants.

This section describes the successful isolation and partial characterization of first-step HeLa mutants resistant to toyocamycin, tubercidin and 6-MeMPR. The results suggest that in human cells the mechanism of action of and characteristics of resistance toward these adenosine analogues parallels those observed in other cell types and involves the cellular phosphorylating enzyme, adenosine kinase.

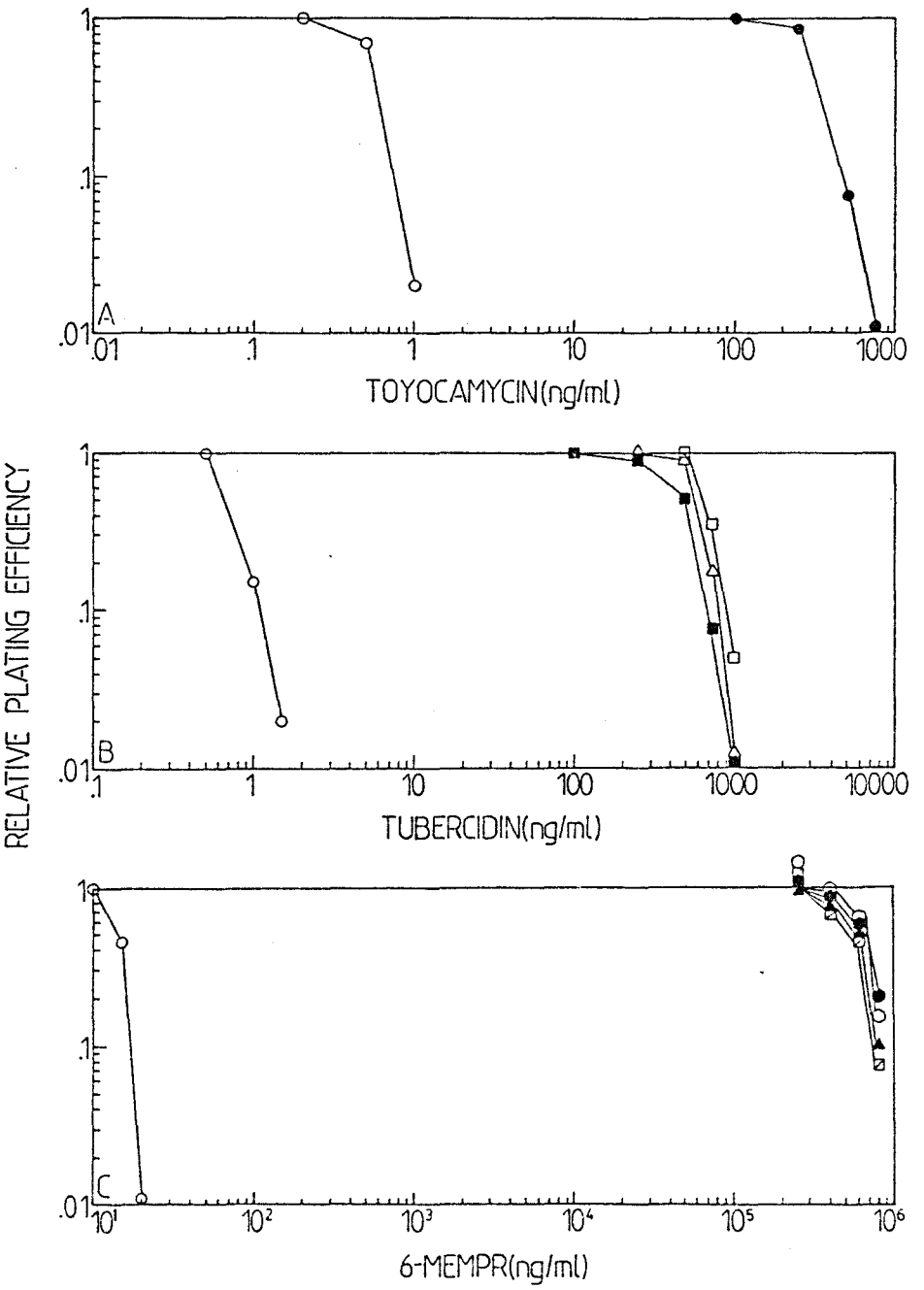
3.1.1 Results

3.1.1.1 Selection of First-Step Mutants from HeLa Cells Using Increased Concentrations of Toyocamycin, Tubercidin and 6-Methylmercaptopurine Riboside

Before selecting any mutants resistant to toyocamycin, tubercidin or 6-methylmercaptopurine riboside (6-MeMPR), the sensitivity of the HeLa cells to each of these drugs was determined. Figure 4 illustrates the effect of increasing concentrations of each adenosine analogue on the relative plating efficiency of HeLa cells. The D_{10} values of toyocamycin, tubercidin and 6-MeMPR for HeLa cells were 0.74 ng/mL, 1.1 ng/mL and 17.0 ng/mL respectively (Table 4).

Figure 4. Dose-response curves of the parental sensitive cells and the mutants selected as toyocamycin, tubercidin or 6-methylmercaptopurine riboside resistant toward increasing concentrations of the adenosine analogue against which they were selected.
(A) Toyocamycin
(B) Tubercidin
(C) 6-Methylmercaptopurine riboside

○, HeLa cells;
●, TOY^{rI}-2;
□, TUB^{rI}-2;
■, TUB^{rI}-4;
△, TUB^{rI}-5;
▲, 6-$MeMPR^{rI}$-1;
⬡, 6-$MeMPR^{rI}$-2;
⬢, 6-$MeMPR^{rI}$-3;
▨, 6-$MeMPR^{rI}$-4.


RELATIVE PLATING EFFICIENCY
1
.1
.01
A
.01
.1
1
10
100
1000
TOYOCAMYCIN(ng/ml)
B
.1
1
10
100
1000
10000
TUBERCIDIN(ng/ml)
C
10^1
10^2
10^3
10^4
10^5
10^6
6-MEMPR(ng/ml)

Previously, it has been shown that stable CHO mutants exhibit a high degree of resistance (100-to 1000-fold) to these nucleoside analogues (Gupta and Siminovitch, 1978b; Gupta and Singh, 1983). Assuming that similar characteristics would be exhibited by HeLa cell mutants, the drug concentrations used for mutant selection were in large excess (at least 50-fold) of the low drug concentrations to which the HeLa cells were sensitive. The selection of first-step mutants was carried out by plating mutagen-treated (300 µg/mL of EMS for 20 hours in the case of the TOY^{rI} and TUB^{rI} mutants or UV-exposure for 10 seconds in the case of the 6-$MeMPR^{rI}$ mutants; both plated on the eighth day subsequent to mutagen treatment) cultures of HeLa cells in the presence of 50 ng/mL, 500 ng/mL and 5 µg/mL of toyocamycin, tubercidin and 6-MeMPR, respectively. Drug resistant clones to each of these nucleoside analogues were obtained at a similar frequency of approximately $1x10^{-6}$. One toyocamycin resistant clone (TOY^{rI}-2), three tubercidin resistant clones (TUB^{rI}-2, TUB^{rI}-4 and TUB^{rI}-5) and four 6-MeMPR resistant clones (6-$MeMPR^{rI}$-1, 6-$MeMPR^{rI}$-2, 6-$MeMPR^{rI}$-3 and 6-$MeMPR^{rI}$-4) were harvested, grown in non-selective medium and tested for their degree of resistance to their respective selective agent. Figure 4 illustrates the dose-response curves of each type of mutant toward increasing concentrations of each adenosine analogue. Similar results

Table 4. Drug Resistance Patterns of the Toyocamycin, Tubercidin and 6-Methylmercaptopurine Riboside Resistant Mutants Toward Their Selective Nucleoside Analogue

Nucleoside Analogue	D_{10} value of the analogue for HeLa cells[a] (ng/mL)	Mutant cell line	D_{10} value of the analogue for the mutant[a] (ng/mL)	Relative resistance of the mutant cell line[b]
Toyocamycin	0.74	TOY^{rI}-2	460	620
Tubercidin	1.10	TUB^{rI}-2	900	820
		TUB^{rI}-4	700	640
		TUB^{rI}-5	800	730
6-MeMPR	17.0	6-$MeMPR^{rI}$-1	8.0×10^{5}	4.7×10^{4}
		6-$MeMPR^{rI}$-2	$>8.0\times10^{5}$	$>4.7\times10^{4}$
		6-$MeMPR^{rI}$-3	$>8.0\times10^{5}$	$>4.7\times10^{4}$
		6-$MeMPR^{rI}$-4	7.2×10^{5}	4.2×10^{4}

[a] The D_{10} value of the drug toward the cell line represents the concentration of the analogue which reduces the plating efficiency of the cells to 10% of that observed in the absence of any drug.

[b] Assuming the D_{10} value of the analogue toward HeLa cells to be 1, the relative degrees of resistance of the mutant cell lines were determined from the ratios of the D_{10} values of the mutants compared to that of the HeLa cells.

for all the mutants have been obtained in several independent experiments.

As illustrated in Figure 4A, TOY^{rI}-2 exhibited a high degree of resistance towards toyocamycin. This mutant was resistant up to approximately 250 ng/mL toyocamycin but at higher concentrations its plating efficiency decreased sharply. Based on a comparison of the D_{10} values (Table 4), the TOY^{rI}-2 mutant was greater than 600-fold more resistant to toyocamycin than the parental (sensitive) HeLa cells. The three tubercidin resistant mutants (Figure 4B) and the four 6-MeMPR resistant mutants (Figure 4C) also exhibited high degrees of resistance to tubercidin and 6-MeMPR, respectively. Each of the tubercidin mutants was resistant to a concentration of 500 ng/mL tubercidin and the 6-MeMPR mutants were resistant to 6-MeMPR at a concentration of $4x10^5$ ng/mL. In this respect, the relative degrees of resistance of the tubercidin mutants ranged from 600- to 800-fold whereas the 6-MeMPR mutants were greater than $4x10^4$-fold resistant to 6-MeMPR (Table 4).

The drug resistant phenotype of all of these mutants was found to be stably retained upon growth in non-selective medium for greater than one year.

These studies indicate that stable mutants, selected at a similar frequency ($1x10^{-6}$) from a mutagen-treated population of cells, exhibit a high degree (600- to $4x10^4$-

fold) of resistance to the nucleoside analogues: toyocamycin, tubercidin and 6-MeMPR.

3.1.1.2 Cross-Resistance Patterns of the First-Step Mutants Toward Various Nucleoside and Base Analogues

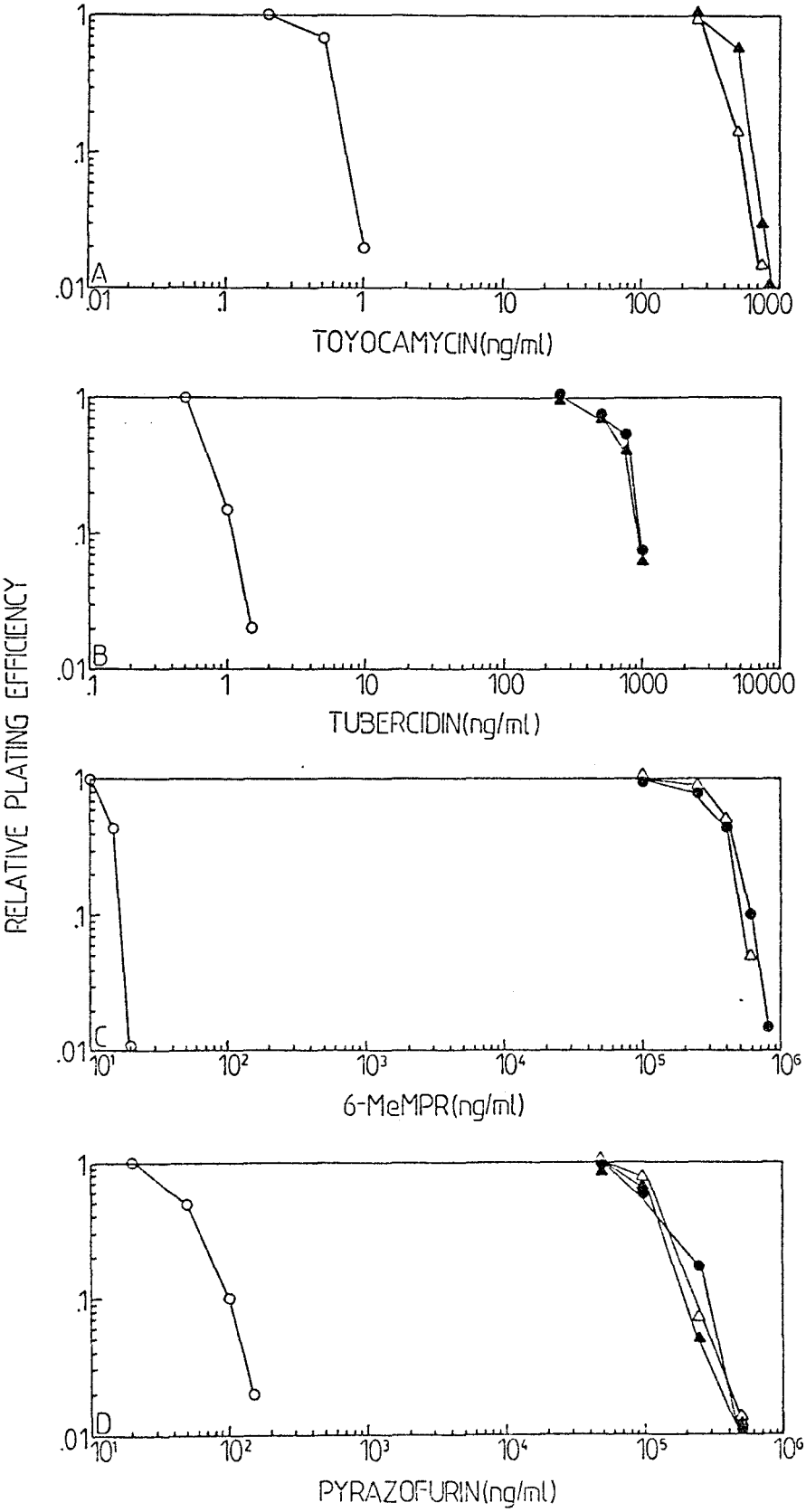
In an effort to determine whether the first-step HeLa mutants resistant to toyocamycin, tubercidin and 6-MeMPR possessed similar patterns of drug resistance, these mutants were tested for their degree of cross-resistance toward nucleoside or base analogues other than the one against which they were initially selected. For this purpose, the relative plating efficiency of each cell line, in the presence of increasing concentrations of seven different anticancer agents, was determined. The drugs employed were: the two pyrrolopyrimidine ribosides, toyocamycin and tubercidin, the adenosine analogues, 6-MeMPR and 6-mercaptopurine riboside (6-MPR), the C-ribonucleoside, pyrazofurin and the purine base analogues, 6-thioguanine and 8-azaadenine. As previously mentioned, toyocamycin, tubercidin and 6-MeMPR are phosphorylated to their respective monophosphate (which is either toxic itself or is a precursor of the toxic di- and triphosphates) via adenosine kinase (Lindberg et al., 1967; Schnebli et al., 1967; Bennett et al., 1966). Similarly, adenosine kinase is responsible for the lethal conversion of pyrazofurin to its toxic 5'-phosphate derivative (Dix et al., 1979). Thus,

those HeLa mutants determined to be resistant to toyocamycin, tubercidin or 6-MeMPR, and therefore possibly defective in adenosine kinase activity, should exhibit cross-resistance to the other AK-activated nucleoside analogues. In contrast, resistance to 6-thioguanine and 6-MPR results from loss or deficiency in the activity of the purine salvage pathway enzyme, hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Bennett et al., 1965; Beaudet et al., 1973; Caskey and Kruh, 1979). Also, mutants resistant to 8-azaadenine are deficient in an alternative purine salvage enzyme, adenine phosphoribosyltransferase (APRT) (Jones and Sargent, 1974; Chasin, 1974). Toyocamycin, tubercidin and 6-MeMPR resistant mutants, which may be affected in AK, were not expected to exhibit any degree of cross-resistance to 6-thioguanine, 6-MPR or 8-azaadenine.

The data presented in Figure 5 illustrates the dose-response curves for the cross-resistance patterns exhibited by one representative toyocamycin, tubercidin or 6-MeMPR resistant mutant toward increasing concentrations of AK-activated nucleoside analogues. Table 5 reveals the cross-resistance data obtained for all mutants in the presence of AK-activated nucleoside analogues as well as in the presence of nucleoside or base analogues activated by other purine salvage enzymes. Similar cross-resistance patterns were exhibited by these mutant cell lines in at

Figure 5. Dose-response curves illustrating the cross-resistance patterns of one representative toyocamycin, tubercidin or 6-methylmercaptopurine riboside resistant mutant toward increasing concentrations of various AK-activated nucleoside analogues.
(A) Toyocamycin
(B) Tubercidin
(C) 6-Methylmercaptopurine riboside
(D) Pyrazofurin

○, HeLa cells;
●, TOY^{rI}-2;
△, TUB^{rI}-2;
▲, $6\text{-}MeMPR^{rI}$-1.


RELATIVE PLATING EFFICIENCY
1
.1
.01
A
.01
.1
1
10
100
1000
TOYOCAMYCIN(ng/ml)
B
.1
1
10
100
1000
10000
TUBERCIDIN(ng/ml)
C
10^1
10^2
10^3
10^4
10^5
10^6
6-MeMPR(ng/ml)
D
PYRAZOFURIN(ng/ml)

least two independent experiments.

As can be seen in Figure 5A, parental (sensitive) HeLa cells were resistant only up to a concentration of 0.35 ng/mL toyocamycin. In contrast, both the TUB^{rI}-2 and 6-MeMPR^{rI}-1 mutants were resistant to toyocamycin up to a concentration of 280 and 350 ng/mL, respectively. The high degree of cross-resistance exhibited by TOY^{rI}-2 and 6-MeMPR^{rI}-1 toward toyocamycin was characteristic of all the HeLa tubercidin and 6-MeMPR resistant mutants. In this respect, the relative degree of resistance toward toyocamycin ranged from 500- to 700-fold for the tubercidin resistant mutants and from 600- to 1000-fold for the 6-MeMPR resistant mutants (Table 5).

Figures 5B, 5C and Table 5 reveal similar cross-resistance patterns for all the mutants toward tubercidin and 6-MeMPR. The TOY^{rI}-2 mutant, which was resistant to tubercidin at a concentration of 400 ng/mL, exhibited a relative degree of resistance greater than 800-fold. This same mutant was resistant up to a concentration of 2.5×10^5 ng/mL 6-MeMPR and therefore displayed a relative degree of resistance toward this drug of greater than 35,000-fold. By comparison, the 6-MeMPR^{rI}-1 mutant, which was resistant to tubercidin at a concentration of 400 ng/mL, was characteristic of all the 6-MeMPR resistant mutants. Collectively, they exhibited a degree of resistance toward

Table 5. Cross Resistance Patterns of Toyocamycin, Tubercidin and 6-Methylmercaptopurine Riboside Resistant Mutants Toward Various Nucleoside and Base Analogues

Nucleoside or base analogue	D_{10} value of the analogue for HeLa cells[a]	Mutant cell line	D_{10} value of the analogue for the mutant[a]	Relative resistance of the mutant cell line[b]
Toyocamycin	0.74 ng/mL	TUB^{rI}-2	540 ng/mL	730
		TUB^{rI}-4	350	470
		TUB^{rI}-5	400	540
		6-$MeMPR^{rI}$-1	640	865
		6-$MeMPR^{rI}$-2	460	620
		6-$MeMPR^{rI}$-3	640	865
		6-$MeMPR^{rI}$-4	780	1055
Tubercidin	1.10 ng/mL	TOY^{rI}-2	940 ng/mL	855
		6-$MeMPR^{rI}$-1	920	840
		6-$MeMPR^{rI}$-2	700	640
		6-$MeMPR^{rI}$-3	900	820
		6-$MeMPR^{rI}$-4	880	800
6-MeMPR	17.0 ng/mL	TOY^{rI}-2	6.0×10^{5} ng/mL	3.5×10^{4}
		TUB^{rI}-2	5.3×10^{5}	3.1×10^{4}
		TUB^{rI}-4	4.7×10^{5}	2.8×10^{4}
		TUB^{rI}-5	5.3×10^{5}	3.1×10^{4}

Nucleoside or base analogue	D_{10} value of the analogue for HeLa cells[a]	Mutant cell line	D_{10} value of the analogue for the mutant[a]	Relative resistance of the mutant cell line[b]
Pyrazofurin	100.0 ng/mL	TOY^{rI}-2	$2.9x10^5$ ng/mL	$2.9x10^3$
		TUB^{rI}-2	$2.3x10^5$	$2.3x10^3$
		TUB^{rI}-4	$1.9x10^5$	$1.9x10^3$
		TUB^{rI}-5	$2.2x10^5$	$2.2x10^3$
		6-$MeMPR^{rI}$-1	$2.0x10^5$	$2.0x10^3$
		6-$MeMPR^{rI}$-2	$2.3x10^5$	$2.3x10^3$
		6-$MeMPR^{rI}$-3	$2.8x10^5$	$2.8x10^3$
		6-$MeMPR^{rI}$-4	$1.4x10^5$	$1.4x10^3$
6-MPR	82.0 ng/mL	TOY^{rI}-2	50 ng/mL	0.6
		TUB^{rI}-2	36	0.4
		TUB^{rI}-4	18	0.2
		6-$MeMPR^{rI}$-1	68	0.8
		6-$MeMPR^{rI}$-2	66	0.8
6-Thio-guanine	$2.9x10^{-7}$ M	TOY^{rI}-2	$1.5x10^{-7}$ M	0.5
		TUB^{rI}-2	$1.6x10^{-7}$	0.6
		TUB^{rI}-4	$<1.0x10^{-7}$	<0.3
		6-$MeMPR^{rI}$-1	$1.6x10^{-7}$	0.6
		6-$MeMPR^{rI}$-2	$1.9x10^{-7}$	0.7

Nucleoside or base analogue	D_{10} value of the analogue for HeLa cells[a]	Mutant cell line	D_{10} value of the analogue for the mutant[a]	Relative resistance of the mutant cell line[b]
8-Aza-	2.8 μg/mL	TOY^{rI}-2	2.6 μg/mL	0.9
adenine		TUB^{rI}-2	2.5	0.9
		TUB^{rI}-4	1.7	0.6
		6-$MeMPR^{rI}$-1	2.7	1.0
		6-$MeMPR^{rI}$-2	2.6	0.9

[a,b] The D_{10} values of nucleoside or base analogues toward the HeLa cells and the mutant cell lines, as well as the relative resistance of the mutant cell lines, were determined as described in the legend of Table 2.

tubercidin which was 700- to 900-fold greater than that of the parental HeLa cells. Similarly, the TUB^{rI} family of mutants was highly resistant to 6-MeMPR and, in general, these mutants exhibited relative degrees of resistance greater than 30,000-fold.

The cross-resistance patterns exhibited by all mutants toward pyrazofurin correlated well with data obtained for cross-resistance toward toyocamycin, tubercidin and 6-MeMPR. As can be seen in Figure 5D, HeLa cells were only resistant to pyrazofurin at a concentration of 25 ng/mL. In comparison, TOY^{rI}-2, TUB^{rI}-2 and 6-$MeMPR^{rI}$-1 were resistant to $6.5x10^4$, $1x10^5$ and $7.5x10^4$ ng/mL pyrazofurin, respectively, and were therefore highly resistant to this C-ribonucleoside. Based on a comparison of the D_{10} values of pyrazofurin for these cell lines (Table 5), the relative degrees of resistance of the toyocamycin, tubercidin and 6-MeMPR resistant families of mutants were, in general, quite high and ranged from 1500- to 3000-fold.

Taken collectively, the relative degrees of resistance exhibited by these mutants toward toyocamycin, tubercidin, 6-MeMPR or pyrazofurin confirm that mutants selected as toyocamycin, tubercidin or 6-MeMPR resistant are highly cross-resistant to other AK-activated nucleoside analogues.

The data illustrating the cross-resistance patterns of selected toyocamycin, tubercidin and 6-MeMPR resistant mutants toward those drugs (6-MPR, 6-thioguanine and 8-azaadenine) activated by purine salvage pathway enzymes other than AK is presented in Table 5. The D_{10} value of 6-MPR toward the parental HeLa cells was 82 ng/mL. By comparison, the D_{10} values of 6-MPR toward TOY^{rI}-2, TUB^{rI}-2 and 4, and 6-$MeMPR^{rI}$-1 and 2 were lower and as a result, the relative degrees of resistance ranged from 0.2- to 0.8-fold. This indicated that these mutants were no more resistant to 6-MPR than the parental HeLa cells. As expected, the observed degree of cross-resistance to 6-thioguanine was quite similar to 6-MPR (since 6-thioguanine is also HGPRT-activated). Compared to the D_{10} value of 2.9×10^{-7} M 6-thioguanine toward HeLa cells, the D_{10} values for the mutants ranged from less than 1.0×10^{-7} M to 1.9×10^{-7} M 6-thioguanine indicating that these mutants were not cross-resistant to this drug. 8-Azaadenine, which is APRT-activated, was the least toxic of all the nucleoside or base analogues studied having a D_{10} toward HeLa cells of 2.8 µg/mL. In general, each of the toyocamycin, tubercidin and 6-MeMPR resistant mutants displayed a D_{10} value for 8-azaadenine similar to that observed for the parental Hela cells. As such, the relative degree of resistance for each of the mutants was close to 1.0.

These results verify that mutants selected as toyocamycin, tubercidin or 6-MeMPR resistant exhibit specific cross-resistance patterns toward certain other AK-activated N- and C-nucleoside analogues but do not display any degree of cross-resistance toward other nucleoside or base analogues that are activated by purine salvage pathway enzymes other than adenosine kinase. In addition, the results of these studies show that, even though all of the mutants exhibit high degrees of resistance to all of the AK-activated nucleoside analogues, their individual phenotype is unique since small differences in the degrees of resistance are observed among mutants resistant to any of these analogues.

3.1.1.3 Adenosine Kinase (AK) Activity in the Cell Extracts of Toyocamycin, Tubercidin and 6-MeMPR Resistant Mutants

Previous studies concerned with the mechanism responsible for the toxic effects elicited by nucleoside analogues have shown that initial phosphorylation of most analogues is essential for cellular lethality. Consequently, mutants resistant to nucleoside analogues exhibit this phenotype as a result of an alteration in the activity of cellular phosphorylating enzymes (Suhadolnik, 1970; Clements, 1975). With respect to the three adenosine analogues involved in this work, it is known that mutants

resistant to normally toxic concentrations of toyocamycin, tubercidin or 6-MeMPR are deficient in or possess an altered activity of adenosine kinase, the enzyme responsible for the conversion of these nucleosides to the mono-phosphorylated form (Bennett et al., 1965; Lindberg et al., 1967; Schnebli et al., 1967; Scholar et al., 1972; McBurney and Whitmore, 1974; Gupta and Siminovitch, 1978b; Gupta and Singh, 1983; Yamanaka et al. 1984). In view of this, it was hypothesized that HeLa mutants resistant to toyocamycin, tubercidin or 6-MeMPR were also defective in adenosine kinase activity. Therefore, the specific activity of adenosine kinase in cell extracts derived from each of the mutant cell lines (and the parental HeLa cells) was determined as outlined in the Methods (Section 2.2.5). Figure 6 graphically illustrates the AK activities present in the extracts of HeLa cells and one representative toyocamycin, tubercidin and 6-MeMPR resistant mutant. Table 6 presents both the absolute and relative AK activity exhibited by all the mutant cell lines. Similar results regarding the AK activities present in the extracts of all of the mutant cell lines were obtained in at least two independent experiments.

As illustrated in Figure 6, the parental HeLa cells phosphorylated (^{3}H)-adenosine in a linear, time-dependent manner for the duration of the assay (120 minutes) and were clearly adenosine kinase activity positive (AK^{+}). By

comparison, however, the TOY^{rI}-2, TUB^{rI}-2 and 6-$MeMPR^{rI}$-1 mutants exhibited extremely low levels of phosphorylation of (^{3}H)-adenosine. In each case, the maximum amount of AK activity exhibited by the mutants was <12% of that exhibited by the parental Hela cells after a 15 minute incubation period and <2% after a 2 hour incubation period (Table 6). Linear regression analysis of the profile of AK activity present in the TOY^{rI}-2, TUB^{rI}-2 and 6-$MeMPR^{rI}$-1 mutant extracts yielded slopes of 0.0061, 0.0075 and 0.0086 nmoles of nucleotide/mg of protein, respectively (the corresponding correlation coefficients were 0.65, 0.62 and 0.77). As such, none of these three mutants showed any significant time-dependent increase in the amount of (^{3}H)-adenosine phosphorylated. This observation was characteristic of all the toyocamycin, tubercidin and 6-MeMPR resistant mutants. Table 6 reveals that the maximum AK activity, relative to HeLa cells, displayed by any of the tubercidin resistant mutants was <2% after a 2 hour incubation period. Similarly, all the 6-MeMPR resistant mutants showed relative AK activities <2% of that exhibited by HeLa cells after 2 hours. Further, for all mutants studied, there was no significant time-dependent increase in the level of phosphorylation of (^{3}H)-adenosine since all activity profiles portrayed slope values (determined by linear regression) which ranged from 0 to 0.0086 nmoles of

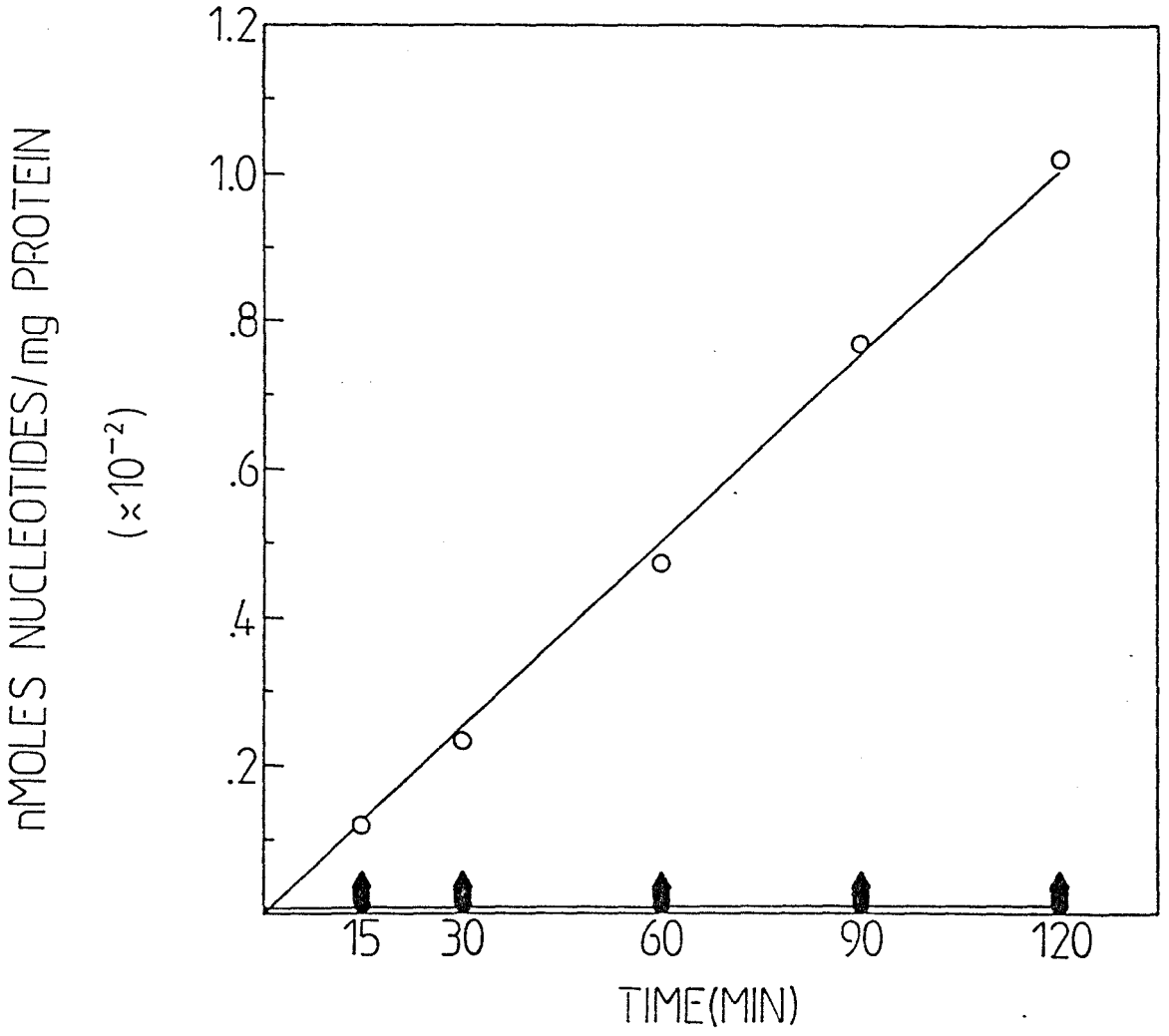


Figure 6. Adenosine kinase activity in the extracts of HeLa cells and one mutant representing each of the toyocamycin, tubercidin and 6-methylmercapto-purine riboside resistant cell lines. The cell extracts used for the assay were prepared by sonication followed by centrifugation at 30,000 x g. The final concentration of (^{3}H)-adenosine (specific activity, 42 Ci/mmole) in the reaction mixture was 4×10^{-5} M. AK activity was quantitated by determining the specific activity of AK at various time intervals for each extract as described in the Methods.

○ , HeLa cells;
■ , TOY^{rI}-2;
▲ , TUB^{rI}-2;
● , 6-MeMPR^{rI}-1.

Table 6. Adenosine Kinase Activity in the Extracts of Parental and Mutant Cell Lines

Cell line	Duration of the assay (min)	nmoles of nucleotide/mg of protein[a] ($\times 10^{-4}$)	Activity relative to Hela cells[b] (%)
HeLa	15	11.91	100.0
	30	23.31	100.0
	60	46.97	100.0
	90	76.69	100.0
	120	102.06	100.0
$TOY^{rI}{}_{-2}$	15	1.29	10.8
	30	0.88	3.8
	60	1.76	3.7
	90	1.88	2.5
	120	1.59	1.6
$TUB^{rI}{}_{-2}$	15	1.13	9.5
	30	2.30	9.9
	60	2.22	4.7
	90	2.35	3.1
	120	2.26	2.2

Cell line	Duration of the assay (min)	nmoles of nucleotide/mg of protein[a] ($x10^{-4}$)	Activity relative to HeLa cells[b] (%)
TUB^{rI}-4	15	1.39	11.7
	30	0.78	3.3
	60	1.17	2.5
	90	1.39	1.8
	120	1.52	1.5
TUB^{rI}-5	15	1.17	9.8
	30	1.39	6.0
	60	1.35	2.9
	90	2.13	2.8
	120	1.43	1.4
6-$MeMPR^{rI}$-1	15	1.00	8.4
	30	0.82	3.5
	60	1.68	3.6
	90	1.95	2.5
	120	1.59	1.6
6-$MeMPR^{rI}$-2	15	1.41	11.8
	30	0.68	2.9
	60	1.36	2.9
	90	1.36	1.8
	120	1.36	1.3

Cell line	Duration of the assay (min)	nmoles of nucleotide/mg of protein[a] ($x10^{-4}$)	Activity relative to HeLa cells[b] (%)
6-MeMPR^{rI}-3	15	1.36	11.4
	30	0.86	3.7
	60	1.45	3.1
	90	1.77	2.3
	120	1.27	1.2
6-MeMPR^{rI}-4	15	1.32	11.1
	30	0.68	2.9
	60	1.27	2.7
	90	1.18	1.5
	120	0.82	0.8

AK activity was quantitated by determining the specific activity of AK at various time intervals for each extract as described in the Methods.

[a] The values presented represent the mean of duplicate assays. The standard error was calculated for the counts per minute of lanthanum chloride precipitable AMP and was found to be less than 10% in all cases. As such, the error in the specific activity of AK at each time point was negligible and omitted from the table.

[b] Assuming the specific activity of AK in HeLa cells to be 100, the relative specific activity of AK in the mutant cell lines was calculated as the ratio of activity in the mutant cells to that of HeLa cells.

nucleotide/mg of protein.

To eliminate the possibility that the extracts derived from each of the mutant cell lines contained an inhibitor of AK activity, cell extracts from the sensitive parental (AK^+) HeLa cells and two representative resistant (AK^-) mutants (TUB^{rI}-2 and 6-$MeMPR^{rI}$-3) were mixed in different proportions (1:1 or 1:2, parent cell:mutant cell extract) and the AK activity determined. As illustrated in Table 7, the parental HeLa cells exhibited a linear, time-dependent increase in the phosphorylation of (^3H)-adenosine. In contrast, the TUB^{rI}-2 and 6-$MeMPR^{rI}$-3 mutant extracts each showed AK activities that were 3.2 and 2.6% respectively of that demonstrated by the HeLa cells after 60 minutes. Furthermore, mixing HeLa cell and TUB^{rI}-2 or 6-$MeMPR^{rI}$-3 mutant extracts, in either equal (1:1) or excess (1:2) proportions, resulted in an observed AK activity that closely approximated that expected from the relative amount of the HeLa cell extract present in the mixture. Thus, these studies exclude the possible presence of an inhibitor of AK activity in the mutant cell extracts.

In summary, all mutants show less than 2% of the AK activity exhibited by parental HeLa cells and are therefore considered adenosine kinase activity minus (AK^-). A similar reduction in AK activity in mutants resistant to toyocamycin, tubercidin and 6-MeMPR has been observed in

Table 7. The Effect of Mutant Cell Extracts on the AK Activity of Parental Cell Extracts

Cell line	Duration of the assay (min)	nmoles of (^{3}H)-AMP produced ($\times 10^{-5}$)	Activity relative to HeLa cells (%)
HeLa	20	11.60	100.0
	40	21.04	100.0
	60	34.40	100.0
TUB^{rI}-2	20	0.58	5.0
	40	0.69	3.3
	60	1.10	3.2
6-$MeMPR^{rI}$-3	20	0.29	2.5
	40	0.23	1.1
	60	0.89	2.6
HeLa + TUB^{rI}-2	20	13.48/13.32	116.2/114.8
(1:1)/(1:2)	40	23.16/23.70	110.1/112.6
	60	38.16/37.40	110.9/108.7
HeLa + 6-$MeMPR^{rI}$-3	20	13.56/13.72	116.9/118.3
(1:1)/(1:2)	40	23.75/23.76	112.9/112.9
	60	37.16/37.80	108.0/109.9

The amount of HeLa cell extract present in each assay was constant. Any possible effect of the mutant cell extract on the AK activity of the HeLa cell extract was tested by adding an equivalent amount (a 1:1 ratio) or twice as much (a 1:2 ratio) of the mutant cell extract. The nmoles of (^{3}H)-AMP produced were quantitated as described in the Methods.

Footnote to Table 7 (cont'd).

[a] The values presented represent the mean of duplicate assays. The standard error calculated for the counts per minute of lanthanum chloride precipitable AMP was found to be less than 10% in every case. As such, the error in the nmoles of (^{3}H)-AMP produced at each time point was negligible and omitted from the table.

[b] Assuming the nmoles of (^{3}H)-AMP produced by the HeLa cell extract to be 100, the relative activity of AK in the mutant or combined HeLa/mutant extracts was calculated as the ratio of activity in the mutant or combined HeLa/mutant extracts to that of HeLa cells.

numerous other cell systems, including those of human origin (Bennett et al., 1966; Yamanaka et al., 1984).

3.1.1.4 (^{3}H)-Adenosine Uptake and Incorporation into Macromolecules by the Mutant Cell Lines

As determined in Section 3.1.1.2, similar cross-resistance patterns were exhibited by all the toyocamycin, tubercidin and 6-MeMPR resistant mutants toward AK-activated nucleoside analogues. This implied that a similar genetic lesion was probably present in each of these mutants. Considering the mechanisms of cellular toxicity elicited by most nucleoside analogues, and the resultant mode of resistance exhibited by various mutants (Suhadolnik, 1970; Clements, 1975), the initial phosphorylating enzyme, adenosine kinase, was likely to be the affected cellular component. Examination of the *in vitro* AK activity present in cell extracts from each mutant revealed that <2% of the AK activity exhibited by parental HeLa cells was present. As such, the genetic lesion present in each of these mutants drastically decreased AK activity and the high degrees of resistance of these mutants toward toyocamycin, tubercidin and 6-MeMPR were likely the result of decreased cellular uptake and incorporation of these nucleosides *in vivo*. This possibility was tested by determining both the *in vivo* uptake and incorporation into macromolecules of (^{3}H)-adenosine by each of the toyocamycin, tubercidin and 6-MeMPR

resistant cell lines. The results of these studies are shown in Figure 7, which illustrates the total uptake (A) and incorporation into macromolecules (B) of (^{3}H)-adenosine by the HeLa, TOY^{rI}-2, TUB^{rI}-2 and 6-MeMPR^{rI}-1 mutant cell lines and Table 8, which shows both the absolute and relative uptake and incorporation into macromolecules of (^{3}H)-adenosine by all the mutants. Similar results for the total uptake and incorporation of (^{3}H)-adenosine by each of the resistant mutants were obtained in at least two independent experiments.

In Figure 7A, the time course of (^{3}H)-adenosine uptake by HeLa cells appeared to be biphasic. Initially (for 15 to 20 minutes), there was a fast rate of uptake of (^{3}H)-adenosine which was followed by a second, slower rate for the remainder of the assay. Figure 7B illustrates that the incorporation of (^{3}H)-adenosine into macromolecules exhibited a lag period compared to the total uptake of (^{3}H)-adenosine. For the initial 40 minutes, there appeared to be a steady, time-dependent rate of (^{3}H)-adenosine incorporation, however, this rate was distinctly slower than, and occurred subsequent to, that seen for total (^{3}H)-adenosine uptake. By comparison, the uptake and incorporation into macromolecules of (^{3}H)-adenosine by the TOY^{rI}-2, TUB^{rI}-2 and 6-MeMPR^{rI}-1 mutants were both greatly

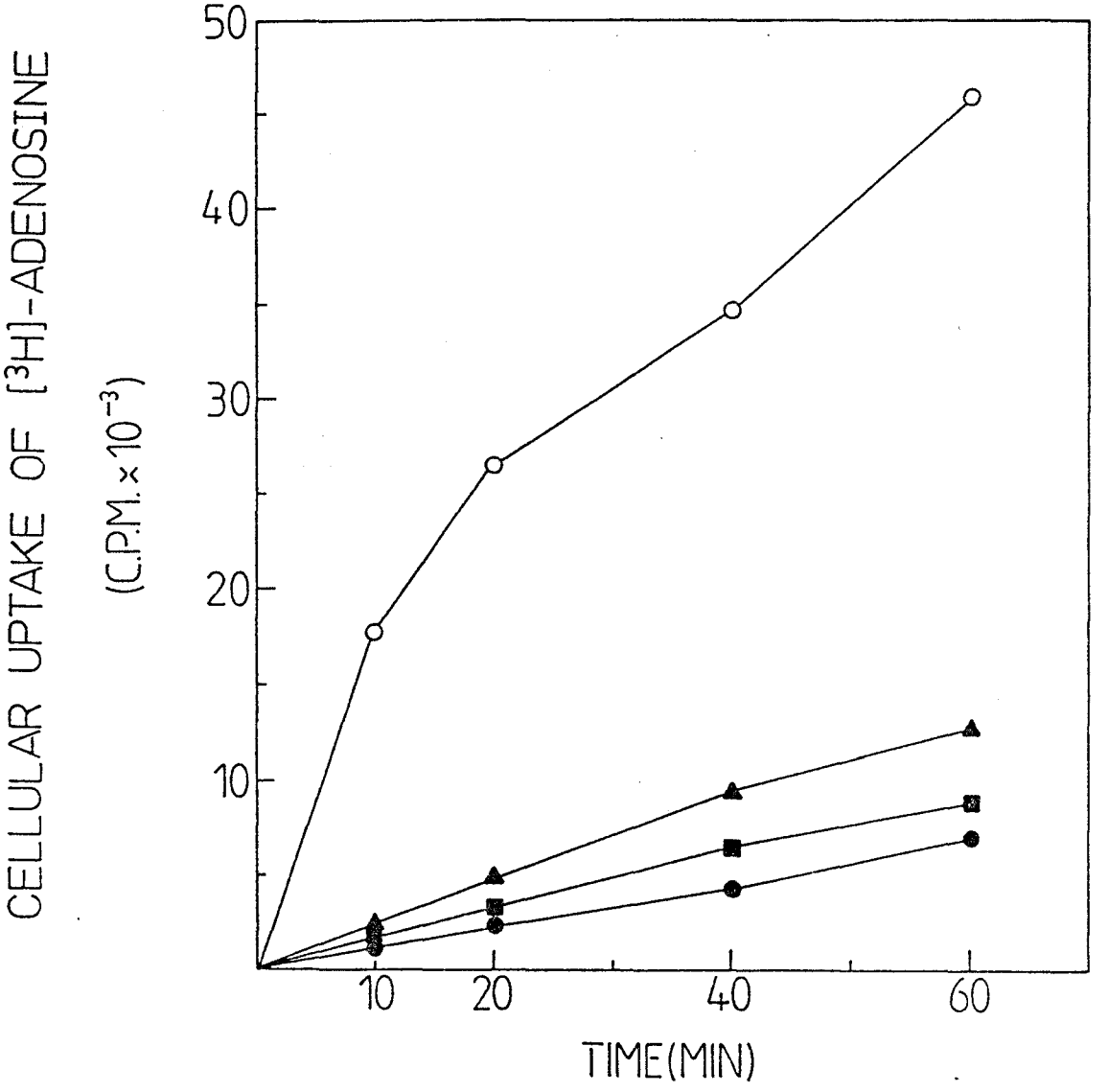


Figure 7. Uptake and incorporation of (^{3}H)-adenosine into macromolecules by HeLa cells and one mutant representing each of the toyocamycin, tubercidin and 6-methylmercaptopurine riboside resistant cell lines. Quantitation of the uptake of (^{3}H)-adenosine was achieved by determining the total amount of radioactivity taken up by the cells. Incorporation of (^{3}H)-adenosine into macromolecules was quantitated by determining the radioactivity present in a TCA-precipitable fraction. The final concentration of (^{3}H)-adenosine (specific activity, 42 Ci/mmole) in the medium was 1 µCi/mL as described in the Methods. A) Uptake of (^{3}H)-adenosine by:
○, HeLa cells; ■, TOY^{rI}-2; ▲, TUB^{rI}-2;
●, 6-MeMPR^{rI}-1.

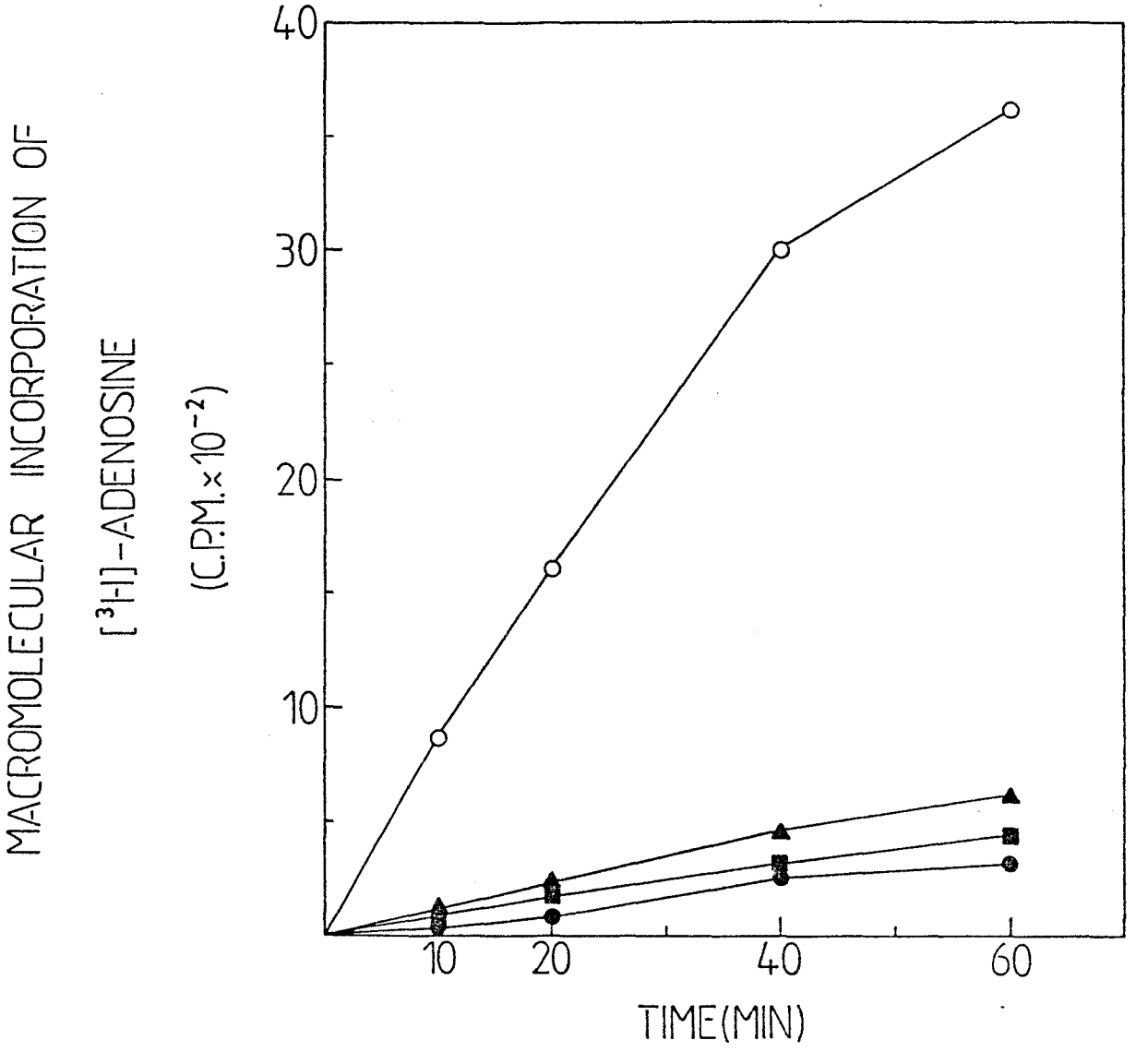


Figure 7. B) Incorporation of (^{3}H)-adenosine into macromolecules by: ○, HeLa cells;
■, TOY^{rI}-2;
▲, TUB^{rI}-2;
●, 6-MeMPR^{rI}-1.

reduced relative to that demonstrated by HeLa cells. TUB^{rI}-2 exhibited the greatest degrees of uptake and incorporation into macromolecules which, after 60 minutes, were 28.0% and 17.6%, respectively, of that shown by HeLa cells. TOY^{rI}-2 showed less (^{3}H)-adenosine uptake and incorporation (the values relative to HeLa cells were 19.2% and 12.6%, respectively) than TUB^{rI}-2 but it was the 6-$MeMPR^{rI}$-1 mutant that exhibited the least degree of either uptake or incorporation with the relative values being 15.3% and 9.1% of that shown by HeLa cells.

The (^{3}H)-adenosine uptake and incorporation exhibited by the TOY^{rI}-2, TUB^{rI}-2 and 6-$MeMPR^{rI}$-1 mutants was characteristic of all the mutants of each type. Table 8 reveals that the family of tubercidin resistant mutants all exhibited degrees of (^{3}H)-adenosine uptake and incorporation into macromolecules which were less than 28.0% and 17.6%, respectively, of that exhibited by HeLa cells. Similarly, the 6-MeMPR resistant mutants all showed values below 15.3% and 10.3% for the relative degrees of uptake and incorporation of (^{3}H)-adenosine, respectively.

These results indicate that these mutants, which are highly resistant to adenosine analogues and possess <2% of the AK activity of the parental cells, exhibit a reduced ability for adenosine uptake and incorporation into macromolecules at a rate that ranges from 9 to 28% and 6 to

Table 8. (^{3}H)-Adenosine Uptake and Incorporation into Macromolecules by the Parental and Mutant Cell Lines

Cell line	Duration of uptake (min)	Total cellular uptake[a] (cpmx10^{-3})	Total cellular uptake relative to HeLa cells[b] (%)	Incorporation into macromolecules[a] (cpmx10^{-2})	Incorporation relative to HeLa cells[b] (%)
HeLa	10	17.86	100.0	8.71	100.0
	20	26.62	100.0	16.14	100.0
	40	34.23	100.0	30.00	100.0
	60	45.88	100.0	35.13	100.0
TOY^{rI}-2	10	1.70	9.5	0.62	7.1
	20	3.35	12.6	1.74	10.8
	40	6.59	19.3	3.22	10.7
	60	8.80	19.2	4.43	12.6
TUB^{rI}-2	10	2.77	15.5	1.20	13.8
	20	5.11	19.2	2.37	14.7
	40	9.66	28.2	4.65	15.5
	60	12.85	28.0	6.20	17.6
TUB^{rI}-4	10	1.70	9.5	0.22	2.5
	20	2.80	10.5	0.73	4.5
	40	5.96	17.4	2.40	8.0
	60	9.03	19.7	2.70	7.7

Cell line	Duration of uptake (min)	Total cellular uptake[a] ($cpmx10^{-3}$)	Total cellular uptake relative to HeLa cells[b] (%)	Incorporation into macromolecules[a] ($cpmx10^{-2}$)	Incorporation relative to HeLa cells[b] (%)
TUB^{rI}-5	10	1.46	8.2	0.27	3.1
	20	2.39	9.0	0.68	4.2
	40	4.75	13.9	2.67	8.9
	60	7.23	15.8	2.73	7.8
6-$MeMPR^{rI}$-1	10	1.49	8.3	0.39	4.5
	20	2.34	8.8	0.83	5.1
	40	4.32	12.6	2.72	9.1
	60	7.02	15.3	3.18	9.1
6-$MeMPR^{rI}$-2	10	1.66	9.3	0.25	2.9
	20	2.52	9.5	0.80	5.0
	40	4.96	14.5	2.66	8.9
	60	4.15	9.0	3.61	10.3
6-$MeMPR^{rI}$-3	10	2.01	11.3	0.25	2.9
	20	2.54	9.5	0.60	3.7
	40	5.43	15.9	1.48	4.9
	60	8.23	17.9	2.31	6.6

Cell line	Duration of uptake (min)	Total cellular uptake[a] ($cpmx10^{-3}$)	Total cellular uptake relative to HeLa cells[b] (%)	Incorporation into macromolecules[a] ($cpmx10^{-2}$)	Incorporation relative to HeLa cells[b] (%)
6-MeMPR^{rI}-4	10	1.81	10.1	0.40	4.6
	20	2.53	9.5	0.82	5.1
	40	3.74	10.9	2.44	8.1
	60	5.94	12.9	2.96	8.4

The uptake and incorporation into macromolecules of (^{3}H)-adenosine by the various cell lines was studied as described in the Methods. The final concentration of (^{3}H)-adenosine (specific activity, 42 Ci/mmole) in special medium was 1 μCi/mL.

[a] The values presented represent the means of duplicate assays. Standard errors were calculated but omitted from the table. In no case was the standard error greater than 10% of the mean indicated.

[b] Assuming the average amount of (^{3}H)-adenosine taken up/incorporated by HeLa cells (for any time period) to be 100, the relative uptake/incorporation in the mutant cell lines was calculated as the ratio of uptake/incorporation by the mutant cells to that of HeLa cells.

13%, respectively, of that shown by the parental, sensitive cells. This implied presence of a "residual" AK activity is consistent with that reported for other adenosine analogue resistant mutants isolated from Chinese hamster ovary (CHO) and rat hepatoma cells (Gupta and Mehta, 1983; Mehta and Gupta, 1983; Plagemann and Wohlhueter, 1983), as well as for human splenic B lymphoid cells (Yamanaka et al., 1984). The observed individual differences in (^{3}H)-adenosine uptake, phosphorylation and incorporation may account for the earlier reported small differences in the degrees of drug and cross-resistance exhibited by each mutant toward the various AK-activated nucleoside analogues (Sections 3.1.1.1 and 3.1.1.2).

3.1.2 Discussion

This section describes the isolation and partial characterization of first-step HeLa cell mutants which exhibit increased resistance toward the nucleoside antibiotics: toyocamycin, tubercidin and 6-MeMPR. The results presented demonstrate that each mutant possesses a biochemical phenotype which is similar to the other TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants and parallels that described for toyocamycin, tubercidin and 6-MeMPR resistant mutants isolated in other cell systems (Bennett et al., 1966; Gupta and Siminovitch, 1978b; Rabin and Gottesman, 1979; Thacker, 1980; Gupta and Singh, 1983; Gupta and Mehta, 1984; Yamanaka et al., 1984).

Several different lines of evidence indicate that the HeLa TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants are genetically and biochemically similar to each other and to those mutants isolated elsewhere:

a) If the genetic lesion responsible for resistance to toyocamycin, tubercidin and 6-MeMPR is similar between these mutants, and therefore represents a common step in the activation of adenosine analogues, similar mutant frequencies should be seen when the selection is carried out in the presence of these nucleosides. This was observed since the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants were obtained in HeLa cells at comparable frequencies (approximately $1x10^{-6}$).

b) Biochemical similarity between these mutants was confirmed by their cross-resistance patterns. It was determined that not only did the toyocamycin, tubercidin and 6-MeMPR resistant mutants exhibit a high degree of resistance toward the selective agent, but they also showed similar, high degrees of cross-resistance toward other AK-activated N- and C-nucleosides (toyocamycin, tubercidin, 6-MeMPR or pyrazofurin). However, the mutants did not exhibit any cross-resistance to other nucleoside or base analogues (6-thioguanine, 6-MPR or 8-azaadenine) which are activated by cellular phosphorylating enzymes other than AK. This demonstrates that the genetic lesion in each of these

mutants is similar and results in a resistant phenotype which is specific only for those nucleoside analogues which require initial phosphorylation by AK to elicit cellular toxicity.

c) All of the mutant cell extracts examined (eight in total) exhibited minimal (<2%) AK activity relative to the parental cells. Thus, each TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutant exhibits a similar phenotype which is deficient in adenosine kinase activity. The subsequent lack of AK-catalyzed phosphorylation of toyocamycin, tubercidin or 6-MeMPR to their respective monophosphate renders mutants highly resistant to these nucleosides.

d) In accordance with their cross-resistance patterns and AK activities, all the mutants showed severely reduced uptake, phosphorylation and incorporation of (^{3}H)-adenosine into macromolecules in vivo. Extrapolating this observation to the adenosine analogues, the in vivo uptake, phosphorylation and incorporation of toyocamycin, tubercidin and 6-MeMPR, which have all been shown to be excellent substrates for AK (Acs et al., 1964; Suhadolnik et al., 1967; Ross and Jaffe, 1972; Zimmerman et al., 1974; Yamanaka et al., 1984), would be severely retarded. This would account for the extremely high degrees of resistance shown by each TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutant.

Thus, the results described in these studies, concerning an adenosine kinase deficiency in toyocamycin, tubercidin and 6-MeMPR resistant HeLa mutants, show that similar to the situation with other purine and pyrimidine analogues (Bennett et al., 1966; Chan et al., 1973; McBurney and Whitmore, 1974; Debatisse and Buttin, 1977; Chan et al., 1978; Chan and Juranka, 1981; Yamanaka et al., 1984), the most common mechanism for cellular resistance to these analogues in human cells results from an alteration in the first biochemical step required for their metabolic activation. Even though specific steps in the mechanism of cellular toxicity elicited by toyocamycin, tubercidin and 6-MeMPR are probably different, it appears that the most common mechanism for resistance in human cells to these, and probably other, adenosine analogues involves a defect in the enzyme, adenosine kinase. Previous results (Bennett et al., 1965; Yamanaka et al., 1984) concerning the mechanism of acquired resistance to adenosine analogues in other human cell systems support this inference.

It appears, then, that various adenosine analogues elicit their cytotoxic effect(s), in numerous cell systems, via a broadly defined "two step" mechanism. The first step is probably universal to most adenosine analogues and involves AK-catalyzed phosphorylation of these drugs to their respective monophosphate. In contrast, the second step

is probably unique to each different adenosine analogue. It would involve a cytotoxic effect, elicited by the appropriate phosphorylated form of the adenosine analogue (either the mono-, di- or triphosphate), on a particular cellular component which is affected predominantly by that adenosine analogue. The first-step mutants isolated in this work, because they are highly cross-resistant to various adenosine analogues and lack AK activity, are defective only in the AK-catalyzed step of this mechanism.

In an effort to increase our understanding of the mechanism of action of toyocamycin, tubercidin and 6-MeMPR in HeLa (human) cells, further studies concerned with the identification of the individual cellular component affected by each of these analogues could be conducted. For this purpose, the selection (from first-step TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants) and biochemical characterization of second-step mutants possessing even further increases in resistance to toyocamycin, tubercidin and 6-MeMPR would be appropriate. From a practical standpoint, however, the selection of second-step mutants resistant to 6-MeMPR may not be possible since the concentrations of 6-MeMPR required would be in the high mg/mL range (6-$MeMPR^{rI}$ mutants are resistant to approximately 1 mg/mL 6-MeMPR). Nevertheless, isolation of second-step mutants resistant to toyocamycin and tubercidin should be feasible since these drugs are toxic at lower

concentrations and first-step mutants are resistant only up to 1 µg/mL toyocamycin or tubercidin. After selection, the second step mutants would be examined for their degree of cross-resistance to other adenosine analogues. Those mutants exhibiting an increase in resistance only to their selective agent should possess an alteration in the second-step of their mechanism of action and no further alteration in the AK-catalyzed phosphorylation step. In other words, the molecular lesion present in these second-step mutants should be very specific and occur at the cellular site which is inhibited by the appropriate phosphorylated derivative of only the selective adenosine analogue. Subsequent to the identification of any such second-step mutants, studies dealing with the in vivo incorporation of the radiolabelled analogue(s) into macromolecules could be performed. These studies would attempt to demonstrate: (a) the intracellular site which is inhibited by the phosphorylated derivative(s) of different adenosine analogues and (b) any differences, between adenosine analogues, that exist with respect to their in vivo mechanism of action in human cells.

3.2 Gel Electrophoretic and Immunoblot Analysis of the Genetic Lesion Present in the TOY^{rI}, TUB^{rI} and 6-MeMPR^{rI} HeLa Mutants

The molecular basis of stable phenotypic variants of cultured mammalian cells is currently under investigation in a number laboratories. One of the most thoroughly investigated classes of drug resistant somatic cell mutants is that involving the salvage enzymes-those enzymes which are responsible for integrating free purine and pyrimidine bases into cellular metabolism. To date, the two most widely studied mammalian purine salvage enzymes have been: (a) hypoxanthine-guanine phosphoribosyltransferase (HGPRT), which is responsible for the synthesis of IMP and GMP from hypoxanthine and guanine respectively, and (b) adenine phosphoribosyltransferase (APRT), which catalyzes the direct synthesis of AMP from adenine and 5-phosphoribosyl-1-pyrophosphate. Both of these enzymes have been implicated in the development of cellular resistance to various purine base analogues (Clements, 1975; Astrin and Caskey, 1976; Caskey and Kruh, 1979).

Previous work concerned with the development of drug resistance to 8-azaguanine and 6-thioguanine [compounds which are converted to their toxic metabolite(s) by HGPRT] and to 8-azaadenine or 2,6-diaminopurine [compounds which are converted to their toxic metabolite(s) by APRT] has

shown that resistant mutants possess an altered HGPRT or APRT activity, respectively (Stutts and Brockman, 1963; Chasin, 1974; Jones and Sargent, 1974; Caskey and Kruh, 1979). Further studies involving a variety of different cell systems and employing numerous drug resistant mutants have revealed the existence of several classes of mutants affected in HGPRT or APRT (Chasin, 1974; Jones and Sargent, 1974; Fenwick et al., 1977, 1984; Caskey and Kruh, 1979; Bradley, 1979,1983; Simon and Taylor, 1983; Turker, 1984). Each class of mutant exhibited a distinct biochemical phenotype characterized by varying levels of HGPRT/APRT activity and the presence or absence of material cross-reacting with antisera specific for HGPRT or APRT. Recently, identification of the nature of the genetic lesion responsible for the drug resistant phenotype exhibited by some of these mutants has been achieved. These studies have successfully demonstrated the existence of specific alterations in the properties of HGPRT or APRT isolated from purine analogue resistant mutants and indicated that this resistance is the consequence of structural (as opposed to regulatory) HGPRT or APRT gene mutations (Beaudet et al., 1973; Chasin, 1974; Jones and Sargent, 1974; Milman et al., 1976,1977; Capecchi et al., 1977; Fenwick et al., 1977; Caskey and Kruh, 1979; Simon and Taylor, 1983).

The results presented in Section 3.1 established that each of the toyocamycin, tubercidin and 6-MeMPR resistant HeLa mutants possessed a genetic lesion that resulted in almost total inactivation of adenosine kinase and consequently, high degrees of resistance toward these nucleoside analogues. Thus, there appears to be a number of similarities in the drug resistant phenotype exhibited by mutants affected in AK, HGPRT or APRT: (a) drug resistance results from an effect on a purine salvage enzyme, (b) the affected enzyme is almost totally inactivated in vitro and in vivo and (c) the resultant mutants are highly resistant to purine base analogues. This suggests that the genetic lesion involved in the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants may also be result of an alteration in the structural gene of AK. In an effort to gain additional insight into the nature of the genetic lesion affecting AK, the toyocamycin, tubercidin and 6-MeMPR resistant mutants were further investigated using SDS-polyacrylamide slab-gel electrophoresis and immunoblot techniques.

The results of these studies demonstrate that, although all the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants have a functionally inactive AK enzyme, each mutant possesses cross-reacting material (CRM) of a similar amount and molecular weight as the AK enzyme present in the parental HeLa cells.

3.2.1 Results

3.2.1.1 SDS-Polyacrylamide Slab-Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis of the Extracts from the TOY^{rI}, TUB^{rI} and 6-MeMPR^{rI} Mutants

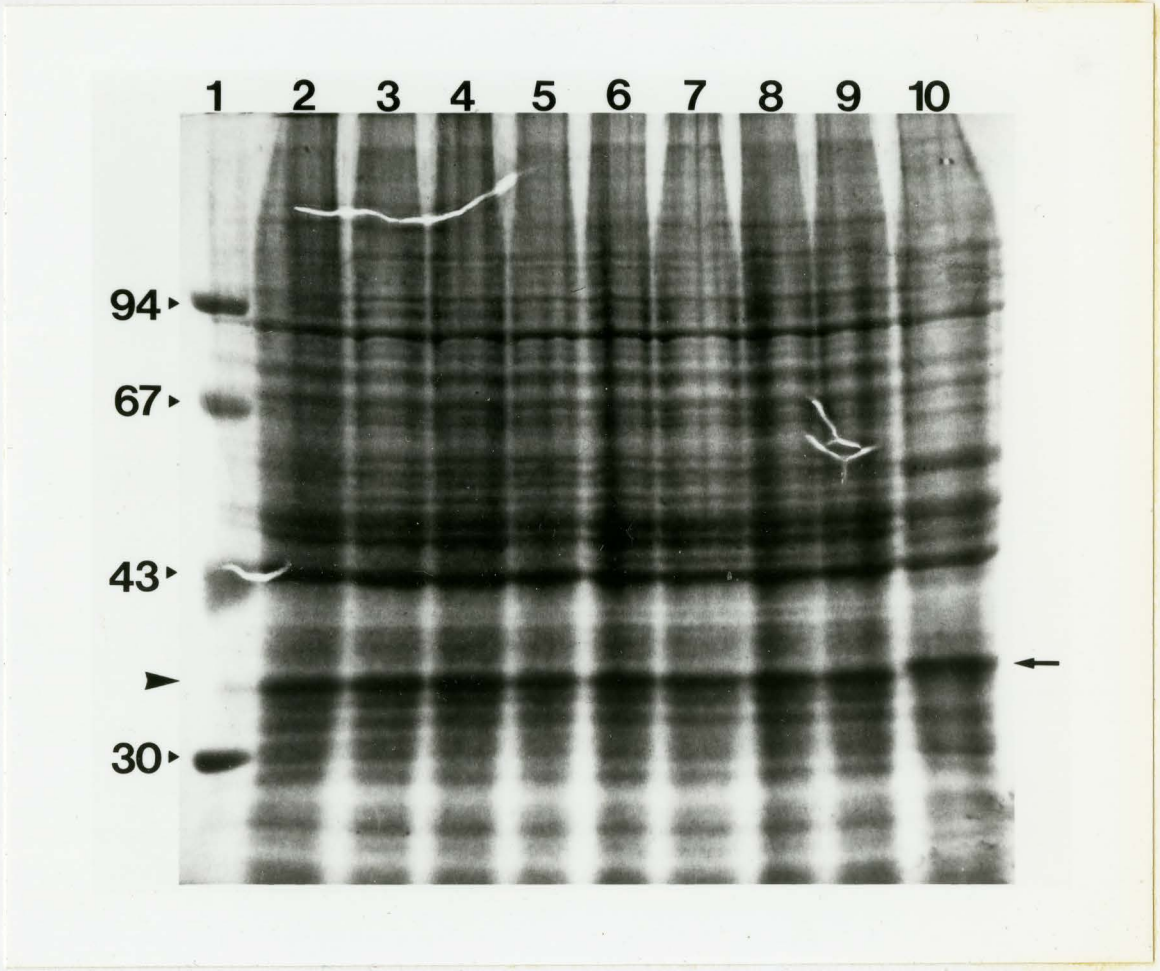
Cell extracts derived from the parental HeLa cells, the HeLa mutants and, to serve as a comparison, CHO/WT cells (which have been characterized in a similar manner elsewhere; Mehta, 1985) were subjected to SDS-PAGE and immunoblot analysis. Alterations in the mutant AK that were sought included: (a) whether this protein was present or absent from the mutants, (b) if material cross-reacting with AK-specific antibody was present, what was its amount and relative molecular mass and (c) was the amount and relative molecular mass of AK from each mutant different from that of the parental HeLa cells or other mutants.

The SDS-polyacrylamide gel presented in Figure 8 is representative of similar gels obtained for each of three independent experiments and shows the proteins present in HeLa, HeLa mutant (excluding 6-MeMPR^{rI}-4) and CHO/WT cell extracts. The arrow placed to the right of lane 10, which represents the proteins present in the CHO/WT cell extract, indicates the migratory position of the AK protein present in CHO/WT cells. Recently, AK from CHO cells has been purified to homogeneity by affinity chromatography and its relative molecular mass reported to be 38,000 (Mehta, 1985).

Figure 8. SDS-polyacrylamide slab gel analysis of the proteins present in whole cell extracts of parental HeLa cells, the different mutant cell lines affected in AK and CHO/WT cells. The crude extract run in each lane corresponds to that amount of protein resulting from the lysis of $2x10^5$ cells as described in the Methods. Proteins in the gel were stained with Coomassie brilliant blue. The molecular weight markers employed were phosphorylase b (M_r=94,000), bovine serum albumin (M_r=67,000), ovalbumin (M_r=43,000) and carbonic anhydrase (M_r=30,000).

Lane 1, relative molecular mass markers $x10^{-3}$;
Lane 2, 20 µL of crude HeLa cell extract;
Lane 3, 20 µL of crude TOY^{rI}-2 cell extract;
Lane 4, 20 µL of crude TUB^{rI}-2 cell extract;
Lane 5, 20 µL of crude TUB^{rI}-4 cell extract;
Lane 6, 20 µL of crude TUB^{rI}-5 cell extract;
Lane 7, 20 µL of crude 6-$MeMPR^{rI}$-1 cell extract;
Lane 8, 20 µL of crude 6-$MeMPR^{rI}$-2 cell extract;
Lane 9, 20 µL of crude 6-$MeMPR^{rI}$-3 cell extract;
Lane 10, 20 µL of crude CHO/WT cell extract;

The arrow indicates the position of the AK protein present in the CHO/WT cell extract and the arrowhead indicates the putative position of the AK protein present in the HeLa cell extract.


1
2
3
4
5
6
7
8
9
10
94
67
43
30

Calculation of the relative molecular mass, based on the migration of the protein standards in lane 1 and assuming log M_r is inversely proportional to relative migration through a polyacrylamide gel, yielded a value of approximately 36,700 for the CHO/WT AK protein band indicated in Figure 8, Lane 10.

The remaining lanes present in Figure 8 represent the proteins present in extracts of the parental HeLa cells (lane 2) and the HeLa TOY^{rI}-2, TUB^{rI}-2,4,5 and 6-$MeMPR^{rI}$-1,2,3 mutants (lanes 3 through 9 respectively). Based upon: (a) the fact that adenosine kinase is found at relatively high levels in numerous tissues (Ho et al., 1968; Krenitsky et al., 1974) and should therefore exhibit a prominent band when a cell extract is electrophoresed through an SDS-polyacrylamide gel, (b) the previously published value for the relative molecular mass of AK isolated from human placenta (range of 37,000-40,000; Andres and Fox, 1979) and the presence of single, very prominent band close to this migratory position and (c) the corresponding similar intensity and location of the CHO/WT AK protein, the band indicated by the arrowhead to the left of lane 1 has been tentatively identified as the AK protein present in the parental HeLa cell extract [this was later confirmed by immunoblot analysis (page 121)]. The relative molecular mass of this protein band, calculated in the same manner as that

for AK found in the CHO/WT cell extract, yielded a value of approximately 35,000 for AK from the parental HeLa cells.

Immunoblot analysis was used to: (a) verify that the protein band present in the SDS-polyacrylamide gel (Figure 8, lane 1) of relative molecular mass 35,000 was AK present in the parental HeLa cell extract and (b) to investigate any possible alteration in AK present in the mutant cell extracts. The rabbit anti-CHO/WT adenosine kinase antibody used in these experiments was a generous gift from Kamal D. Mehta, McMaster University. This antibody specifically immunoprecipitates a protein of relative molecular mass 38,000 from CHO cell extracts and such treatment results in the removal of AK activity from these extracts (Mehta, 1985). Thus, there is strong evidence indicating that this antiserum specifically cross-reacts with AK present in CHO cell extracts.

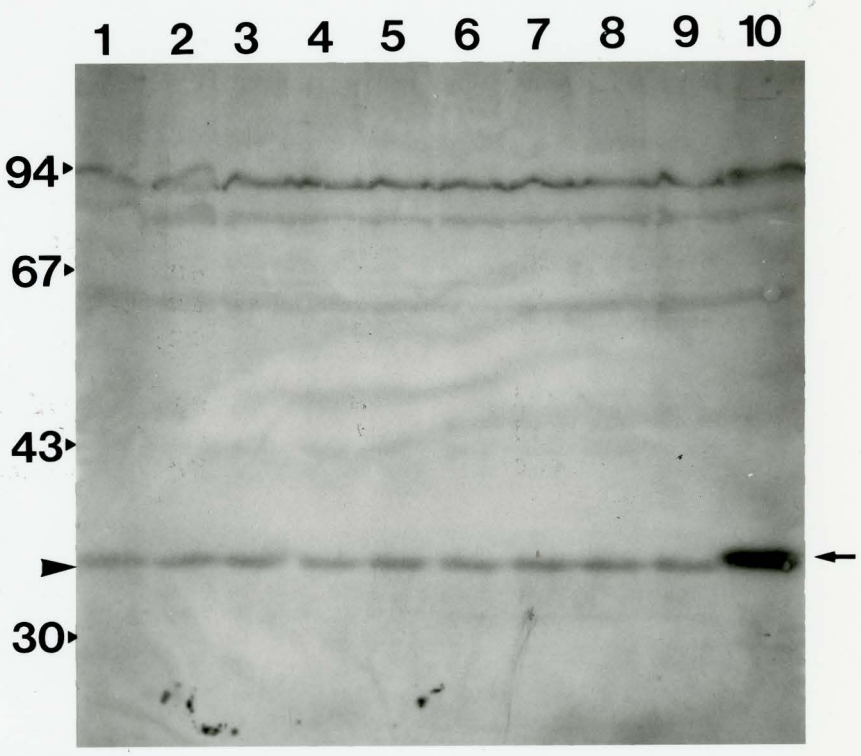
Using this AK-specific antibody, the extracts derived from HeLa cells, HeLa mutants and CHO/WT cells were examined for the presence of cross-reacting material. The resulting immunoblot, which is representative of three similar blots obtained in independent experiments, is presented in Figure 9. Lane 10, which represents the proteins present in the CHO/WT cell extract, reveals the presence of major CRM (indicated by the arrow) of a similar relative molecular mass to that calculated previously for

the CHO/WT AK protein (M_r=36,700). This band should represent CHO/WT adenosine kinase. Similarly, lane 1, which represents the proteins present in the parental HeLa cell extract, contained a major cross-reacting band of relative molecular mass approximately equal to 35,000 (indicated by the arrowhead). This strongly suggests that the protein band in Figure 8, which was tentatively identified as AK, is indeed AK present in the parental HeLa cell extract. As well, the TOY^{rI}-2 (lane 2), TUB^{rI}-2,4 and 5 (lanes 3,4 and 5 respectively) and 6-$MeMPR^{rI}$-1,2,3 and 4 mutants (lanes 6,7,8 and 9 respectively) all contained a major band of CRM that had a relative molecular mass of approximately 35,000. It must be noted that, in addition to the major cross-reacting band found in each extract, there were other minor cross-reacting bands present in the high relative molecular mass region of the immunoblot. Whether such bands arose from specific antibody-protein interactions (ie. possible precursor proteins to the mature AK protein) or non-specific binding is not clear. However, it is interesting to note that these bands were present in both the HeLa parental and mutant cell extracts, as well as in the CHO/WT cell extract. Similar cross-reacting proteins are found in CHO mutants, defective in AK activity, that are resistant to other purine nucleoside analogues (Mehta, 1985).

Figure 9. Immunoblotting of the proteins present in whole cell extracts of parental HeLa cells, the different mutant cell lines affected in AK and CHO/WT cells. Following SDS-polyacrylamide slab gel electrophoresis of the proteins present in crude extracts of each of the different cell lines, electrophoretic transfer of the proteins to a nitrocellulose membrane was carried out. The nitrocellulose membrane was then treated with thirty times diluted AK specific antibody and subsequently with (^{125}I)-labelled IgG F(ab')$_2$ fragment as described in the Methods.

Lane 1, HeLa cell extract;
Lane 2, TOY^{rI}-2 cell extract;
Lane 3, TUB^{rI}-2 cell extract;
Lane 4, TUB^{rI}-4 cell extract;
Lane 5, TUB^{rI}-5 cell extract;
Lane 6, 6-MeMPR^{rI}-1 cell extract;
Lane 7, 6-MeMPR^{rI}-2 cell extract;
Lane 8, 6-MeMPR^{rI}-3 cell extract;
Lane 9, 6-MeMPR^{rI}-4 cell extract;
Lane 10, CHO/WT cell extract;

The arrow indicates the position of AK in the CHO/WT cell extract and the arrowhead indicates the position of AK in the HeLa and mutant cell extracts.


1 2 3 4 5 6 7 8 9 10
94
67
43
30

3.2.2 Discussion

Since its discovery in yeast (Caputto, 1951; Kornberg and Pricer, 1951), adenosine kinase has been partially purified from a number of mammalian sources (Lindberg et al., 1967; Schnebli et al., 1967; Murray, 1968; Lindberg, 1969; Divekar and Hakala, 1971; Henderson et al., 1972; Namm and Leader, 1974) and purified to homogeneity from brewer's yeast (Leibach et al., 1971). This has led to several reports describing the molecular weight of AK isolated from numerous systems. Adenosine kinase found in the parental HeLa cell extract has a relative molecular mass of approximately 35,000 as determined by SDS-PAGE and immunoblot analysis in this work. This is in close agreement with that reported for AK isolated from brewer's yeast (Leibach et al., 1971), mouse erythrocytes (M_r=36,000; Schmidt et al., 1974), rat heart (DeJong, 1977), rabbit liver (M_r=34,000 $\pm$2,000; Miller et al., 1979) and the only other human system investigated thus far, human placenta (M_r=37,000-40,000; Andres and Fox, 1979). However, a relative molecular mass of 35,000 for HeLa AK differs substantially from that reported elsewhere for AK isolated from rabbit liver (M_r=23,000; Lindberg et al., 1967) and L1210 murine leukemia cells (M_r=56,000; Chang et al., 1980).

Analysis of the SDS-polyacrylamide gel and immunoblot of cell extracts derived from parental HeLa cells

and each of the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ cell lines provides strong evidence that the genetic lesions present in these mutants are of a similar nature. The inferences that can be made concerning the nature of the genetic lesion affecting AK are:

a) Since all of the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants had a major protein that cross-reacted with AK-specific antibody, the genetic lesion allowed each mutant to retain its ability to express the AK gene. This implies that resistance exhibited by human (HeLa) cells toward AK-activated nucleosides is due to the production of a functionally inactive AK enzyme and is not the result of gene inactivation or chromosomal loss that renders the cells AK deficient. Thus, the mutants are defective in AK activity and not in expression of the AK gene.

b) Since each mutant appeared to contain a similar amount of CRM compared to other mutants and the parental HeLa cells (as judged by visual inspection of the band intensity of the autoradiogram), the genetic lesion probably did not involve a regulatory type of genetic alteration. A mutation in a regulatory region would be expected to result in the absence of the corresponding gene product from the cell or production of the gene product in abnormal quantities. Neither possibility was observed for the toyocamycin, tubercidin or 6-MeMPR resistant mutants.

c) Each of the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants possessed a protein with an electrophoretic mobility which was similar to that of AK from parental HeLa cells. This indicates that AK from each mutant has a relative molecular mass similar to that of the parental HeLa cells (M_r=35,000). Thus, the genetic lesion in each mutant is probably not of the nonsense or deletion type. Each of these mutations would result in a substantially truncated gene product as a result of either premature chain termination or the simple absence of several amino acid residues from the gene product. This is contrary to that indicated by the immunoblot. The only way that the genetic lesion affecting AK could be of the nonsense or deletion type, and yield the observed results, is if the nonsense mutation is close to the C-terminus of the gene product or if the deletion is quite small. The likelihood of these possibilities is remote considering that the mutagens used to create these lesions (EMS or UV-light) induce primarily base substitutions.

d) A mutation in a structural gene is characterized by: i) an occurrence at low frequency, with the mutant phenotype usually remaining stable in the absence of selection, ii) an increased occurrence of the variant phenotype after exposure to mutagens and iii) an association of the phenotypic change with the production of an altered gene product (Clements, 1975). All of the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants

exhibited these properties and therefore fit this classification. Realizing this, the genetic lesion possessed by each mutant is probably a missense type of mutation present in the structural gene for AK.

Previously, it has been suggested that the mutational event(s) leading to toyocamycin or tubercidin resistance in CHO cells involves not only deletion mutations, but also chromosomal loss and rearrangement (Gupta and Siminovitch, 1978b; Rabin and Gottesman, 1979). In further support of this, Eves and Farber (1981) observe that in 50% of the AK mutants found in the CAK mouse cell line only one allele of the linked ES-10 allele was expressed. They conclude from this that the occurrence of these mutants is associated with the loss of part of chromosome 14. Contrastingly, the results reported here, which show that each toyocamycin, tubercidin and 6-MeMPR resistant mutant possesses an equivalent amount of CRM as the parental cells, strongly suggest that AK-deficient human (HeLa) cell mutants have not undergone any deletion, chromosomal loss or rearrangement type events. Rather, the genetic alteration affecting AK that led to increased resistance toward AK-activated nucleoside analogues was probably a missense type of mutation in the structural gene for AK. The proposed occurrence of a missense mutation in a structural gene leading to increased resistance toward

purine nucleoside analogues is reported elsewhere, in other cellular systems, for the structural gene of AK (Mehta, 1985), the structural gene of HGPRT (Caskey and Kruh, 1979) and the structural gene of APRT (Simon and Taylor, 1983).

Further proof of a missense type of mutation in the structural gene of AK could be achieved using two-dimensional (2-D) gel electrophoretic and immunoblot analysis of (^{35}S)-methionine labelled total cellular protein. Initially, the total protein of HeLa cells would be labelled with (^{35}S)-methionine, subjected to 2-D gel electrophoresis, transferred to nitrocellulose and autoradiography used to identify potential reference spots/regions corresponding to major cellular proteins. Subsequently, identification of the spot/region corresponding to the parental AK protein [or mutant AK protein(s)] present in 2-D gels could be accomplished by comparing the autoradiograms of the same 2-D blot before and after treatment with AK-specific antibody followed by (^{125}I)-labelled goat anti-rabbit IgG. In this way, comparison of the spots/regions present in the 2-D protein profile of any of the mutant AK proteins with that of the parent AK protein could be accomplished. The presence of a mutant AK protein with a slightly altered electrophoretic mobility in the first-dimension (the IEF or charge dimension) and a similar electrophoretic mobility in the second-dimension (the SDS-

PAGE or relative molecular mass dimension) would be consistent with the proposal that a missense type of alteration in the structural gene of AK is responsible for each of the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutant phenotypes.

3.3 Quantitative Mutagenesis at the Adenosine Kinase (AK) Locus in Human (HeLa) Cells

Our awareness of the widespread occurrence of mutagens/carcinogens stems from a number of biological test systems which employ a variety of organisms and use numerous end points for detection. Much of the work is carried out in microbial systems, such as those employing Salmonella typhimurium (Ames et al., 1975; Hollstein et al., 1979; Maron and Ames, 1983; Ames, 1984), however, assay systems based on cultured mammalian cells have added a new dimension to mutagen/carcinogen screening (Hollstein et al., 1979; Kilbey et al., 1984). Of the various mammalian mutagen screening systems which have been developed, those which measure the induction of mutations at specific genetic loci provide a direct and quantifiable measure of the mutagenic activity of any agent. In this respect, mutants which confer resistance to specific drugs provide useful end points for the identification of various types of genetic changes that occur within a cell. A large number of well characterized drug resistant genetic selection systems, which appear suitable for studies in quantitative mutagenesis (including screening for mutagenic agents), have been developed (Clive et al., 1972; Arlett, 1977; Friedrich and Coffino, 1977; O'Neill et al., 1977; Clive et al., 1979; Hsie et al., 1980; Gupta and Siminovitch, 1980a; Gupta and Goldstein, 1981;

Hsie et al., 1981; Gupta and Singh, 1982; Singh and Gupta, 1982; Gupta and Singh, 1983; Singh and Gupta, 1983; Gupta, 1984). Of these systems, those involving drug resistant markers which select for genetic alterations affecting purine and pyrimidine salvage pathway enzymes have proven to be extremely useful for mutagen/carcinogen screening studies. These markers include: (a) resistance to 6-thioguanine (Thg^r) or 8-azaguanine which results from the loss or deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Arlett, 1977; Caskey and Kruh, 1979), (b) resistance to 5-bromodeoxyuridine ($BUdR^r$) or 5-fluorodeoxyuridine which results from a loss of thymidine kinase (TK) (Clive et al., 1972, 1979), (c) resistance to 8-azaadenine (AA^r) which results from a deficiency in the enzyme adenine phosphoribosyltransferase (APRT) (Chasin, 1974; Jones and Sargent, 1974) and (d) resistance to 6-methylmercaptopurine riboside ($6\text{-}MeMPR^r$) or tubercidin (TUB^r) which results from a loss of adenosine kinase (AK) (Gupta and Singh, 1983; Gupta, 1984). Since mammalian cells possess the ability to synthesize purines and pyrimidines through de novo pathways, the aforementioned salvage pathway enzymes are not essential for growth under normal conditions (Caskey and Kruh, 1979). Thus, these drug resistant markers should, in theory, be capable of detecting a variety of different types of genetic lesions, including

those which may cause loss or inactivation of these functions (ie. base substitutions, frameshifts, additions, deletions, chromosome breakage or rearrangement) and be of great use in studies related to quantitative mutagenesis.

Since one aim of the various short term assays of mutagenicity and/or carcinogenicity is to predict the specific risk of human beings to different agents, human cells in culture, which retain many of the genetic and biochemical characteristics of humans, present themselves as a logical model for the systematic investigation of mutagenic changes that occur in human cells in vivo. Further, due to the genetic and metabolic differences that exist between humans and other organisms, it has been proposed that test systems possessing human specificity are necessary for mutagen and/or carcinogen screening (Berky and Sherrod, 1977; Jacobs and DeMars; 1984). In this respect, quantitative and qualitative mutagenesis data derived from cultured human cells, together with data concerning DNA-mutagen interactions and DNA repair in human cells, should help to increase our understanding of the mutagenic changes that occur in human cells in vivo.

Consequently, the development of a pertinent mutagen and/or carcinogen screening system may involve the use of: (a) drug resistant markers that serve as end points indicative of a loss or alteration in the activity of either

the purine or pyrimidine salvage enzymes and (b) human cells in culture. Ideally, this combination would produce a test system which possesses the ability to detect a wide variety of different types of genetic alterations that may occur in human cells in vivo.

Unfortunately, the use of human cells in this respect has been restricted due to the paucity of accurate and reliable selective markers that can serve as end points in such systems. As yet, the only drug resistant markers of the aforementioned type that have been applied to quantitative mutagenesis studies in human cells include resistance to 6-thioguanine (Thg^r; HGPRT locus; Jacobs and DeMars, 1984) and resistance to 8-azaadenine (AA^r; APRT locus; Steglich and DeMars, 1982; Jacobs and DeMars, 1984). Other drug resistant markers, including resistance to α-amanitin (Ama^R) which affects RNA polymerase II, resistance to 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (Drb^R) which inhibits mRNA synthesis, resistance to ouabain (Oua^R) which affects membrane Na^+/K^+ ATPase and resistance to diptheria toxin (Dip^r) which inhibits protein synthesis, have recently extended the quantitative analysis of mutations in human cells to other areas of the genome (Mankowitz et al., 1974; Buchwald and Ingles, 1976; Buchwald, 1977; Gupta and Siminovitch, 1980b; Gupta and Goldstein, 1981). However, in view of the essential nature

of the affected functions, these markers are expected to detect only those types of genetic lesions which alter function without completely destroying activity. Thus, predominantly base substitutions would be detected using these genetic markers thereby limiting their usefulness for the detection of various different acting mutagens and/or carcinogens. As a result, there is a need to develop additional accurate and reliable drug resistant markers that can serve as end points for the detection of different types of genetic alterations in quantitative mutagenesis studies involving human cells.

Previous work (Gupta and Singh, 1983) has revealed that the selection system for AK^- mutants provides a valuable genetic marker for quantitative mutagenesis studies in CHO cells. Since the results obtained concerning the selection and the genetic and biochemical characterization of AK^- mutants in HeLa cells (Section 3.1) paralleled those obtained by Gupta and Singh (1983), the present work was undertaken in an attempt to determine the ability of the AK^- mutant selection system to serve as an end point for quantitative mutagenesis studies in human cells. The initial work performed in this regard involved the use of the HeLa cell system. HeLa cells offer certain favourable characteristics including: (a) their ease of growth in either suspension culture or on solid surfaces, (b) their

relatively rapid growth rate (doubling time=18 to 20 hours), (c) their high (>80%) plating efficiency, (d) their human origin and (e) their transformed phenotype (allows for full characterization of spontaneous or induced mutants because of their "infinite" life span). Unfortunately, they also present two major drawbacks. These are: (a) their transformed phenotype - the validity of using a permanent cell line as a model for in vivo processes is questionable since the complex changes that occur upon transformation are not well understood and (b) the instability of their already aneuploid karyotype. Consequently, the thrust of this work was not the development of a mutagen and/or carcinogen screening system involving HeLa cells, but rather determination of the ability of the AK locus to serve as an additional drug resistant marker for quantitative mutagenesis studies involving human cells. HeLa cells were used only as a pilot system in this regard. The eventual goal (assuming the response of the AK locus in HeLa cells to be a favourable one) was to determine whether the AK locus could serve the same purpose when human cells exhibiting highly stable, diploid karyotypes (normal human diploid fibroblasts) were used for mutagenesis studies.

In this respect, efforts were made to determine: (a) which nucleoside analogue (toyocamycin, tubercidin or 6-MeMPR) is most appropriate for the selection of AK^- mutants,

(b) the optimum concentration of this analogue for AK^- mutant selection, (c) the optimal cell density or concentration for selection of drug resistant colonies and (d) the optimum time subsequent to mutagenesis for maximum expression of the AK^- mutant phenotype. Having determined these appropriate selective conditions, the concentration-response of the AK locus after treatment with different mutagenic agents (ethylmethanesulfonate, ICR-170 and ultraviolet-light) was investigated.

The results of these studies indicate that the AK locus may be useful as an additional drug resistant marker for the study of quantitative mutagenesis in human cells.

3.3.1 Results

3.3.1.1 Characteristics of the Toyocamycin, Tubercidin and 6-MeMPR Resistant Hela Cell Mutants

To determine which of the three nucleoside analogues involved in this work (toyocamycin, tubercidin or 6-MeMPR) would best be suited for use in quantitative mutagenesis studies involving human (Hela) cells, it was first necessary to establish whether similar mutants were being selected by each of these drugs. For this purpose, selections were performed in the presence of toyocamycin, tubercidin or 6-MeMPR to determine whether drug resistant mutants were obtained at similar frequencies, exhibited similar cross-resistance patterns and possessed similar AK activities,

independent of the nucleoside analogue against which they were originally selected.

To study the effect of each of these analogues on the mutant frequency, the selection of drug resistant mutants from both nonmutagenized and mutagenized (300 µg/mL of EMS for 20 hours; plated on the eighth day subsequent to mutagen treatment) HeLa cells was carried out in the presence of toyocamycin, tubercidin and 6-MeMPR. In nonmutagenized cells, resistant colonies were obtained at a similar frequency (approximately $1x10^{-7}$) when selected against any one of the nucleoside analogues. The frequency of drug resistant mutants in the EMS-treated HeLa cells was between 8- and 10-fold greater than that observed for the control population. Observed mutation frequencies in mutagenized HeLa cells were approximately $7.6x10^{-7}$, $7.6x10^{-7}$ and $1.1x10^{-6}$ in the presence of toyocamycin, tubercidin and 6-MeMPR, respectively. Thus, mutants resistant to either of these three nucleoside analogues were obtained at comparable frequencies from either control or mutagenized cultures.

Subsequently, in an effort to determine whether the resistant colonies obtained using these three nucleoside analogues possessed similar drug resistant phenotypes, a number of toyocamycin, tubercidin and 6-MeMPR resistant HeLa mutants were picked, grown in nonselective medium and tested

for their degree of cross-resistance toward AK-activated nucleoside analogues other than the one against which they were originally selected. The results of these studies are presented in Table 5 (Section 3.1.1.2). They show that not only did the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants exhibit a high degree of resistance toward their selective agent, but they also exhibited similar, high degrees of cross-resistance toward various AK-activated N- and C-nucleosides (toyocamycin, tubercidin, 6-MeMPR and pyrazofurin). Thus, all of these mutants were similar with respect to their drug resistant phenotype.

Lastly, to establish whether the HeLa mutants resistant to these drugs were affected in the same function, the level of AK activity present in the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants, as compared to parental HeLa cells, was determined. The results of these studies (Table 6, Section 3.1.1.3) showed that all of the eight resistant clones examined were found to possess minimal (<2%) AK activity, relative to parental cells, in cell extracts. Therefore, these mutants were also similar in their biochemical nature.

Taken collectively, the cross-resistance studies, the AK deficiency of all of the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants and the selection of AK^{-} mutants at comparable frequencies independent of the selective nucleoside analogue indicated that similar mutants were being selected by

toyocamycin, tubercidin or 6-MeMPR. Thus, any of these three nucleoside analogues can be used for the selection of AK^- HeLa mutants in quantitative mutagenesis studies. Despite this, all further work was carried out using 6-MeMPR for the following reasons. First, 6-MeMPR was readily available compared to the other analogues which were either not available commercially (toyocamycin) or were available at a significantly increased cost (tubercidin). Second, it has been reported in CHO cells (Gupta and Singh, 1983) that as the concentration of various selective drugs (toyocamycin, tubercidin, 6-MeMPR or pyrazofurin) was increased over a 10- to 20-fold range, a slight decrease (up to 2-fold) in the frequency of AK^- mutants in both control and mutagenized cell populations was observed. However, this observed decrease in mutant frequency as the selective drug concentrations were raised, was minimal in the case of 6-MeMPR. Therefore, 6-MeMPR was chosen in those studies for work with CHO cells and in the present studies for work with Hela cells. Lastly, since resistance to 6-MeMPR has been used as an end point for quantitative mutagenesis studies in CHO cells (Gupta and Singh, 1983), its use in Hela cells allowed a direct comparison between the mutagenic response of the AK locus in CHO and Hela cells.

3.3.1.2 Optimal Conditions for Studying the Mutagenic Response of the AK Locus

Before any genetic selection system can be employed for quantitative mutagenesis, knowledge of certain selective parameters is essential. These include: (a) the optimal selective drug concentration, (b) the optimal cell density or concentration during selection and (c) the optimum time required for maximum expression of the mutant phenotype following mutagen treatment.

The selective drug concentration should be chosen such that all of the sensitive cells are killed and virtually no background growth is observed in any dishes. If visible background growth is unavoidable, it should not in any way interfere with the scoring or analysis of the mutants. Furthermore, any of the clones selected at this drug concentration, which are subsequently isolated and analyzed, should be affected in the appropriate function (in this case, AK activity) and should maintain their drug resistant characteristics even in the absence of selective pressure. A 6-MeMPR concentration of 5.0 µg/mL fulfilled these requirements. Since the D_{10} value of 6-MeMPR for Hela cells was approximately 0.017 µg/mL (Table 4, Section 3.1.1.1), a concentration of 5.0 µg/mL killed all of the sensitive (AK^+) cells. The small amount of visible background present in the dishes did not interfere with the scoring of resistant clones, especially when AK^- mutants were allowed to form aggregates (colonies) of 40 or more

cells. Any of the mutant colonies (6-MeMPR^{rI}-1, 6-MeMPR^{rI}-2, 6-MeMPR^{rI}-3 or 6MeMPR^{rI}-4) that were picked, grown in non-selective medium and subsequently analyzed for the nature of their drug resistant phenotype were found: (a) to be greater than 40,000-fold resistant to 6-MeMPR, (b) to possess minimal (<2%) AK activity relative to parental sensitive cells and (c) to exhibit a drug resistant phenotype that remained stable upon growth in the absence of 6-MeMPR for more than one year (Section 3.1). The use of a selective 6-MeMPR concentration which was significantly less or greater than 5.0 µg/mL was not justified. 6-MeMPR at a concentration of 1.0 µg/mL was insufficient to maintain the background growth at a required minimum level and this interfered with the scoring of 6-MeMPR^{r} mutants. Although the use of 6-MeMPR at 10.0 µg/mL achieved results which were comparable to those obtained with 5.0 µg/mL of 6-MeMPR, excess drug provided no additional benefit and was, therefore, not necessary.

The optimal cell density or concentration used during mutant selection is one that allows the analysis of a large number of cells without loss in mutant recovery (due to cross-feeding effects) or difficulty in scoring the resultant drug resistant mutants. To determine whether the recovery of AK^{-} mutants was affected by cell density, reconstruction experiments were carried out. This involved

plating both a constant number (200) of Hela resistant (AK^-) cells and different numbers of Hela sensitive (AK^+) cells in the presence of 5.0 µg/mL of 6-MeMPR and determining the recovery of AK^- mutant cells. Variation in the number of sensitive (AK^+) Hela cells from $1x10^5$ to $2x10^6$ per P-100 dish (60 cm^2 area) had no significant effect on the recovery of either the TOY^{rI}-2, TUB^{rI}-4 or 6-$MeMPR^{rI}$-3 mutants since in every case the percent relative cloning efficiency of the AK^- cells was close to 100 (Table 9). However, since a certain degree of variability in the percent relative cloning efficency of AK^- cells in the presence of AK^+ cells was found in these reconstruction experiments, additional work was performed to verify that there was no significant effect of cell density on the recovery of AK^- mutants. For this purpose, Hela cells were mutagenized (300 µg/mL of EMS for 20 hours which resulted in approximately 60% cell killing) and plated at different cell densities (eight days subsequent to mutagen treatment) in the presence of 6-MeMPR (5.0 µg/mL) to determine the frequency of AK^- mutants. The results revealed that the AK^- mutant frequency was approximately $1.3x10^{-6}$, independent of the cell concentration at the time of plating. This verified that cell density did not affect the recovery of AK^- mutants.

Lastly, although no significant effect on AK^- mutant recovery occurred when $2x10^6$ sensitive (AK^+) Hela cells were

Table 9. Reconstruction Experiments to Show the Absence of Effect of Cell Density of the Recovery of AK^- Mutants

Mutant	No. of sensitive (AK^+) cells	No. of resistant (AK^-) cells	No. of AK^- mutant colonies observed[a]	% relative cloning efficiency of AK^- cells[b]
TOY^{rI}-2	$5x10^5$	-	0	
	$1x10^6$	-	0	
	$2x10^6$	-	0	
	-	200	140.3 ± 3.8	
	$1x10^5$	200	145.7 ± 4.5	104.8
	$2x10^5$	200	141.7 ± 8.5	101.0
	$5x10^5$	200	154.7 ± 10.1	110.3
	$1x10^6$	200	156.0 ± 8.7	111.2
	$2x10^6$	200	147.3 ± 10.0[c]	105.0
TUB^{rI}-4	$5x10^5$	-	0	
	$1x10^6$	-	0	
	$2x10^6$	-	0	
	-	200	117.5 ± 4.8	
	$1x10^5$	200	122.3 ± 7.1	104.1
	$2x10^5$	200	119.0 ± 6.2	101.2

Mutant	No. of sensitive (AK^+) cells	No. of resistant (AK^-) cells	No. of AK^- mutant colonies observed[a]	% relative cloning efficiency of AK^- cells[b]
TUB^{rI}-4 (con't.)	$5x10^5$	200	115.7±3.0	98.5
	$1x10^6$	200	127.7±1.5	108.7
	$2x10^6$	200	125.0±11.1[c]	106.4
6-$MeMPR^{rI}$-3	$5x10^5$	-	0	
	$1x10^6$	-	0	
	$2x10^6$	-	0	
	-	200	151.3±4.3	
	$1x10^5$	200	148.0±5.1	97.8
	$2x10^5$	200	148.1±3.4	97.9
	$5x10^5$	200	153.1±5.0	101.2
	$1x10^6$	200	162.7±5.2	107.5
	$2x10^6$	200	164.6±9.6[c]	108.9

The indicated numbers of sensitive (AK^+) and resistant (AK^-) cells were plated in 100-mm-diameter dishes in special medium containing 5 µg/mL of 6-MeMPR as described in the Methods. The number of cells plated was based upon cell count measurements with a Coulter counter. After 11 to 12 days in the presence of 5 µg/mL of 6-MeMPR, the number of observed AK^- mutant colonies was recorded.

[a] The values presented represent the means ± standard deviations.

[b] Assuming the number of AK^- mutant colonies observed in the absence of AK^+ cells to be 100, the relative cloning

Footnote to Table 9 (cont'd).

efficiency of AK^- cells was calculated as the ratio of the number of AK^- mutant colonies observed in the presence of AK^+ cell to that observed in the absence of AK^+ cells.

[c] At this cell density, accurate scoring of the AK^- mutant colonies was difficult (see Results, Section 3.3.1.2).

plated in the presence of 200 AK^- cells, the background of dead or non-growing cells present at this cell density (and only this density) interfered with the precise scoring of viable AK^- mutants and was not appropriate for the 6-MeMPR mutant selection. Consequently, $1x10^6$ cells per P-100 dish was chosen as the appropriate cell density for the selection of Hela AK^- mutants.

The time required for maximum expression of the mutant phenotype must be determined before any genetic marker can be used for quantitative mutagenesis. Generally, expression time is that period subsequent to mutagenesis during which the mutation frequency in a cell culture reaches a maximum stable plateau and remains there. To determine the optimal expression time for AK^- Hela mutants, a culture of actively growing Hela cells was divided into three portions: one portion served as the control (nonmutagenized) cell population, another was treated with 2.0 µg/mL of MNNG for 4 hours (resulted in about 35% cell survival) and the last portion was exposed to 350 µg/mL of EMS for 16 hours (resulted in about 40% cell survival). At various times after mutagen treatment, the MNNG-treated, EMS-treated and control populations were plated in the presence of 6-MeMPR (5.0 µg/mL) and the frequency of the AK^- mutants determined. Figure 10 presents the results of these studies. As indicated in Figure 10A, only a minimal increase

in the frequency of AK^- mutants was observed in the MNNG-treated culture for the initial 3 days. Subsequently, the AK^- mutant frequency increased significantly and reached a maximum around day 8 or 9. The mutant frequency, after reaching this level, remained constant for at least the next 3 or 4 days. Similar results were obtained when the maximum expression time for AK^- mutants was investigated using the EMS-treated culture (Figure 10B). In this case, the AK^- mutant frequency remained relatively low for the first 3 days, increased to a maximum at approximately day 8 and plateaued at this mutant frequency for at least 4 subsequent days. In contrast to either the MNNG- or EMS-treated Hela cells, the nonmutagenized (control) culture showed no increase in the AK^- mutant frequency during a similar 12 day period. Thus, the time of maximum expression of the 6-MeMPR mutant phenotype was approximately 8 days post-mutagenesis.

In summary, these results indicate that the optimum selective parameters that should be used for quantitative mutagenesis studies involving Hela cell resistance to 6-MeMPR are: (a) selective concentration: 5.0 μg/mL 6-MeMPR, (b) cell density: $1x10^6$ cells/P-100 dish and (c) expression time: 8 days.

3.3.1.3 The Concentration-Response of the AK Locus to Mutagenic Agents

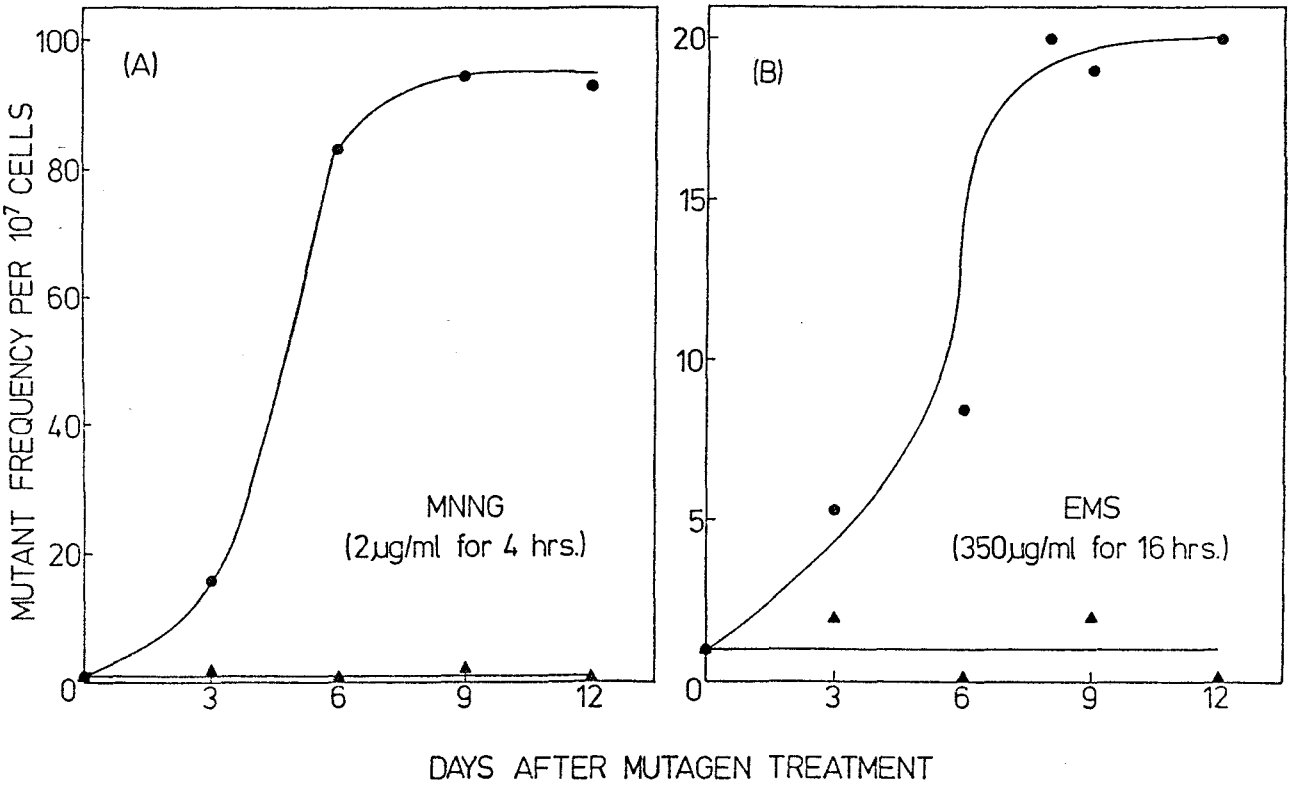


Figure 10. Expression time requirement for the AK^- mutants after mutagen treatment. The profile of the expression period for the 6-methylmercaptopurine riboside resistant phenotype was determined by selecting AK^- mutants in special medium supplemented with 5.0 µg/mL of 6-MeMPR at various times subsequent to mutagenesis.
(A) MNNG
(B) EMS

● , mutagen-treated culture;
▲ , control, untreated culture.

Having determined the optimum conditions for the selection of AK^- HeLa mutants, the concentration-response of the AK locus after treatment with various mutagenic agents was investigated. However, before any chemical is examined for mutagenicity, it is important to determine the concentration range over which it is biologically effective since various physical and chemical agents differ greatly in their toxic and mutagenic potencies. As there appears to be a good correlation between the toxic and mutagenic concentrations of various chemicals (Clive et al., 1979), one criterion that has been used to indicate biological effectiveness is the effect of a chemical on cell survival. In general, it is considered that the concentrations of a chemical that reduce cell survival to between 90 and 10% provide an appropriate range for studying mutagenicity (Arlett, 1977; O'Neill et al., 1977; Clive et al., 1979; Carver et al., 1980; Gupta and Siminovitch, 1980a; Gupta and Singh, 1982; Gupta, 1984). Therefore, if a chemical does not elicit a mutagenic response after reducing cell survival to approximately 10%, it is usually considered nonmutagenic in that particular test system.

The agents used in this work, including the alkylating agent, ethylmethanesulfonate (EMS), the heterocyclic DNA-intercalating agent, ICR-170 and ultraviolet (UV)-light (λ=254nm), possess mutagenic activity

in a variety of assay systems (Searle, 1984). To determine their biologically effective concentration range toward HeLa cells, the effects of various concentrations of these chemicals (or radiation) on HeLa cell survival were analyzed in two independent experiments and the results are presented in Table 10. The appropriate concentration ranges for mutagenesis of HeLa cells using EMS, ICR-170 and UV-light were determined to be 100 to 400 µg/mL, 0.25 to 2.0 µg/mL and 47.0 to 235.0 ergs/mm^2 respectively.

Knowing this, the concentration-response of the AK locus after treatment by these agents was investigated. HeLa cells were exposed to three or four different concentrations of each mutagenic agent which resulted in between 10 and 90% cell killing (as determined from Table 10) and after an 8 day expression period, the mutagenized cultures were plated separately at a cell density of $1x10^6$ cells/P-100 dish in the presence of 5.0 µg/mL of 6-MeMPR to select for AK^- mutants. The results presented in Figure 11(A-C) show that for the three mutagenic agents examined, there existed a nearly linear concentration-dependent increase in the frequency of mutants at the AK locus. Similar results were obtained with these mutagens in two independent experiments (except for EMS where only one experiment was done).

Table 10. Determination of the Biologically Effective Concentration Range of Various Mutagens Toward HeLa Cells

Mutagenic agent	Duration of treatment of HeLa cells	Concentration or dose of the mutagen administered	Surviving fraction[a] (%)
EMS	20 hours	100 µg/mL	92.1
		200	81.5
		300	55.6
		400	11.2
ICR-170	16 hours	0.25 µg/mL	88.4
		0.5	77.4
		1.0	39.6
		2.0	15.6
		3.0	6.9
UV-light	2 seconds	47.0 ergs/mm^2	76.8
	4	94.0	67.3
	6	141.0	58.3
	8	188.0	38.7
	10	235.0	17.9
	15	352.5	5.2

The indicated concentration or dose of each mutagen was used to treat $1x10^7$ actively growing HeLa cells for the duration indicated. Subsequently, 250 mutagenized cells representing each concentration/dose were plated in regular medium and

Footnote to Table 10 (cont'd).

the number of surviving colonies determined after growth for 11 to 12 days.

[a] The values presented represent the mean number of HeLa cells surviving mutagen treatment as determined by duplicate experiments. The variation in the number of surviving colonies between experiments was minimal and the standard error was therefore omitted from the table.

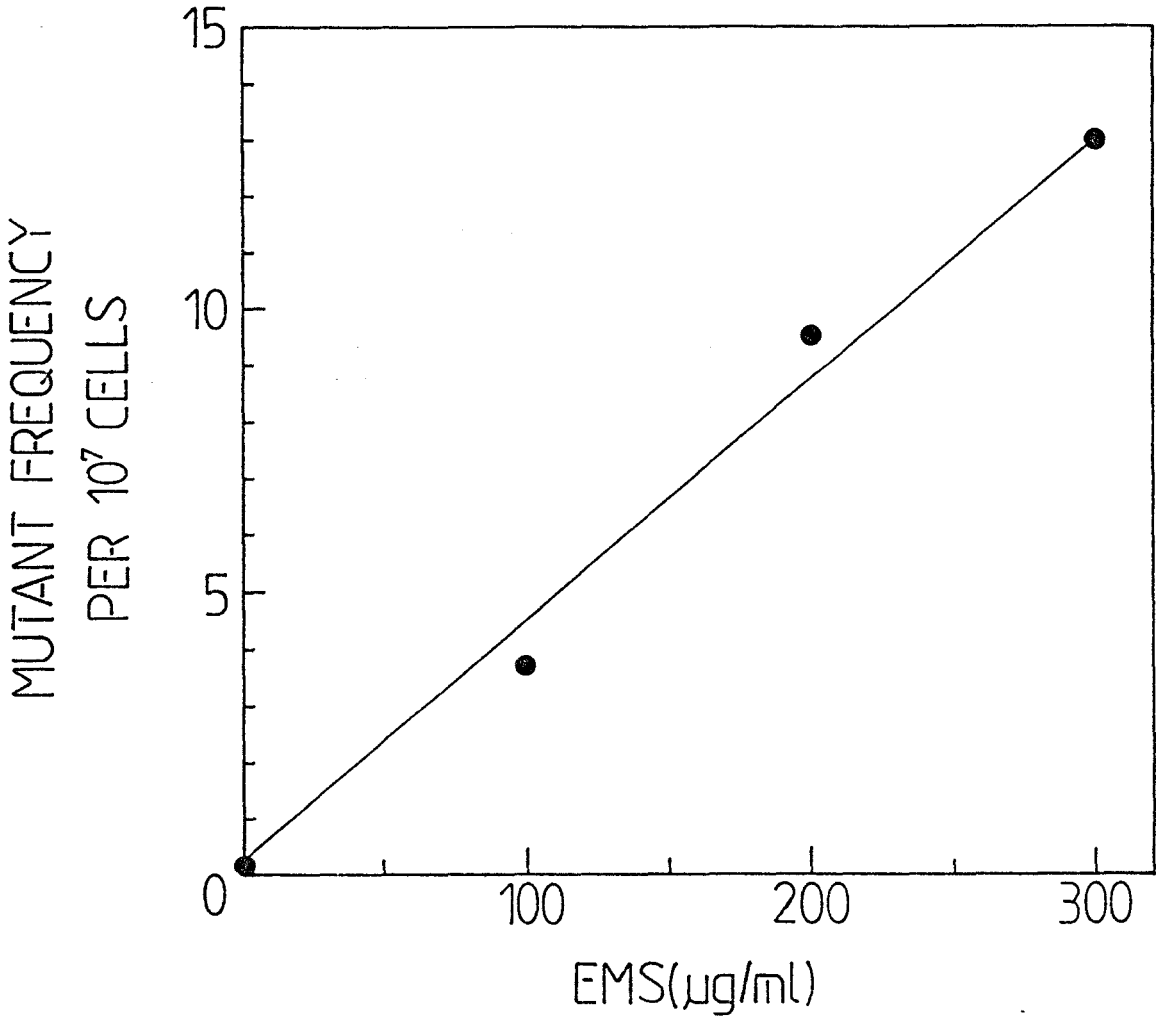


Figure 11. The effect of different concentrations of various mutagenic agents on the frequencies of AK^- mutants in the HeLa cell line. HeLa cells were exposed to three or more different concentrations of the mutagenic agent which resulted in between 10 and 90% cell killing, and after an 8 day expression period, the mutagenized cells were plated in the presence of 6-MeMPR (5.0 µg/mL) to select for AK^- mutants as described in the Methods.
A) The concentration response of the AK locus to ethylmethanesulfonate (EMS). The values represent the mutant frequencies determined from plating 10^7 mutagenized Hela cells/mutagen concentration.

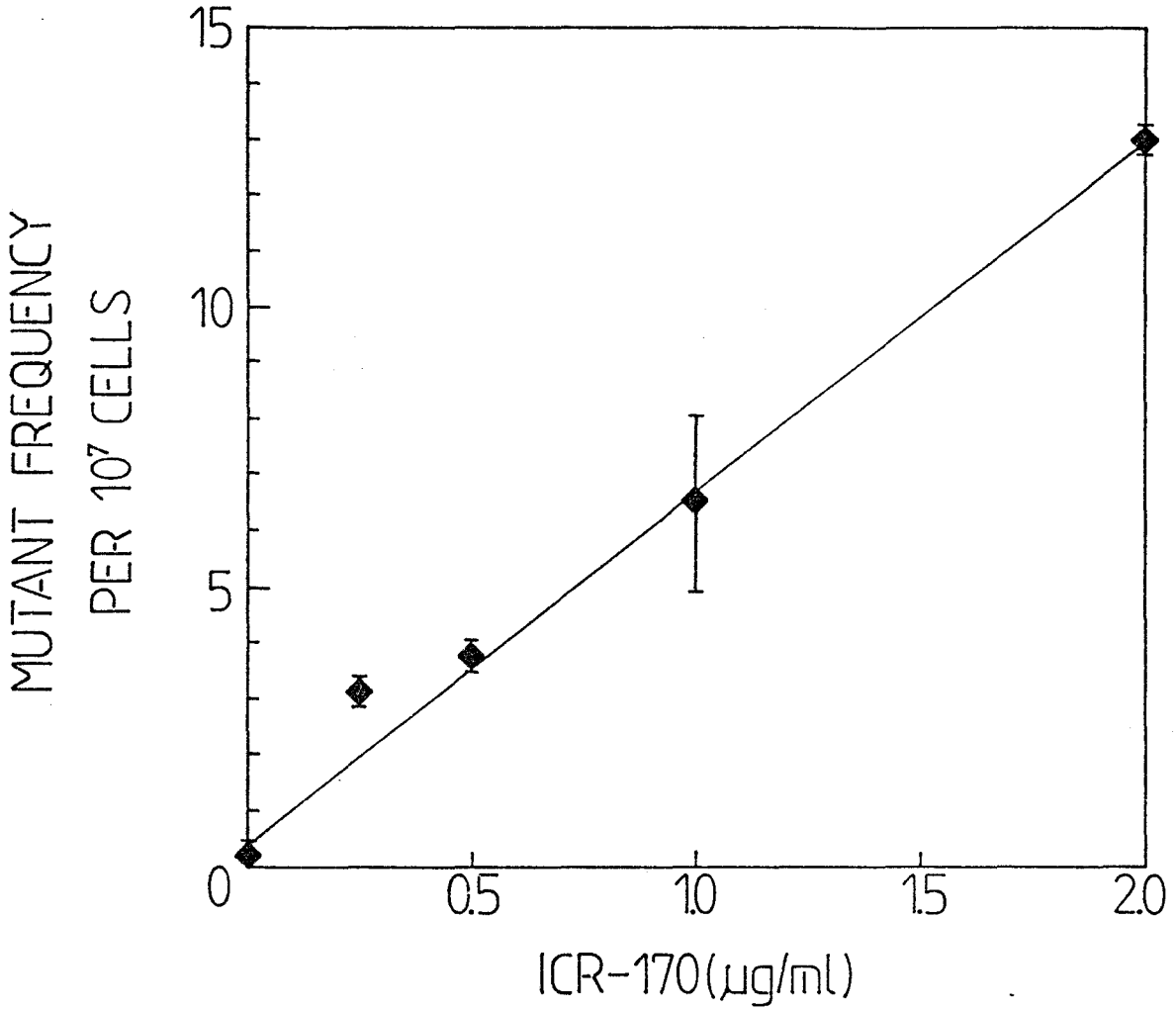


Figure 11. B) The concentration response of the AK locus to ICR-170. The values represent the mean mutant frequencies ($\pm$ standard errors) determined from plating 10^7 mutagenized Hela cells/mutagen concentration in duplicate experiments.

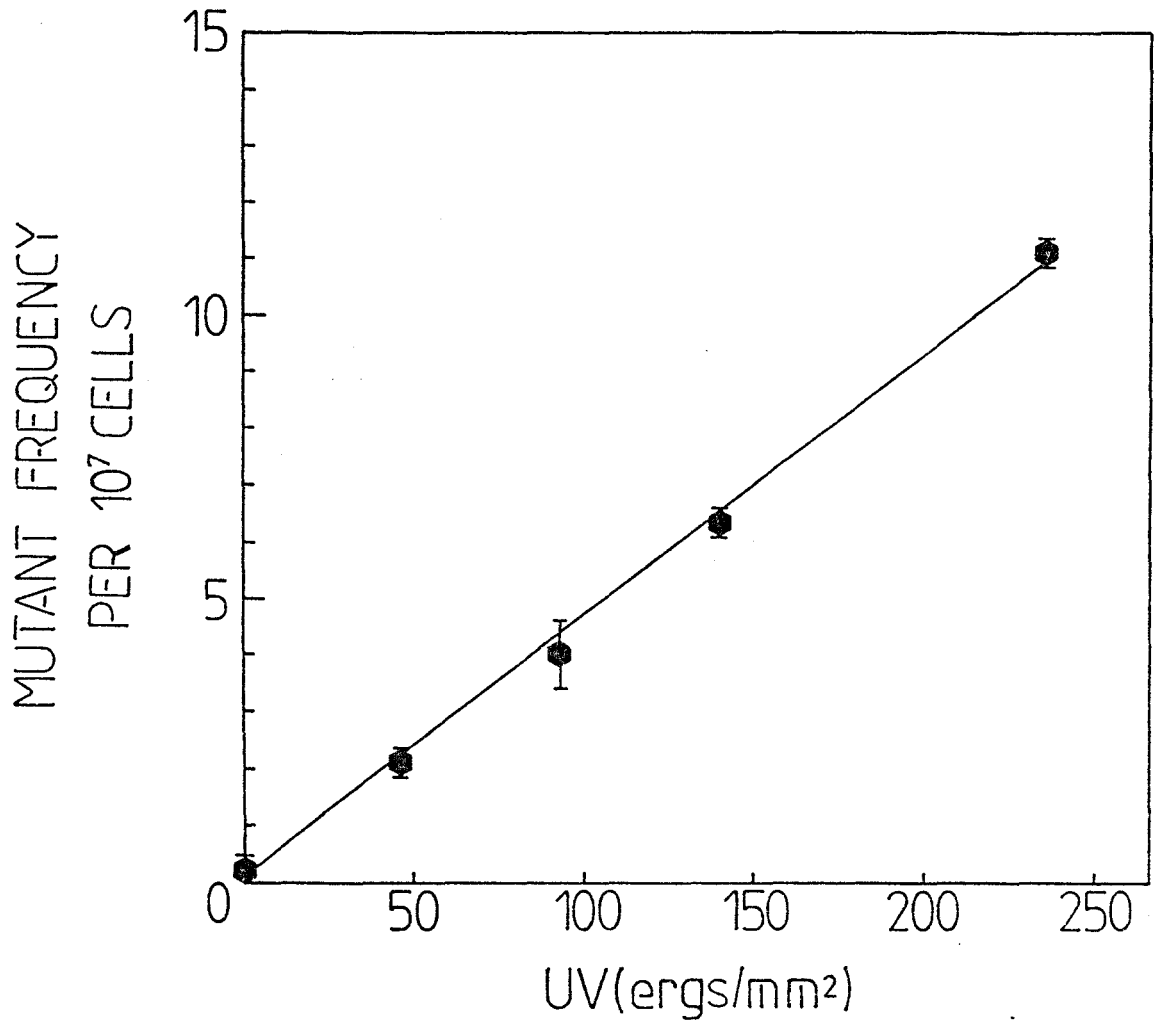


Figure 11. C) The concentration response of the AK locus to ultraviolet (UV)-light. The values represent the mean mutant frequencies ($\pm$ standard errors) determined from plating 10^7 mutagenized Hela cells/mutagen concentration in duplicate experiments.

3.3.2 Discussion

From the point of view of quantitative mutagenesis and mutagen detection studies, an ideal genetic marker should possess the following characteristics: (a) it should be readily selectable, with all the resultant clones showing similar genetic and biochemical phenotypes, (b) it should require a relatively short period of phenotypic expression and (c) it should be responsive to a variety of different acting mutagens in a dose-dependent manner. Various drug resistant genetic markers that meet these requirements have been developed in other mammalian systems (Hollstein et al., 1979; Ki et al., 1984). However, there still exists a need to develop accurate and reliable drug resistant genetic markers which satisfy these criteria when used as end points for quantitative mutagenesis purposes in human cells. Consequently, the objective of the present work was to investigate the potential of the AK locus, which has shown great promise in related work in CHO cells (Gupta and Singh, 1983), to serve as a useful drug resistant genetic marker for studies related to mutagenesis in human cells. This work, therefore, describes the optimal conditions for the selection of AK^- mutants and the utility of the AK locus for quantitative mutagenesis studies in a "model" human system, HeLa cells.

The results presented concerning the similar mutant frequency, cross-resistance patterns and AK activity deficiency of all the TOY^{rI}, TUB^{rI} and 6-$MeMPR^{rI}$ mutants indicate that similar mutants were selected in HeLa cells using either toyocamycin, tubercidin or 6-MeMPR. Thus, selection for genetic alterations at the AK locus in human cells satisfies at least one criterion of an ideal genetic marker; that is, drug resistant mutants are readily selectable and exhibit similar phenotypes.

In theory, any of the three aforementioned nucleoside analogues could be used for the selection of AK^- HeLa mutants since similar mutants would be obtained in each case. However, for several reasons, 6-MeMPR was chosen as the selective agent and its optimum selective concentration was found to be 5.0 µg/mL. Having determined this, two other selective parameters, the optimum cell density during selection and the optimum time required for the expression of the mutant phenotype following mutagen treatment, were investigated. A concentration of $1x10^6$ HeLa cells/P-100 dish was optimal for AK^- mutant selection since no cross-feeding effects were observed at this cell density. The absence of any cross-feeding effects in a selection system for AK^- mutants has been reported previously (Gupta and Singh, 1983). In this system, there was no effect of cell density on the recovery of CHO (AK^-) mutants at cell concentrations of up to $5x10^5$ cells/P-100 dish.

The optimum expression time for the AK^- phenotype after mutagen treatment was determined in two independent experiments (involving either EMS or MNNG as the mutagenic agent) to be approximately 8 days. Although this expression time is significantly longer than that reported for other genetic markers in human cells (Ama^R=4 days, Buchwald and Ingles, 1976; Drb^R=5 days, Gupta and Siminovitch, 1980b; Oua^R=2 generations, Buchwald, 1977; Dip^r=5 days, Gupta and Siminovitch, 1978a), it is similar to that reported for the 6-$MeMPR^r$ (AK^-) marker in CHO cells (Gupta and Singh, 1983; Gupta, 1984) and for other markers, Thg^r and AA^r ($HGPRT^-$ and $APRT^-$), in human diploid fibroblasts which are indicative of genetic alterations in purine salvage pathway enzymes (Jacobs and DeMars, 1984). In addition, this expression period appears to be consistent with the time required for preexisting salvage enzymes (either AK, HGPRT or APRT) to be reduced to the very low level necessary before the mutant phenotype will appear (Gupta and Siminovitch, 1980a; Jacob and DeMars, 1984). Thus, selection for mutations at the AK locus in human cells appeared to satisfy the second criterion of a good genetic marker; that is, phenotypic expression occurred within a relatively short period subsequent to mutagenesis.

The final criterion used to evaluate the ability of the AK locus to serve as a drug resistant end point for

quantitative mutagenesis studies in human cells was the concentration-response of this locus to treatment with different mutagenic agents. Paralleling the earlier work of Gupta and Singh (1983), the three direct acting mutagens chosen for this purpose were EMS, ICR-170 and ultraviolet-light. The mutagenic activity of each of these agents has been previously characterized as follows: (a) in mammalian cells, EMS has been shown to ethylate primarily the N-7 moiety of guanine which results in predominantly G:C to A:T transitions or less frequently G:C to C:G (or T:A) transversions (Malling and de Serres, 1969; Freifelder, 1983), (b) ICR-170 is a heterocyclic DNA-intercalating agent that has been shown, at least in prokaryotic systems, to be a very effective frameshift mutagen and frequently to induce base pair substitutions (Drake and Baltz, 1976; Fuscoe et al., 1979) and (c) in mammalian cells, the predominant genetic lesions induced in DNA by UV-irradiation are thymine dimers. These dimers are removed by the error-free excision repair process or, when this is saturated, by other error-prone repair processes that induce numerous genetic changes including base substitutions (transitions), frameshifts and deletions (Trosko et al., 1965; Hanawalt et al., 1979).

Using these mutagenic agents, the results show that the frequency of AK^- HeLa mutants increased in a nearly linear concentration-dependent manner. Even though the 6-

MeMPR^r mutants were rare in nonmutagenized HeLa cells (frequency ranges from 0.5 to 1.0x10^{-7}), treatment of HeLa cells with these mutagens at the concentrations indicated enhanced the frequency of such mutants at least 10-fold. The frequency of 6-MeMPR^r mutants in HeLa cells is thus similar to that observed in human diploid fibroblasts resistant to α-amanitin (Buchwald and Ingles, 1976), DRB (Gupta and Siminovitch, 1980b) and ouabain (Buchwald, 1977) but lower than that observed for resistance to diptheria toxin (Gupta and Goldstein, 1981) and 6-thioguanine (Jacobs and Demars, 1984). This response of the AK locus to EMS, ICR-170 and UV-light indicated that this selection system, like that reported for 6-MeMPR^r in CHO cells (Gupta and Singh, 1983) and Thg^r/AA^r in human diploid fibroblasts (Jacobs and DeMars, 1984), was capable of detecting not only base substitution types of alterations, but frameshifts and possibly deletions as well. The detection of different types of genetic alterations by the 6-MeMPR^r selection system was expected. This results because AK (like HGPRT/APRT) is a purine salvage pathway enzyme that is not essential for growth under normal conditions and any genetic lesion that results in loss or inactivation of AK (by mutations in either the structural gene or regulatory region) will not affect cell viability and should be detected.

Collectively, the results of this work reveal that the 6-MeMPR^r selection system shows many useful attributes for quantitative mutagenesis studies involving human cells in culture. These include:

(a) 6-MeMPR^r mutants were obtained spontaneously in human (HeLa) cells in culture [the mutation frequency was low however (0.5 to 1.0×10^{-7})],

(b) mutants obtained were >40,000-fold more resistant to 6-MeMPR than were parental HeLa cells,

(c) all the 6-MeMPR resistant clones examined were found to possess stable drug resistant phenotypes in the absence of drug,

(d) the mutants were affected in a known phenotype that has been well characterized (Bennett et al., 1966; Thacker, 1980; Gupta and Singh, 1983; Yamanaka, 1984),

(e) the selection system was free from cell density or cross-feeding effects such that up to 1×10^6 cells could be plated/P-100 dish for selection purposes,

(f) after mutagen treatment, a relatively short time period was required for maximum expression of the mutant phenotype,

(g) the frequency of 6-MeMPR^r mutants increased in a linear dose-dependent manner upon treatment of HeLa cells with a number of direct acting mutagens,

(h) AK is dispensible under normal conditions and therefore all mutations that sufficiently reduce AK activity, even to complete absence, can be detected as viable mutants.

As such, it appeared that selection for genetic alterations at the AK locus provided a valuable system for studies concerned with quantitative mutagenesis in human cells.

Due to their favourable growth characteristics in culture, HeLa cells were used to test the potential of the AK locus serving as an end point for quantitative mutagenesis studies involving human cells. However, future development of a mutagen and/or carcinogen screening system possessing human specificity, which is based upon the response of the AK locus, would best involve the use of normal, human diploid fibroblasts. The use of these cells offers two very important advantages over the use of HeLa cells in culture. First, normal human fibroblasts are not transformed and do not transform spontaneously to established permanent cell lines. Therefore, they should provide a better system representing the in vivo human processes. Second, in contrast to the unstable, aneuploid karyotype of HeLa cells, normal human fibroblasts possess highly stable, diploid karyotypes which are better suited to mutagenesis studies. Thus, use of the 6-MeMPR^r drug resistant marker as an end point indicative of mutation at the AK locus in normal, human diploid fibroblasts should create a screening system that possesses the ability to accurately identify those compounds which may be mutagenic and/or carcinogenic to the human population.

3.4 Drug Resistance and Biochemical Studies of the First- and Second-Step HeLa Mutants Resistant to Puromycin

As discussed in the Introduction, the details of the mechanism of puromycin-induced inhibition of protein synthesis are relatively well established. However, the same is not true for the development of cellular resistance to this aminoacyl-tRNA analogue. Affinity labelling studies have been used by several investigators in an attempt to identify ribosomal proteins from both prokaryotes and eukaryotes with which puromycin interacts (Pongs et al., 1973; Stahl et al., 1974; Cooperman et al., 1975; Grant et al., 1983; Weitzmann and Cooperman, 1985). Although these efforts have defined certain puromycin-binding ribosomal proteins, they have not contributed greatly to understanding the interaction of puromycin with the ribosomal peptidyl-transferase centre and have not contributed at all to understanding the development of cellular resistance to puromycin. Alternatively, somatic cell mutants have been used to identify the ribosomal proteins with which drugs interact and thereby interfere with protein synthesis. For example, Chinese hamster cells resistant to emetine have an alteration in the ribosomal protein S20, implying that this is the protein to which emetine binds to inhibit protein synthesis in sensitive cells (Boersma et al., 1979; Reichenbacher and Caskey, 1979). It has been this approach,

the genetic and biochemical study of somatic cell mutants, that has provided most of the information concerning the development of cellular resistance to puromycin.

Studies of the genetic basis of puromycin resistance were first undertaken by Leiberman and Ove (1959a,b). They proposed that in mouse fibroblasts two levels of puromycin resistance (each having a different mutation rate) existed and that mutants were the result of pre-existing variants in the population rather than induced by the selective agent. However, Harris (1967) showed that in pig kidney cells puromycin resistance could result from both genetic and unstable, non-genetic variation. In the case of non-genetic variation, resistance was increased in proportion to cell density. In an extension of this work, Cass (1972) showed that the biochemical basis of non-genetic resistance to puromycin at high cell densities was associated with a decreased incorporation of puromycin. Subsequently, colchicine resistant CHO cells were isolated and found to be cross-resistant to puromycin (Ling, 1975). These variants were found to possess an altered membrane glycoprotein which lowered the rate of passive diffusion of colchicine and other molecules including puromycin. Paralleling this, Morrow et al. (1980) have isolated puromycin resistant clones from V79 Chinese hamster cells. Resistance in these variants was unstable in the absence of puromycin, appeared

to be due to a lowered uptake of puromycin and was inversely proportional to puromycin uptake rate. A recent report dealing with the cellular development of puromycin resistance (Fallon and Stollar, 1982) involves the characterization of one first-step and two second-step stable puromycin resistant clones isolated from a cultured mosquito cell line. The first-step and one second-step mutant were found to be puromycin resistant as a direct result of decreased membrane permeability to this drug. However, the other second-step mutant was unique among all the puromycin resistant variants/mutants isolated to date since several criteria (puromycin transport, cross-resistance to colchicine, sensitivity of in vitro protein synthesis by mutant cell extracts to puromycin) suggested that puromycin resistance was the result of an alteration at the level of the cellular protein synthetic apparatus.

It appears that the development of cellular resistance to puromycin is in almost every instance associated with an alteration in membrane permeability that reduces drug uptake. However, it has been possible to isolate puromycin resistant mutants that appear to be affected at the level of protein synthesis (Fallon and Stollar, 1982). Unfortunately, further characterization of the nature of the specific alteration affecting membrane permeability or the protein synthetic machinery has not been

achieved. This, in conjunction with the fact that no puromycin resistant human cell mutants have been reported, led us to attempt to isolate and characterize puromycin resistant HeLa cell mutants using a genetic approach. Efforts were made to: (a) select first-step puromycin resistant HeLa mutants, (b) select more highly puromycin resistant second-step HeLa mutants from the first-step mutants, (c) determine the degrees of resistance and cross-resistance of these first- and second-step mutants to various anticancer agents and (d) determine whether the lesion responsible for puromycin resistance in these mutants affects the membrane permeability of cells to this and other drugs or specifically alters some component of the cellular protein synthetic apparatus.

The present work describes the successful isolation and partial characterization of first- and second-step HeLa mutants resistant to puromycin. The results suggest that in human cells the most common mechanism responsible for the development of cellular resistance to puromycin involves the occurrence of a genetic lesion which nonspecifically reduces the membrane permeability of cells to puromycin and other unrelated anticancer agents.

3.4.1 Results

3.4.1.1 Selection of First- and Second-Step Mutants from HeLa Cells Using Increased Concentrations of Puromycin

Before selecting any mutants resistant to puromycin, the sensitivity of HeLa cells to this drug was determined. Figure 12 shows that these cells were resistant up to a concentration of approximately 0.06 µg/mL puromycin but at higher concentrations (between 0.07 and 0.12 µg/mL) their plating efficiency decreased drastically so that the D_{10} value of puromycin for HeLa cells was approximately 0.10 µg/mL. At puromycin concentrations greater than 0.12 µg/mL, no surviving cells were observed.

The selection of puromycin resistant mutants from human (HeLa) cells was carried out such that the resistance exhibited by any mutant was at least 2-fold. This approach seemed logical since puromycin resistant mosquito and Chinese hamster cell mutants have been shown to exhibit between a 2- to 7-fold and 10-fold increase, respectively, in resistance to this drug (Morrow et al., 1980; Fallon and Stollar, 1982).

Mutant selection was attempted in both mutagen-treated (400 µg/mL of EMS for 20 hours; plated on the third day subsequent to mutagen treatment) and untreated HeLa cells using 0.25 µg/mL of puromycin. In nonmutagenized cultures, no resistant colonies were observed when a total

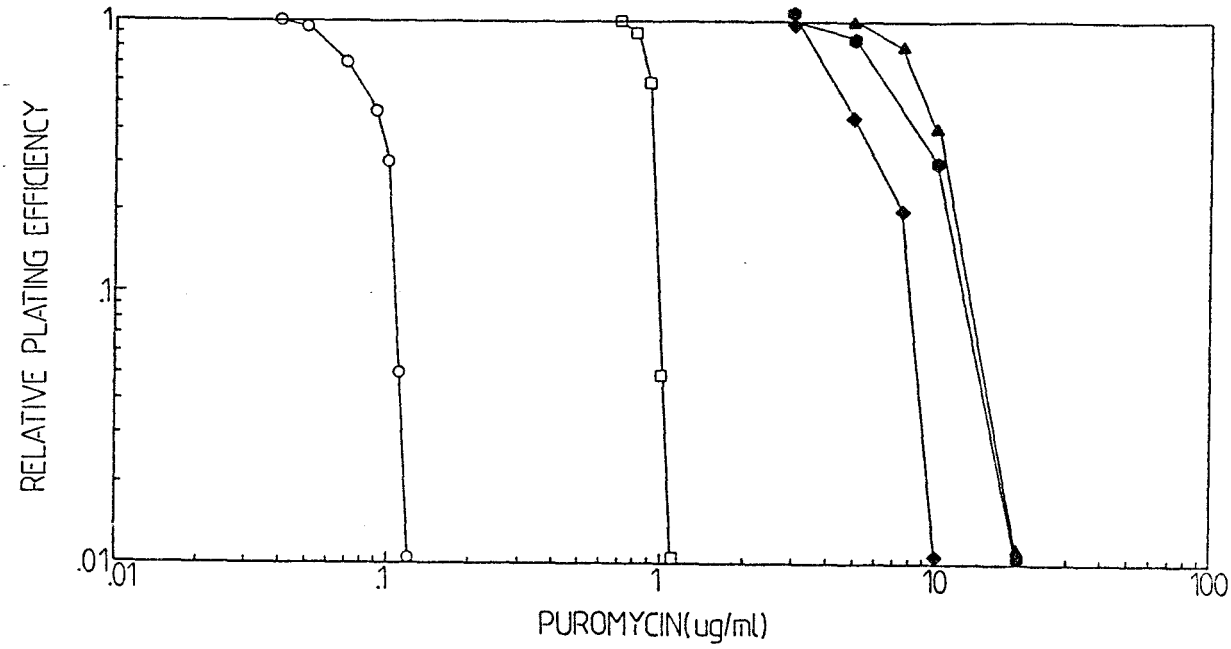


Figure 12. Dose-response curves of the parental sensitive cells and the first- and second-step puromycin resistant mutants in the presence of increasing concentrations of puromycin. The relative plating efficiencies of the various cell lines in the presence of different drug concentrations were determined by measuring the degree of resistance in 24-well dishes, as described in the Methods.
○ , HeLa cells;
□ , PUR^{rI}-27;
▲ , PUR^{RII}-26D;
⬢ , PUR^{rII}-27C;
◆ , PUR^{rII}-28B.

of $1.6x10^7$ viable cells were plated (mutation frequency $<6.3x10^{-8}$) whereas pretreatment with EMS (400 µg/mL) resulted in the appearance of resistant colonies at a frequency of approximately $5.0x10^{-7}$. Three individual colonies, isolated from a single experiment, were picked, grown in non-selective medium and tested for their degree of resistance to puromycin. Of these, only one proved to be significantly (>3-fold) more resistant to puromycin than the parental (sensitive) cells. The dose-response curve of this mutant (PUR^{rI}-27) toward puromycin is shown in Figure 12. It indicates that the D_{10} value of puromycin toward the PUR^{rI}-27 mutant was approximately 0.98 µg/mL which meant that this mutant was about 10-fold more resistant to puromycin than the parental HeLa cells. The resistance of this mutant to puromycin was found to be stably retained upon growth in non-selective medium for more than one year.

The PUR^{rI}-27 mutant was used to select second-step mutants that exhibited an even higher degree of resistance to puromycin. For this purpose, both mutagenized (350 µg/mL of EMS for 20 hours; plated on the third day subsequent to mutagenesis) and nonmutagenized PUR^{rI}-27 cells were plated in the presence of 10 µg/mL of puromycin. From nonmutagenized cells, one highly resistant colony was obtained (mutation frequency approximately $3.3x10^{-7}$) whereas pretreatment with EMS (350 µg/mL) resulted in the appearance

of highly puromycin resistant colonies at a frequency of about 1.4×10^{-6}. A total of three second-step colonies (PUR^{rII}-26D, derived from nonmutagenized PUR^{rI}-27 cells and PUR^{rII}-27C and 28B, derived from mutagenized PUR^{rI}-27 cells) were picked, grown in the presence of 10 μg/mL puromycin (since the stability of their resistant phenotype was unknown) and tested for their degree of resistance to puromycin. The dose-response curves of each of these mutants toward puromycin are presented in Figure 12. The D_{10} values of puromycin toward PUR^{rII}-26D, PUR^{rII}-27C and PUR^{rII}-28B were approximately 13.0 μg/mL, 12.5 μg/mL and 8.0 μg/mL, respectively, which indicated that these mutants were 130-, 125- and 80-fold more resistant to puromycin, respectively, than HeLa cells. Similar estimates of D_{10} for these first- and second-step mutants have been obtained in at least three independent experiments.

3.4.1.2 Cross-resistance Patterns of First- and Second-Step Puromycin Resistant Mutants to Various Anticancer Agents

Earlier work with mammalian cell mutants resistant to various anticancer agents (emetine, ouabain, podophyllotoxin, taxol, vinblastine, VM26 and VP16-213) has shown that the cross-resistance of these mutants toward other drugs and inhibitors can provide important information regarding the mechanism of action of and development of resistance to a

particular drug, as well as information concerning the nature of the genetic lesion present in the mutant cells (Gupta et al., 1980; Gupta, 1981, 1983a,b,d, 1985). Therefore, the cross-resistance patterns of the first-step puromycin resistant mutant (PUR^{rI}-27) and the three second-step puromycin resistant mutants (PUR^{rII}-26D, PUR^{rII}-27C and PUR^{rII}-28B) toward various anticancer agents were determined.

The anticancer agents examined included: (a) drugs that directly interact with DNA (aclacinomycin A, actinomycin D, adriamycin, bisantrene, cis-Platin, daunomycin, ellipticine, mithramycin, mitomycin C and mitoxantrone), (b) drugs that affect microtubule function and other cellular processes (colchicine, maytansine, taxol, vinblastine, harringtonine, VM26 and VP16-213) and (c) drugs that are base analogues or antimetabolites (ara-C, 5-fluorouracil, methotrexate and MGBG). Tables 11 and 12 reveal the cross-resistance patterns of the PUR^{rI}-27, PUR^{rII}-26D, PUR^{rII}-27C and PUR^{rII}-28B mutants toward these agents.

With reference to the drugs that directly interact with DNA, the PUR^{rI}-27 mutant exhibited partial cross-resistance to aclacinomycin A (2.7-fold), bisantrene (3.5-fold), cis-Platin (2.0-fold) and mitomycin C (1.6-fold) and a high degree of cross-resistance to actinomycin D (16.0-

fold), adriamycin (18.5-fold), daunomycin (9.9-fold), mithramycin (7.9-fold) and mitoxantrone (119.2-fold), relative to Hela cells. Ellipticine was the only anticancer agent of this type toward which PUR^{rI}-27 exhibited normal sensitivity. Relative to PUR^{rI}-27, the second-step puromycin resistant mutants maintained their sensitivity to ellipticine and their partial resistance to aclacinomycin A (except for PUR^{rII}-27C which was 10-fold cross-resistant), cis-Platin and mitomycin C. However, compared to the PUR^{rI}-27 mutant, their relative degree of cross-resistance increased toward bisantrene (2.9- to 6.9-fold), actinomycin D (1.6- to 11.3-fold), adriamycin (3.8- to 9.2-fold), daunomycin (6.0- to 10.7-fold), mithramycin (>3.3-fold) and mitoxantrone (2.9- to 8.1-fold).

The PUR^{rI}-27 mutant also exhibited significant cross-resistance toward the microtubule poisons (colchicine, maytansine, taxol and vinblastine), the protein synthesis inhibitor, harringtonine and VM26 and VP16-213, compounds which affect DNA metabolism. Relative to Hela cells, this mutant was found to be 8.8-, 5.4-, 21.5- and 8.6-fold resistant to colchicine, maytansine, taxol and vinblastine, respectively, as well as 3.9-fold resistant to harringtonine and greater than 5.0-fold resistant to VM26 and VP16-213. Second-step mutants exhibited a further increase in the degree of cross-resistance to each of these anticancer

Table 11. Cross Resistance Patterns of the First-Step Puromycin Resistant Mutant (PUR^{rI}-27) Toward Various Anticancer Agents

Anticancer agent	D_{10} value of the drug for HeLa cells[a] (ng/mL)	D_{10} value of the drug for PUR^{rI}-27[a] (ng/mL)	Relative resistance of PUR^{rI}-27[b]	Comment[c]
Aclacinomycin A	3.1	8.3	2.7	PR
Actinomycin D	0.1	1.6	16.0	R
Adriamycin	2.0	37.0	18.5	R
Bisantrene	0.74	2.6	3.5	PR
cis-Platin	150.0	300.0	2.0	PR
Daunomycin	3.6	35.5	9.9	R
Ellipticine	130.0	115.0	0.9	N
Mithramycin	20.0	157.9	7.9	R
Mitomycin C	26.5	43.0	1.6	PR
Mitoxantrone	5.2	620.0	119.2	R
Colchicine	2.1	18.5	8.8	R
Maytansine	0.076	0.41	5.4	R
Taxol	1.65	35.5	21.5	R
Vinblastine	0.64	5.5	8.6	R
Harringtonine	36.0	140.0	3.9	PR

Anticancer agent	D_{10} value of the drug for HeLa cells[a] (ng/mL)	D_{10} value of the drug for $PUR^{rI}-27$[a] (ng/mL)	Relative resistance of $PUR^{rI}-27$[b]	Comment[c]
VM26	10.0	64.0	6.4	R
VP16-213	185.0	920.0	5.0	R
ara-C[d]	28.0	27.0	1.0	N
5-Fluorouracil[d]	300.0	500.0	1.7	PR
Methotrexate[d]	4.0	5.0	1.3	N
MGBG	1.7×10^{-6}M	1.7×10^{-6}M	1.0	N

[a] The D_{10} value of a drug toward a cell line represents the concentration of the drug which reduces the plating efficiency of the cells to 10% of that observed in the absence of any drug. The D_{10} values of various drugs for the HeLa and mutant cell lines were obtained by determining the relative plating efficiencies of the cell lines in the presence of different drug concentrations.

[b] Assuming the D_{10} values of various drugs toward HeLa cells to be 1, the relative degree of resistance of the mutant cell line was obtained as the ratio of the D_{10} value of the mutant cell line to the HeLa cell line.

[c] Depending on the degree of cross-resistance (or sensitivity) toward a given drug, the behaviour of the $PUR^{rI}-27$ mutant was classified as N=normal (degree of resistance between 0.7- and 1.3-fold), PR=partially resistant (degree of resistance between 1.4- and 4.0-fold), R=resistant (degree of resistance >4.0-fold) or S=sensitive (degree of resistance <0.7-fold).

[d] The degree of resistance toward these drugs was determined in α-MEM minus nucleosides (special) medium supplemented with 10% dialyzed fetal calf serum.

drugs. Their relative increases in cross-resistance were 5.8- to 11.6-fold for colchicine, 3.8- to 12.2-fold for maytansine, 7.3- to 10.4-fold for taxol, 3.6- to 13.6-fold for vinblastine, 2.3- to 9.6-fold for harringtonine, 4.1- to 10.3-fold for VM26 and 4.2- to 10.8-fold for VP16-213 over that exhibited by the PUR^{rI}-27 mutant.

In contrast to most of the aforementioned drugs, base analogues and antimetabolites were agents toward which the first- and second-step puromycin resistant mutants exhibited normal sensitivity relative to Hela cells. In this regard, the PUR^{rI}-27 mutant showed no cross-resistance toward ara-C, methotrexate and MGBG and only a slight degree of cross-resistance (1.7-fold) toward 5-fluorouracil. By comparison, the PUR^{rII} mutants were normally sensitive to ara-C, 5-fluorouracil and MGBG and slightly cross-resistant (1.2- to 1.9-fold) to methotrexate.

These studies revealed that the first- and second-step puromycin resistant mutants were cross-resistant to a wide variety of unrelated anticancer agents. Some of these drugs (colchicine, daunomycin, VM26 and VP16-213) were examined because many drug resistant mammalian cell mutants, which exhibit decreased membrane permeability, show increased resistance to these compounds (Bech-Hansen et al., 1976; Ling et al., 1979; Gupta, 1983d, 1985). Therefore, it appears that the 10-fold increase in resistance to puromycin

Table 12. Cross-Resistance Patterns of the Second-Step Puromycin Resistant Mutants Toward Various Anticancer Agents

Anticancer agent	D_{10} value of the drug for HeLa cells[a] (ng/mL)	Relative resistance[b] of PUR^{rII-} 26D	PUR^{rII-} 27C	PUR^{rII-} 28B	Comment[c]
Aclacinomycin A	3.1	1.6	10.0	3.0	PR[e]
Actinomycin D	0.1	26.0	180.0	50.0	R
Adriamycin	2.0	70.0	170.0	88.5	R
Bisantrene	0.74	10.1	24.3	13.0	R
cis-Platin	150.0	1.8	3.5	1.3	PR
Daunomycin	3.6	104.2	105.6	58.9	R
Ellipticine	130.0	0.7	1.2	0.8	N
Mithramycin	20.0	26	26	26	R
Mitomycin C	26.5	1.7	2.4	1.8	PR
Mitoxantrone	5.2	346.2	961.5	365.4	R
Colchicine	2.1	69.5	102.4	51.0	R
Maytansine	0.076	20.4	66.0	38.2	R
Taxol	1.65	127.3	224.2	157.6	R
Vinblastine	0.64	31.3	117.2	46.9	R
Harringtonine	36.0	9.0	37.5	11.1	R

Anticancer agent	D_{10} value of the drug for HeLa cells[a] (ng/mL)	Relative resistance[b] of PUR^{rII}- 26D	PUR^{rII}- 27C	PUR^{rII}- 28B	Comment[c]
VM26	10.0	41.5	66.0	26.5	R
VP16-213	185.0	21.6	54.1	21.1	R
ara-C[d]	28.0	1.0	0.8	1.1	N
5-Fluorouracil[d]	300.0	1.0	1.1	0.9	N
Methotrexate[d]	4.0	1.9	1.6	1.2	PR[e]
MGBG	1.7×10^{-6}M	0.9	1.2	0.9	N

[a,b] The D_{10} values of the anticancer agents toward the HeLa and mutant cell lines, as well as the relative degree of resistance of the mutant cell lines, were determined as described in the legend of Table 11.

[c] The degree of cross-resistance exhibited by the PUR^{rII}-26D, PUR^{rII}-27C and PUR^{rII}-28B mutants was classified in the same manner as that for the PUR^{rI}-27 mutant: N=normal (degree of resistance between 0.7- and 1.3-fold), PR= partially resistant (degree of resistance between 1.4- and 4.0-fold), R=resistant (degree of resistance >4.0-fold) or S=sensitive (degree of resistance <0.7-fold).

[d] The degree of resistance toward these drugs was determined in special medium supplemented with 10% dialyzed fetal calf serum.

[e] For these drugs, the degree of cross-resistance was classified according to the behaviour of two of the three second-step mutants.

exhibited by the PUR^{rI}-27 mutant, as well as the further 8- to 13-fold increase in puromycin resistance shown by the PUR^{rII} mutants, probably results from a lesion that non-specifically affects the membrane permeability of these mutants to puromycin and other unrelated anticancer agents.

3.4.1.3 Uptake of (^{3}H)-Daunomycin by the Parental and Puromycin Resistant Cell Lines

Previous work involving various drug resistant mutants has shown that mutants which are decreased in membrane permeability exhibit substantially reduced uptake of (^{3}H)-daunomycin (Gupta, 1983b,d, 1985). Since the cross-resistance studies with both the first- and second-step puromycin resistant mutants indicate that these mutants probably exhibit a decrease in their membrane permeability toward various compounds, the uptake of (^{3}H)-daunomycin by the parental and mutant cell lines was examined. As revealed in Table 13, the initial rates of (^{3}H)-daunomycin uptake by PUR^{rI}-27, PUR^{rII}-26D, PUR^{rII}-27C and PUR^{rII}-28B mutants were approximately 68%, 42%, 34% and 47%, respectively, of that exhibited by parental (sensitive) HeLa cells. Thus, there appears to be a direct correlation between the significantly lower initial rates of (^{3}H)-daunomycin uptake and the increased degree of puromycin resistance shown by each mutant. In addition, the uptake of (^{3}H)-daunomycin by the PUR^{rI}-27 mutant was approximately 33% of that shown by the

Table 13. Uptake of (^{3}H)-Daunomycin by HeLa Cells and the First- and Second-Step Puromycin Resistant Mutants

Cell line	Duration of uptake (min)	(^{3}H)-daunomycin uptake/10^6 cells[a] (cpmx10^{-3})	Uptake relative to HeLa cells[b]
HeLa	10	6.24±0.24	100
	20	9.30±0.37	100
	40	10.66±0.10	100
	60	12.81±0.21	100
PUR^{rI}-27	10	4.22±0.09	67.6
	20	5.76±0.11	62.0
	40	4.60±0.07	43.1
	60	4.27±0.34	33.3
PUR^{rII}-26D	10	2.62±0.14	41.9
	20	2.45±0.16	26.4
	40	2.47±0.55	23.1
	60	2.16±0.28	16.9
PUR^{rII}-27C	10	2.11±0.07	33.8
	20	2.17±0.13	23.4
	40	2.46±0.14	23.1
	60	2.19±0.23	17.1

Cell line	Duration of uptake (min)	(^{3}H)-daunomycin uptake/10^6 cells[a] (cpmx10^{-3})	Uptake relative to HeLa cells[b]
PUR^{rII}-28B	10	2.93±0.14	47.0
	20	3.30±0.09	35.4
	40	2.87±0.35	27.0
	60	2.89±0.23	22.6

The cellular uptake of (^{3}H)-daunomycin by the various cell lines was studied as described in the Methods. The final concentration of (^{3}H)-daunomycin (specific activity, 1.5 Ci/mmole) in regular growth medium was $2x10^{-7}$M.

[a] Means ± standard errors of duplicate assays are presented.

[b] Assuming the average amount of (^{3}H)-daunomycin taken up by HeLa cells (for any time period) to be 100, the relative uptake in the mutant cell lines was calculated as the ratio of uptake by the mutant cells to that of HeLa cells.

parental HeLa cells during a 60 minute incubation period. By comparison, after 60 minutes, the amount of (^{3}H)-daunomycin taken up by the PUR^{rII}-26D, PUR^{rII}-27C and PUR^{rII}-28B mutants was 17%, 17%, and 22%, respectively, of that exhibited by the parental HeLa cells. This lower intracellular accumulation of (^{3}H)-daunomycin in the resistant lines indicates that an effectively lower internal concentration of puromycin is probably responsible for resistance. Similar results were obtained with each of these cell lines in two independent experiments.

The fact that the first- and second-step puromycin resistant mutants showed significantly reduced uptake of (^{3}H)-daunomycin (with respect to rate of uptake and amount accumulated) strongly suggests that the biochemical lesion present in these mutants affects their cellular permeability to various drugs.

3.4.2 Discussion

Although the alterations present in the first- and second-step mutants have not been fully characterized, the results obtained suggest that: (a) the initial isolate, PUR^{rI}-27, was resistant due to a decreased permeability to puromycin and (b) the PUR^{rII} mutants developed increased resistance to this antibiotic as a result of a second, probably similar, membrane alteration which further reduced their membrane permeability to puromycin. The several lines of evidence that support these inferences are:

(a) Relative to HeLa cells, the PUR^{rI}-27 mutant showed at least partial (1.3- to 4.0-fold), if not a high degree (>4.0-fold) of resistance toward certain drugs that either intercalate or interact directly with DNA (aclacinomycin A, actinomycin D, adriamycin, bisantrene, cis-Platin, daunomycin, mithramycin, mitomycin C and mitoxantrone), affect microtubule function/assembly (colchicine, maytansine, taxol and vinblastine), inhibit protein synthesis (harringtonine) or interfere with DNA metabolism (VM26 and VP16-213). Thus, the first-step mutant exhibited significant degrees of cross-resistance to one related (harringtonine) and a wide variety of other unrelated anticancer agents. These results, in conjunction with those of previous work which show that cross-resistance toward colchicine, daunomycin, VM26 and VP16-213 is associated with an alteration in the membrane permeability of drug resistant mutants (Bech-Hansen et al., 1976; Ling et al., 1979; Gupta, 1983d, 1985) including mutants resistant to puromycin (Fallon and Stollar, 1982), strongly suggest that the lesion present in the first-step mutant affects its membrane permeability to puromycin and other agents.

(b) Studies with the second-step mutants showed that they exhibited a significant further increase in resistance to several of these same unrelated agents. The PUR^{rII}-26D, PUR^{rII}-27C and PUR^{rII}-28B mutants showed an approximate 2-

to 9-fold increase in resistance over the PUR^{rI}-27 mutant toward actinomycin D, adriamycin, bisantrene, daunomycin, mithramycin, mitoxantrone, colchicine, maytansine, taxol, vinblastine, harringtonine, VM26 and VP16-213.

(c) Relative to the parental HeLa cells, both the initial rate of (^{3}H)-daunomycin uptake and the intracellular concentration of (^{3}H)-daunomycin after 60 minutes were significantly reduced in the PUR^{rI}-27 mutant and further reduced in the PUR^{rII}-26D, PUR^{rII}-27C and PUR^{rII}-28B mutants. These findings are in agreement with those reported by Morrow et al. (1980) which show that puromycin resistance in V79 Chinese hamster cell mutants is the result of a membrane defect that reduces both the initial rate of (^{3}H)-puromycin uptake by greater than 45% and the intracellular concentration of (^{3}H)-puromycin (after a 60 minute incubation) by greater than 70%.

To date, this is the first report of puromycin resistance in cultured human cells and the first report, in any system, of mutants resistant to these concentrations of puromycin (up to 13.0 μg/mL). The results suggest that, similar to the situation reported for other puromycin resistant mutants (Morrow et al., 1980; Fallon and Stollar, 1982), the most common mechanism for the development of cellular resistance to puromycin in Hela cells involves an alteration in membrane permeability that reduces drug

uptake. Since puromycin is a protein synthesis inhibitor, it might be expected that some of the mutants selected for puromycin resistance would represent an alteration in the cellular protein synthetic apparatus rather than membrane permeability. However, this was not the case with the one first-step and three second-step mutants characterized herein. At present, there has only been one report of a second-step puromycin resistant mutant that has resulted from an alteration at the level of the machinery necessary for translation (Fallon and Stollar, 1982). Additional information concerning the mechanism of action and development of resistance towards puromycin in human cells could be obtained if mutants affected in their protein synthetic apparatus were isolated. It may be that such puromycin resistant mutants are so rare, in comparison to those of the membrane permeability type, that a much larger group of mutants need be examined to obtain one affected in protein synthesis. Alternatively, the selection protocol used by Fallon and Stollar (1982), in which mutants are selected in the presence of the detergent, Tween 80, in order to maximize the recovery of clones affected in their protein synthetic apparatus and minimize the recovery of clones with an altered membrane permeability, could be employed to select puromycin resistant human cell mutants that are directly affected in protein synthesis.

A model for the mechanism of acquired resistance to colchicine by CHO cells, proposed by Ling (1975), may be of some relevance to the present work. Ling (1975) suggested that certain membrane proteins, such as the glycoprotein (M_r=165,000) that appears in the membranes of colchicine resistant mutants but not parental cell membranes, are modulators of membrane fluidity (mmf proteins). The molecular conformation of these proteins may regulate membrane permeability to a variety of compounds and hence determine resistance or cross-resistance exhibited by mutants. As yet, no attempt has been made to obtain definitive evidence that would allow us to make substantiated statements suggesting that a similar genetic lesion, which alters or induces protein(s) that modulate membrane fluidity, is responsible for the puromycin resistant of these HeLa mutants. However, it is interesting to note that the colchicine resistant CHO mutants reported by Ling (1975) are cross-resistant to puromycin and other anticancer agents, as are our puromycin resistant HeLa mutants. In an effort to increase our understanding of the development of puromycin resistance in human (HeLa) cells, further studies concerned with the identification of any affected membrane component(s) present in the first- and second-step mutants should be conducted. Initially, an attempt should be made to determine the lipid composition of

the parental and mutant plasma membranes in an effort to detect any notable differences in their cholesterol: phospholipid ratios or their percent unsaturated fatty acids that might account for altered mutant membrane permeability. Additionally, the protein composition of the parental and mutant plasma membranes could be analyzed by SDS-polyacrylamide gel electrophoresis in an attempt to detect any possible correlation between the appearance of a new mutant cell membrane glycoprotein and increased resistance to puromycin.

3.5 Drug Resistance and Biochemical Studies of the First- and Second-Step HeLa Mutants Resistant to Taxol

As discussed in the Introduction, taxol exhibits potent antileukemic and tumor inhibitory properties (Wani et al., 1971; Kisner et al., 1983) in addition to novel growth inhibitory effects toward numerous eukaryotic cell types (Lataste et al., 1984). Consequently, there is considerable interest in this microtubule stabilizing agent. At present, however, the mechanism of action of and development of resistance toward taxol are not totally understood.

One approach that should provide insight into both these areas combines genetics with biochemistry and morphology. The introduction of a specific genetic lesion into cells, followed by characterization of the nature of this lesion and its physiological consequence (drug resistance), allows one to study the role of cellular constituents in determining the phenotypic character of a mutant cell. In an attempt to use such an approach, several investigators (including ourselves) have isolated mutants exhibiting varying degrees of resistance toward the cytotoxic effects of taxol (Cabral et al., 1981, 1983; Warr et al., 1982; Gupta, 1983d; Cabral, 1983; Gupta, 1985; Roy and Horwitz, 1985). Cabral et al. (1981) have reported the isolation of a 2- to 3-fold taxol resistant CHO cell line which possesses an electrophoretic variant α-tubulin. This

alteration in α-tubulin appears to confer taxol resistance to this mammalian cell line. These investigators (Cabral, 1983; Cabral et al., 1983) have also described a taxol resistant CHO cell line that requires the continuous presence of taxol for normal cell division to occur. No changes in α- or β-tubulin were observed in this cell line. It was hypothesized that this mutant lacked a factor necessary for mitosis and taxol was able to substitute for it. Other work (Warr et al., 1982; Gupta, 1983d, 1985) has resulted in the isolation of stable 6- to 11-fold taxol resistant CHO mutants. Gupta (1983d) has suggested the existence of two different classes of taxol resistant CHO mutants. The first class, which probably possesses a genetic lesion that affects a microtubule-related component, shows cross-resistance to vinblastine but enhanced sensitivity to other microtubule inhibitors. In contrast, the second class is highly resistant to various microtubule inhibitors, as well as other different-acting drugs, and shows decreased cellular uptake of (^{3}H)-daunomycin. Thus, this genetic lesion probably non-specifically affects the membrane permeability of various drugs. Recently, Roy and Horwitz (1985) have reported the development of an 800-fold taxol resistant murine cell line that exhibits limited cross-resistance to both microtubule-related and other unrelated drugs. Interestingly, this work revealed a strong

correlation between the appearance of a M_r=135,000 phosphoglycoprotein in the plasma membrane and resistance to taxol. It was suggested that the appearance of this high molecular weight membrane glycoprotein may influence permeability to taxol.

This work was attempted in an effort to understand further the mechanism of action and development of resistance to taxol in human cells. Efforts were made: (a) to select first-step taxol resistant HeLa mutants, (b) once first-step mutants were isolated, to determine whether it was possible to select more resistant second-step HeLa mutants, (c) to determine the degrees of resistance and cross-resistance to various anticancer agents of any first- or second-step mutants and (d) to determine whether these mutants possess a lesion that specifically affects a microtubule-related cellular component or nonspecifically affects the membrane permeability of cells to various drugs.

The present studies describe the successful isolation and partial characterization of first- and second-step HeLa mutants resistant to taxol. The results suggest that, in human cells, the development of cellular resistance to taxol may result from two distinctly different mechanisms. One possible mechanism involves a biochemical lesion that specifically affects a microtubule-related cellular component. The second mechanism involves a lesion

which nonspecifically affects the membrane permeability of cells to various microtubule-related and unrelated drugs.

3.5.1 Results

3.5.1.1 Selection of First- and Second-Step Taxol Resistant Mutants from HeLa Cells

The effect of taxol on the relative plating efficiency of HeLa cells was determined as a preliminary step to selecting mutants resistant to this drug. Figure 13, which illustrates the dose-response curve, indicates that this microtubule poison was highly cytotoxic to human cells at a very low concentration. HeLa cells were resistant to a taxol concentration of about 1.0 ng/mL but their relative plating efficiency decreased sharply at higher drug concentrations. The D_{10} value of taxol for HeLa cells was 1.6 ng/mL and at taxol concentrations greater than 2.0 ng/mL, no viable colonies were observed.

The selection of first-step taxol resistant mutants from this human cell line was carried out by plating both mutagen-treated (400 µg/mL of EMS for 20 hours; plated on the fourth day subsequent to mutagen treatment) and nonmutagenized HeLa cells in the presence of 5.0 ng/mL taxol. Any mutants obtained would then be at least 2- to 3-fold more resistant to taxol than the parent cells. This approach seemed plausible since first-step taxol resistant mutants that have been isolated from CHO cells were found to

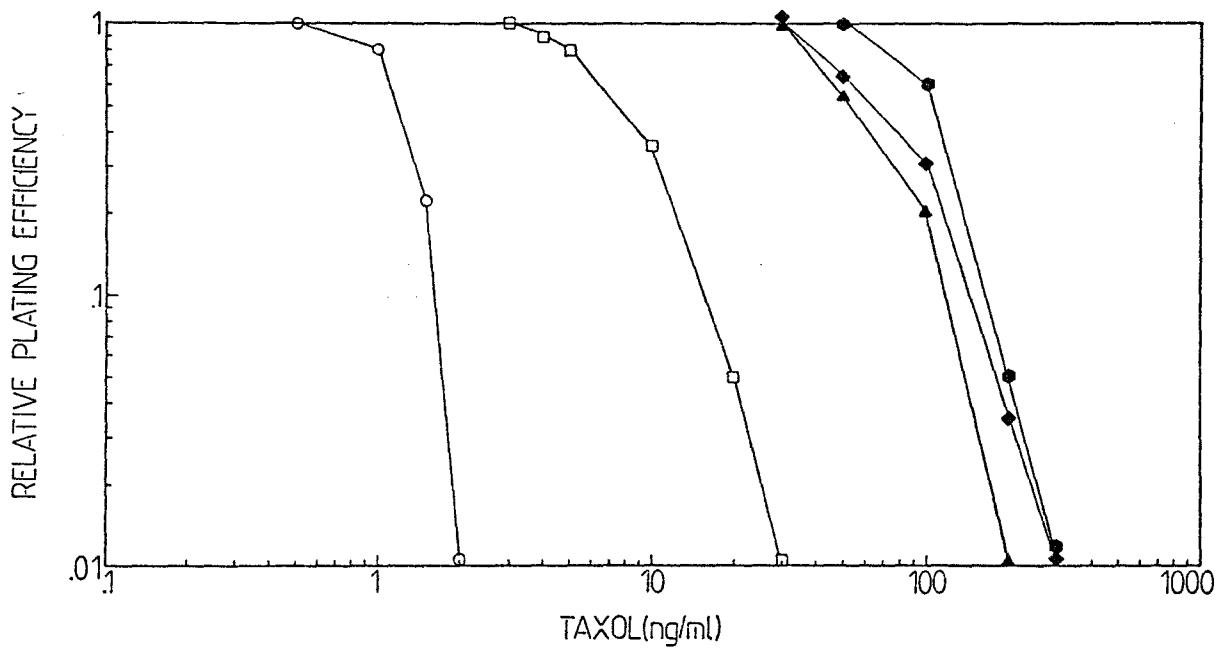


Figure 13. Dose-response curves of the parental sensitive cells and the first-and second-step taxol resistant mutants in the presence of increasing concentrations of taxol. The relative plating efficiencies of the various cell lines in the presence of different drug concentrations were determined by measuring the degree of resistance in 24-well dishes, as described in the Methods.

○ , HeLa;
□ , TAX^{RI}-24A;
▲ , TAX^{RII}-6;
⬢ , TAX^{RII}-7D;
◆ , TAX^{RII}-7G.

be between 2- and 11-fold resistant to this drug (Cabral et al., 1981; Warr et al., 1982; Gupta, 1983d; Cabral, 1983). In non-mutagenized cells, no taxol resistant colonies were observed from plating a total of 11.2×10^6 viable cells. This implied that the frequency of mutation to taxol resistance in these cells was less than 8.9×10^{-8}. In contrast, pretreatment of HeLa cells with EMS (400 μg/mL) resulted in the appearance of taxol resistant colonies at a frequency of approximately 1×10^{-6}. Six colonies obtained from two independent experiments were picked, grown in non-selective medium and tested for their degree of resistance to taxol. Of these, two mutants proved to be significantly (>3-fold) more resistant to taxol than the parental cells. The dose-response curve of the most resistant mutant (TAX^{RI}-24A) is presented in Figure 13. It shows that the D_{10} value of taxol for this mutant was approximately 15.5 ng/mL. Therefore, the TAX^{RI}-24A mutant was almost 10-fold more resistant to taxol than the parental HeLa cells. The drug resistant phenotype of this mutant was to be stably retained upon growth in the absence of taxol for greater than one year.

The TAX^{RI}-24A mutant was subsequently used to select second-step mutants that exhibited an even greater resistance to taxol. Both mutagenized (350 μg/mL of EMS for 20 hours; plated on the third day subsequent to mutagenesis) and nonmutagenized TAX^{RI}-24A cells were plated in the

presence of 200 ng/mL taxol for this purpose. However, at this taxol concentration, no second-step mutants were found. Consequently, a new population of TAX^{RI}-24A cells was mutagenized as before and plated in the presence of taxol at a concentration of 100 ng/mL. In nonmutagenized cultures, resistant colonies were obtained at a frequency of approximately 7.0×10^{-7} whereas pretreatment with EMS (350 μg/mL) resulted in the appearance of second-step taxol resistant colonies at a frequency of about 2.4×10^{-6}. Six second-step taxol mutants were picked, grown in 100 ng/mL taxol (since the stability of their taxol resistant phenotype was unknown) and tested for their degree of resistance to taxol. The dose-response curves of the three most resistant mutants (TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G) toward taxol can be seen in Figure 13. The D_{10} values of taxol toward TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G were approximately 115 ng/mL, 165 ng/mL and 145 ng/mL, respectively. Thus these mutants were 72-, 103- and 91-fold more resistant to taxol than HeLa cells. Similar results for the first- and second-step taxol resistant mutants were obtained in at least three independent experiments.

3.5.1.2 Cross-Resistance Patterns of First- and Second-Step Taxol Resistant Mutants to Various Anticancer Agents

Since taxol belongs to the family of microtubule poisons but its mechanism of action (a microtubule stabilizer) appears to be unique within this family, it was of interest to determine whether the development of resistance to taxol resulted in concomitant resistance to other mitotic inhibitors. Therefore, the cross-resistance of each of the TAX^{RI} and TAX^{RII} mutants toward colchicine, maytansine and vinblastine was determined. As can be seen in Table 14, the TAX^{RI}-24A mutant was 1.8-fold resistant to both colchicine and vinblastine and 1.6-fold resistant to maytansine compared to the parental HeLa cells. Use of the TAX^{RI}-24A mutant to generate second-step mutants which were approximately 10-fold more resistant to taxol resulted in a corresponding increase in the cross-resistance to other microtubule poisons. In this respect, Table 15 shows that the TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G mutants were at least 10-fold resistant to colchicine, 9-fold resistant to maytansine and 20-fold resistant to vinblastine relative to Hela cells.

To determine whether the genetic lesion present in the first- or second-step mutants was specifically altering the sensitivity of cells to microtubule poisons or non-specifically altering the sensitivity of cells to a variety of different-acting drugs through an effect on membrane permeability, the cross-resistance of these mutants to other

Table 14. Cross Resistance Patterns of the First-Step Taxol Resistant Mutant (TAX^{RI}-24A) Toward Various Anticancer Agents

Anticancer agent	D_{10} value of the drug for HeLa cells[a] (ng/mL)	D_{10} value of the drug for TAX^{RI}-24A[a] (ng/mL)	Relative resistance of TAX^{RI}-24A[b]	Comment[c]
Colchicine	2.1	3.8	1.8	PR
Maytansine	0.076	0.125	1.6	PR
Vinblastine	0.64	1.14	1.8	PR
Daunomycin	3.6	4.2	1.2	N
Puromycin	74.0	90.0	1.2	N
VM26	10.0	11.7	1.2	N
VP16-213	185.0	240.0	1.3	N
Aclacinomycin A	3.1	2.9	0.9	N
Actinomycin D	0.1	0.1	1.0	N
Adriamycin	2.0	2.6	1.3	N
Bisantrene	0.74	0.85	1.1	N
cis-Platin	150.0	130.0	0.9	N
Ellipticine	130.0	120.0	0.9	N
Mithramycin	20.0	20.3	1.0	N

Anticancer agent	D_{10} value of the drug for HeLa cells[a] (ng/mL)	D_{10} value of the drug for TAX^{RI}-24A[a] (ng/mL)	Relative resistance of TAX^{RI}-24A[b]	Comment[c]
Mitomycin C	26.5	25.0	0.9	N
Mitoxantrone	5.2	30.0	5.8	R
Harringtonine	36.0	29.0	0.8	N
ara-C[d]	28.0	25.0	0.9	N
5-Fluorouracil[d]	300.0	295.0	1.0	N
Methotrexate[d]	4.0	3.7	0.9	N
MGBG	1.7×10^{-6}M	1.7×10^{-6}M	1.0	N

[a] The D_{10} value of a drug toward a cell line represents the concentration of the drug which reduces the plating efficiency of the cells to 10% of that observed in the absence of any drug. The D_{10} values of various drugs for the HeLa and mutant cell lines were obtained by determining the relative plating efficiencies of the cell lines in the presence of different drug concentrations.

[b] Assuming the D_{10} values of various drugs toward HeLa cells to be 1, the relative degree of resistance of the mutant cell line was obtained as the ratio of the D_{10} value of the mutant cell line to the HeLa cell line.

[c] Depending on the degree of cross-resistance (or sensitivity) toward a given drug, the behaviour of the TAX^{RI}-24A mutant was classified as N=normal (degree of resistance between 0.7- and 1.3-fold), PR=partially resistant (degree of resistance between 1.4- and 4.0-fold), R=resistant (degree of resistance >4.0-fold) or S=sensitive (degree of resistance <0.7-fold).

Footnote to Table 14 (cont'd).

[d] The degree of resistance toward these drugs was determined in α-MEM minus nucleosides (special) medium supplemented with 10% dialyzed fetal calf serum.

drugs, whose mechanism of action involves cellular components other than microtubules, was investigated. The compounds that were examined in this regard included the protein synthesis inhibitor puromycin (Yarmolinsky and de la Haba, 1959), the DNA-intercalating agent daunomycin (Theologides et al., 1968) and VM26 and VP16-213, semisynthetic derivatives of the microtubule inhibitor podophyllotoxin which exert their cytotoxic effect on DNA metabolism not microtubule assembly/function (Gupta, 1983b). These drugs were chosen because mutants affected in membrane permeability are known to exhibit cross-resistance to these agents (Bech-Hansen et al., 1976; Ling et al., 1979; Gupta, 1983b,d, 1985). Table 14 shows that, relative to Hela cells, TAX^{RI}-24A exhibited no cross-resistance to daunomycin, puromycin, VM26 or VP16-213. In contrast, the second-step mutants were approximately 5-fold cross-resistant to daunomycin, VM26 and VP16-213 and at least 15-fold cross-resistant to puromycin. These results suggest that the initial 10-fold increase in taxol resistance exhibited by the TAX^{RI}-24A mutant results from a lesion that specifically affects a microtubule-related component. However, the further 7- to 10-fold increase in resistance to taxol shown by the TAX^{RII} mutants appears to result from a lesion that nonspecifically affects the membrane permeability of these cell lines to taxol and various other unrelated anticancer

Table 15. Cross-Resistance Patterns of the Second-Step Taxol Resistant Mutants Toward Various Anticancer Agents

Anticancer agent	D_{10} value of the drug for HeLa cells[a] (ng/mL)	Relative resistance[b] of TAX^{RII}-6	TAX^{RII}-7D	TAX^{RII}-7G	Comment[c]
Colchicine	2.1	13.3	20.0	11.9	R
Maytansine	0.076	9.5	13.2	12.1	R
Vinblastine	0.64	25.0	25.8	20.0	R
Daunomycin	3.6	7.6	8.3	5.6	R
Puromycin	74.0	16.2	17.6	14.9	R
VM26	10.0	8.5	15.0	4.9	R
VP16-213	185.0	4.3	6.5	4.3	R
Aclacinomycin A	3.1	1.6	2.6	1.1	PR[e]
Actinomycin D	0.1	13.0	16.0	7.4	R
Adriamycin	2.0	13.0	17.5	17.0	R
Bisantrene	0.74	2.4	2.4	2.2	PR
cis-Platin	150.0	0.7	0.5	0.4	S[e]
Ellipticine	130.0	1.0	1.0	0.9	N
Mithramycin	20.0	7.9	11.3	5.0	R

Anticancer agent	D_{10} value of the drug for HeLa cells[a] (ng/mL)	Relative resistance[b] of TAX^{RII}-6	TAX^{RII}-7D	TAX^{RII}-7G	Comment[c]
Mitomycin C	26.5	0.9	0.8	0.8	N
Mitoxantrone	5.2	134.6	134.6	96.2	R
Harringtonine	36.0	2.0	3.6	1.9	PR
ara-C[d]	28.0	0.5	0.4	0.5	S
5-Fluorouracil[d]	300.0	0.6	0.6	0.8	S[e]
Methotrexate[d]	4.0	0.8	0.9	1.0	N
MGBG	1.7×10^{-6}M	0.9	0.9	0.9	N

[a,b] The D_{10} values of the anticancer agents toward the HeLa and mutant cell lines, as well as the relative degree of resistance of the mutant cell lines, were determined as described in the legend of Table 14.

[c] The degree of cross-resistance exhibited by the TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G mutants was classified in the same manner as that for the TAX^{RI}-24A mutant: N=normal (degree of resistance between 0.7- and 1.3-fold), PR= partially resistant (degree of resistance between 1.4- and 4.0-fold), R=resistant (degree of resistance >4.0-fold) or S=sensitive (degree of resistance <0.7-fold).

[d] The degree of resistance toward these drugs was determined in special medium supplemented with 10% dialyzed fetal calf serum.

[e] For these drugs, the degree of cross-resistance was classified according to the behaviour of two of the three second-step mutants.

agents.

The development of drug resistance is one of the major causes for the failure of chemotherapy in the treatment of various kinds of cancer. In an effort to prevent the development of drug resistance to a single agent, combination chemotherapy, which employs a variety of different drugs, is used in the hope that resistance to a drug combination will not readily occur (Devita and Schein, 1973; Dorr and Fritz, 1980). Taxol has been shown to possess antitumor activity toward P388 leukemia, B16 melanoma and human Mx-1 mammary tumor xenografts insulated under the subrenal capsule of nude mice and has been undergoing clinical trials since April 1983 (Kisner et al., 1983). Thus, it was of interest to determine whether the TAX^{RI} and TAX^{RII} mutants would exhibit cross-resistance to other anticancer agents. The anticancer agents examined included those that directly interact with DNA (aclacinomycin A, actinomycin D, adriamycin, bisantrene, cis-Platin, ellipticine, mithramycin, mitomycin C and mitoxantrone), the protein synthesis inhibitor (harringtonine) and various base analogues and antimetabolites (ara-C, 5-fluorouracil, methotrexate and MGBG). The results of the cross-resistance studies of the TAX^{RI}-24A and the three TAX^{RII} mutants for these drugs are presented in Table 14 and Table 15 respectively. As can be seen, the sensitivity of the TAX^{RI}-

24A mutant was very similar to that exhibited by the parental HeLa cells toward the cytotoxic effects of most of the anticancer agents examined (aclacinomycin A, actinomycin D, adriamycin, ara-C, bisantrene, cis-Platin, ellipticine, 5-fluorouracil, harringtonine, methotrexate, MGBG, mithramycin and mitomycin C). However, this first-step mutant did show cross-resistance (5.8-fold) toward the DNA intercalating agent, mitoxantrone. Conversely, the cross-resistance patterns of the second-step taxol resistant mutants were significantly different from those of TAX^{RI}-24A. Relative to HeLa cells, the TAX^{RII} mutants were partially resistant to aclacinomycin A (1.1- to 2.6-fold), bisantrene (2.2- to 2.4-fold) and harringtonine (1.9- to 3.6-fold) and significantly resistant to actinomycin D (7.4- to 16.0-fold), adriamycin (13.0-fold to 17.5-fold) and mithramycin (5.0- to 11.3-fold), all of which were drugs to which the TAX^{RI}-24A mutant showed normal sensitivity. In addition, the TAX^{RII} mutants were significantly more cross-resistant to mitoxantrone (96.2- to 134.6-fold compared to HeLa cells) than TAX^{RI}-24A. Interestingly, the TAX^{RII} mutants were somewhat more sensitive to ara-C (2.0- to 2.5-fold), cis-Platin (1.4- to 2.5-fold) and 5-fluorouracil (1.3- to 1.7-fold) than the parental or first-step mutant cells. For the remaining drugs (ellipticine, methotrexate, MGBG and mitomycin C), the sensitivity of the TAX^{RII}-6,

TAX^{RII}-7D and TAX^{RII}-7G mutants was the same as that exhibited by the TAX^{RI}-24A mutant and HeLa cells.

3.5.1.3 Uptake of (^{3}H) Daunomycin by the Parental and Taxol Resistant Cell Lines

The cross-resistance studies with the first-step taxol resistant cell line indicated that the TAX^{RI}-24A mutant probably possessed a genetic lesion which specifically affected a microtubule-related cellular component. By comparison, the TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G mutants, which are likely affected in this same microtubule component since they were derived from TAX^{RI}-24A, exhibited cross-resistance patterns that suggested their further increase in resistance to taxol was the result of a lesion that nonspecifically affected the membrane permeability of these mutants to various compounds. To test this possibility directly, the uptake of (^{3}H)-daunomycin by these cell lines was determined. (^{3}H)-Daunomycin uptake was examined because earlier work has shown that taxol and other drug resistant mutants affected in membrane permeability exhibit substantially reduced uptake of this drug (Gupta, 1983b,d, 1985). The results of the present studies are found in Table 16. They indicate that the initial rate of (^{3}H)-daunomycin uptake by the TAX^{RI}-24A mutant was approximately 94% of that shown by HeLa cells whereas the corresponding

Table 16. Uptake of (^{3}H)-Daunomycin by HeLa Cells and the First- and Second-Step Taxol Resistant Mutants

Cell line	Duration of uptake (min)	(^{3}H)-daunomycin uptake/10^6 cells[a] (cpmx10^{-3})	Uptake relative to HeLa cells[b]
HeLa	10	6.24±0.24	100
	20	9.30±0.37	100
	40	10.66±0.10	100
	60	12.81±0.21	100
TAX^{RI}-24A	10	5.84±0.20	93.7
	20	8.47±0.23	91.1
	40	9.07±0.12	85.1
	60	10.94±0.56	85.4
TAX^{RII}-6	10	4.83±0.12	77.4
	20	5.96±0.23	64.1
	40	6.29±0.53	59.1
	60	7.00±0.30	54.6
TAX^{RII}-7D	10	4.21±0.48	67.4
	20	5.02±0.20	54.0
	40	5.55±0.12	52.0
	60	5.38±0.53	42.0

Cell line	Duration of uptake (min)	(^{3}H)-daunomycin uptake/10^6 cells[a] (cpmx10^{-3})	Uptake relative to HeLa cells[b]
TAX^{RII}-7G	10	4.90±0.47	78.6
	20	5.88±0.47	63.3
	40	5.91±0.25	55.5
	60	6.18±0.26	48.2

The cellular uptake of (^{3}H)-daunomycin by the various cell lines was studied as described in the Methods. The final concentration of (^{3}H)-daunomycin (specific activity, 1.5 Ci/mmole) in regular growth medium was $2x10^{-7}$M.

[a] Means ± standard errors of duplicate assays are presented.

[b] Assuming the average amount of (^{3}H)-daunomycin taken up by HeLa cells (for any time period) to be 100, the relative uptake in the mutant cell lines was calculated as the ratio of uptake by the mutant cells to that of HeLa cells.

initial uptake rates for TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G were 77%, 67% and 79% of that shown by parental cells. Thus, in contrast to the first-step mutant, the second-step mutants appear to show a significantly lower initial rate of (^{3}H)-daunomycin uptake. Additionally, these results reveal that the uptake of (^{3}H)-daunomycin by the TAX^{RI}-24A mutant was approximately 85% of that exhibited by the parental HeLa cells after a 60 minute incubation. In contrast, the TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G mutants showed a 45%, 58% and 52% reduction in (^{3}H)-daunomycin uptake respectively after a 60 minute incubation. Thus, reduced intracellular accumulation of taxol may be responsible for the increased resistance of the second-step mutants but is probably not responsible for the increased resistance of TAX^{RI}-24A. Similar results were obtained in two independent experiments with these cell lines.

These results suggest that the lesion present in the first-step taxol resistant mutant does not affect its permeability to various drugs but altered membrane permeability is most likely responsible for the resistance characteristic of the second-step taxol resistant mutants.

3.5.2 Discussion

Although the nature of the lesion present in the first-step taxol resistant mutant has not been fully characterized, the results presented in this work strongly

suggest that the TAX^{RI}-24A mutant is specifically affected in a microtubule-related cellular component. Several lines of evidence that support this inference are:

(a) The TAX^{RI}-24A mutant exhibited at least partial cross-resistance to each of the other microtubule inhibitors examined (colchicine, maytansine and vinblastine).

(b) The cross-resistance of this mutant was specific to microtubule inhibitors as no cross-resistance was observed to a wide variety of different anticancer agents that act on other cellular targets.

(c) No cross-resistance was exhibited by the TAX^{RI}-24A mutant toward daunomycin, puromycin, VM26 or VP16-213, compounds for which cross-resistance has previously been associated with altered membrane permeability in drug-resistant mutants, including those resistant to taxol (Gupta, 1983d, 1985; Roy and Horwitz, 1985).

(d) The TAX^{RI}-24A mutant exhibited only a 6% reduction in the initial rate of (^{3}H)-daunomycin uptake and only a 14% reduction in the intracellular accumulation of (^{3}H)-daunomycin after 60 minutes. This reduction is comparable to that reported by Gupta (1983d) who found that a taxol resistant first-step CHO cell mutant, which was thought to be altered in a microtubule component, exhibited an approximate 7% reduction in the accumulation of (^{3}H)-daunomycin after a 45 minute incubation.

In contrast to the data obtained for the TAX^{RI}-24A mutant, the results found for the second-step mutants, TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G, suggest that the increased resistance toward taxol exhibited by these mutants is the result of a second lesion that affects the membrane permeability to taxol and other unrelated anticancer drugs nonspecifically. The lines of evidence supporting this hypothesis are:

(a) The TAX^{RII} mutants exhibited at least a 10-fold increase in the degree of cross-resistance to each of the microtubule inhibitors and a concomitant increase in cross-resistance toward several DNA intercalating agents and protein synthesis inhibitors to which the TAX^{RI}-24A mutant exhibited normal sensitivity.

(b) In contrast to the TAX^{RI}-24A mutant which showed no cross-resistance to agents indicative of an alteration in membrane permeability, the TAX^{RII} mutants exhibited a significant (>4-fold) increase in cross-resistance to daunomycin, puromycin, VM26 and VP16-213. Similar cross-resistance patterns of taxol resistant mutants affected in their membrane permeability have been reported earlier (Gupta, 1983d, 1985; Roy and Horwitz, 1985).

(c) The TAX^{RII}-6, TAX^{RII}-7D and TAX^{RII}-7G mutants exhibited significant reductions in both the initial rate of uptake (decreased 22 to 32%) and the intracellular accumulation

(decreased 45 to 58%) of (^{3}H)-daunomycin. This reduction in uptake agrees with that reported by Gupta (1983d) who found that a first-step CHO cell mutant resistant to taxol, which was affected in its membrane permeability to various drugs, exhibited an approximate 78% reduction in (^{3}H)-daunomycin uptake.

This is the first report of taxol resistance in cultured human cells. The results suggest that, similar to the situation reported for other taxol resistant mutants (Cabral et al., 1981; Gupta, 1983d; Roy and Horwitz, 1985), the development of resistance to taxol in human cells may result from two distinctively different mechanisms. One mechanism appears to involve a biochemical lesion that specifically alters a microtubule-related cellular component and in this work results in the 10-fold increase in taxol resistance exhibited by the TAX^{RI}-24A mutant. Alternatively, a second mechanism, which is responsible for the further increase in resistance exhibited by the TAX^{RII} mutants, involves a genetic lesion which affects the membrane permeability of cells to taxol and other microtubule-related and unrelated drugs nonspecifically.

Previous studies by Cabral et al. (1981) suggest that an alteration in α-tubulin can confer taxol resistance on a mammalian (CHO) cell line. Although no attempt has as yet been made to determine whether the biochemical

alteration present in the TAX^{RI}-24A mutant is at all similar, it is possible that this first-step HeLa mutant is affected in α-tubulin. Two dimensional gel electrophoretic analysis of the proteins present in the TAX^{RI}-24A mutant, compared to those present in the parental cells, should provide insight into this issue. The appearance of an altered/extra α-tubulin spot in the TAX^{RI}-24A mutant would provide strong evidence that in human cells taxol resistance may result from a specific alteration in one of the major microtubule components, α-tubulin.

Roy and Horwitz (1985) have reported the isolation of an 800-fold taxol resistant murine cell line that is cross-resistant to colchicine, vinblastine, puromycin, adriamycin and actinomycin D. They show that the drug resistant phenotype of this mutant is the result of decreased membrane permeability to taxol and is correlated with the appearance of a new phosphoglycoprotein (M_r=135,000) in the mutant plasma membrane. This glycoprotein is absent from the parental cell line and from the resistant cells after growth in the absence of taxol. Consequently, the proposal of Ling (1975) concerning the mechanism of acquired resistance to colchicine in CHO cells may be applicable to the development of cellular resistance by human cells toward both puromycin (Section 3.4.2) and taxol. Further, it may be that a general mechanism by which

sensitive cells develop resistance to specific anticancer agents involves decreased drug permeability resulting from an alteration in or the appearance of certain high molecular weight membrane glycoproteins which modulate membrane fluidity. In support of this, cross-resistance to structurally unrelated drugs, decreased drug permeability and the appearance of new glycoproteins in the plasma membrane has been described in drug resistant Chinese hamster cells (Carlsen et al., 1977; Garman and Center, 1982; Center, 1983; Garman et al., 1983) and in drug resistant human leukemic lymphoblasts (Beck et al., 1979). Further work with the TAX^{RII} mutants should parallel the membrane characterization studies proposed for the PUR^{rI} and PUR^{rII} mutants (Section 3.4.2) in an attempt to identify a correlation between the appearance of a new mutant cell membrane glycoprotein and increased resistance to taxol.

Taxol is currently being investigated as an anticancer agent in human clinical trials (Kisner et al.,1983). Similarly, many of the other compounds that have been used in the present cross-resistance studies are either employed as or are being investigated as anticancer drugs. Work related to their mechanism of action has led to the classification of these anticancer agents into three distinct groups (Gupta, 1985). With respect to this, the results of the present cross-resistance studies involving

the TAX^{RII} HeLa mutants are in agreement with the classification scheme of Gupta (1985). Additionally, they provide information regarding drug combinations to which pleiotropic drug resistance in human cells should not readily develop and may therefore prove more useful in the treatment of different types of cancer.

Group 1 compounds, as defined by Gupta (1985), are those to which cellular resistance could develop (and in the case of the HeLa TAX^{RII} mutants, does develop) simultaneously. This group includes most DNA intercalating agents (aclacinomycin A, actinomycin D, adriamycin, bisantrene, daunomycin, mithramycin and mitoxantrone), antimitotic inhibitors (colchicine, maytansine and vinblastine), protein synthesis inhibitors (harringtonine and puromycin), VM26 and VP16-213. In contrast, Group 2 compounds are those to which the HeLa TAX^{RII} mutants (and Gupta's TAX^{R}-2 CHO mutant) exhibit no significant degree of cross-resistance. This group includes the DNA alkylating agent, mitomycin C and the antimetabolites, methotrexate and MGBG. Lastly, Group 3 compounds are those for which the sensitivity of the HeLa TAX^{RII} (CHO TAX^{R}-2) mutants is enhanced in comparison to parental (normal) cells. This group includes ara-C, 5-fluorouracil and cis-Platin. In almost every case, the classification of compounds based upon their cytotoxic effect toward HeLa TAX^{RII} mutants was

in direct agreement with the classification scheme of Gupta (1985). Only one compound, the DNA intercalating agent, ellipticine, differed. Gupta (1985) observed a 1.5-fold increase in cross-resistance toward ellipticine exhibited by the CHO $TAX^{R}{}_{-2}$ mutant (therefore, it belonged to Group 1) whereas the present results found that TAX^{RII} mutants were normally sensitive to ellipticine (therefore, ellipticine belonged to group 2). Nevertheless, it is obvious that the difference in observed cross-resistance (1.5-fold vs. 1.0-fold) is not large.

These cross-resistance studies, corroborate those of Gupta (1985) and similarly suggest a number of important considerations concerning the use of these agents in the chemotherapy of human cancer: (a) the use of a combination of more than one agent from the Group 1 compounds would not prove helpful in preventing the development of pleiotropic drug resistance by human cancer cells since resistance to these drugs may occur simultaneously, (b) drug combinations involving Group 1 and 2 compounds should be useful in the prevention of pleiotropic drug resistance and treatment of cancer since simultaneous resistance to these drugs should not occur, (c) drug combinations involving Group 1 and 3 compounds should be the most useful in the prevention of pleiotropic drug resistance and the treatment of cancer since resistance to one agent would enhance the cytotoxic

effect of the other agents and (d) possibly the most effective drug combinations for the treatment of human cancer would include compounds from Groups 1, 2 and 3.

4. CONCLUSIONS

The work presented in this thesis has dealt with the isolation and characterization of Hela cell mutants resistant to a number of different anticancer agents.

Hela mutants resistant to the purine nucleoside analogues were selected at similar frequencies (approximately $1x10^{-6}$) using toyocamycin, tubercidin or 6-MeMPR. The drug resistant phenotype of these mutants appears to be quite similar since each mutant exhibited a high degree of cross-resistance to various AK-activated nucleoside analogues and showed greatly reduced cellular uptake and macromolecular incorporation of adenosine *in vivo*. Cell extracts derived form these mutants contained less than 2% of the adenosine kinase activity present in parental Hela cell extracts, a finding which is consistent with their cross-resistance patterns and reduced adenosine uptake and incorporation. These results indicate that in human cells the *in vivo* cytotoxic effects of toyocamycin, tubercidin and 6-MeMPR are dependent upon the initial phosphorylation of these nucleoside analogues by adenosine kinase. Further, the principal mechanism responsible for resistance to these drugs in human cells involves a significant reduction in adenosine kinase activity; specifically, defective synthesis of lethal metabolite(s),

as a direct consequence of reduced intracellular adenosine kinase activity, results in resistance.

Further insight into the nature of the genetic lesions present in the toyocamycin, tubercidin and 6-MeMPR mutants was gained using SDS-polyacrylamide gel electrophoretic and immunoblot analysis. Adenosine kinase present in the Hela cell extracts was shown to have a molecular weight of approximately 35,000. Each mutant possessed a major protein that cross-reacted with antibody specific for adenosine kinase indicating that these mutants retained their ability to synthesize the adenosine kinase gene product. Since this cross-reacting band was of similar intensity for each mutant and the parental Hela cell extract, the genetic lesion in these mutants does not involve an alteration in the regulatory region of the adenosine kinase gene. Further, because the cross-reacting band present for each mutant has a similar electrophoretic mobility to that of parental Hela cell adenosine kinase, the alteration affecting adenosine kinase is probably not of the nonsense or deletion type. Thus, these results strongly suggest that the lesion present in each mutant involves a missense type of mutation present in the structural gene for adenosine kinase.

Investigation into the utility of the AK^- mutant selection system for studies related to quantitative

mutagenesis in human cells revealed a number of positive attributes. These include: (a) 6-MeMPR resistant Hela mutants could be obtained spontaneously at a low frequency, (b) the mutants isolated were stable in the absence of drug and highly resistant to 6-MeMPR, (c) the selection system was free from cell density or cross-feeding effects, (d) a relatively short period was required for maximum expression of the mutant phenotype and (e) the frequency of 6-MeMPR resistant Hela mutants increased in a linear, dose-dependent manner upon treatment of Hela cells with direct-acting mutagens. Also, because adenosine kinase is not essential for cell viability, the AK locus should be responsive to any type of genetic alteration. Taken collectively, these attributes dictate that the adenosine kinase locus should provide a valuable system for quantitative mutagenesis in human cells.

Selection for puromycin resistant Hela mutants yielded first- and second-step mutants that were approximately 10- and 100-fold more resistant to puromycin, respectively, than parental Hela cells. Cross-resistance and drug uptake studies suggest that in human cells resistance to puromycin results from a genetic lesion which nonspecifically reduces the membrane permeability of cells to puromycin and other unrelated anticancer agents. Thus, the mechanism of acquired resistance exhibited by these mutants involves defective drug uptake/transport.

First- and second-step taxol resistant Hela mutants were found to be approximately 10- and 100-fold more resistant to taxol, respectively, than parental Hela cells. Subsequent cross-resistance and uptake studies with these mutants suggest that in human cells two distinctly different mechanisms may confer resistance to taxol. In the first-step mutant, the genetic lesion that results in resistance appears to affect a microtubule-related cellular component specifically. Alternatively, in the second-step mutants, the genetic lesion responsible for resistance nonspecifically affects the membrane permeability of these cells to various microtubule-related and unrelated drugs. Thus, the possible mechanisms of acquired resistance to taxol in human cells involve either an alteration in the intracellular target site for taxol or defective drug uptake/transport.

Further studies that could be conducted with each of these HeLa cell mutants have been suggested (Section 3.)

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