INTERACTIONS OF MITOXANTRONE WITH CYTOKERATIN IN HUMAN AND MURINE TUMOR CELLS

by

Patricia Ann Bauman

A Dissertation Submitted to the Faculty of the

COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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Order Number 9408390

Interactions of mitoxantrone with cytokeratin in human and murine tumor cells

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The University of Arizona, 1993

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Patricia Ann Bauman

entitled INTERACTIONS OF MITOXANTRONE WITH CYTOKERATIN IN HUMAN AND MURINE TUMOR CELLS

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Dr. William S. Dalton	9-17-93 Date
Dr. G. Tim Bowden	9/17/93 Date
Dr. Roger Miesfeld	9/17/93 Date
Dr. Klaus Brendel	9-17-93 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Dr. Anne E. Cress	9-17-93 Date

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ACKNOWLEDGEMENTS

Many individuals and groups are to be recognized and thanked for their scientific, technical, financial and personal support, beginning with my advisory committee. Drs. Anne Cress, Bill Dalton, Tim Bowden, Roger Miesfeld and Klaus Brendel have all been immensely helpful throughout the process. I also want to thank the laboratory groups of Bill Dalton, Tim Bowden and Gene Gerner for their collective input, especially their generosity with equipment and supplies. Discussions with Margaret Tome have been particularly helpful. Further, I wish to acknowledge the American Association of University Women for their financial contribution to my doctoral research.

Individually, I would like to acknowledge those people who have taught me specific techniques that were instrumental to my research. These include Joanne Finch, Kirsten Kurath, Jesse Martinez, Robin Roberts, Dave Stringer, Larry Vernetti and Maggie Wallon. Derall Willis initiated me on the Macintosh computers, and Isaac Rabinovitz has endeavored to keep me interested (with some success, I might add). Anne Cress and Colette Witkowski have also spent many an hour teaching me about photography and microscopy. Thanks to all.

The greatest debt of gratitude goes to my colleagues in the Cress Lab. I can't imagine a better group for thoughtful criticism, creative problem solving and overall support. Special thanks to Anne, Vickie Peck, Isaac, Robin, Stringer, Dorothea von Bredow and Colette.

Finally, I thank my husband and most loyal supporter, Kevin Gerrish for delighting in the joys and commiserating during the frustrations of the last few years.

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ABSTRACT

The prolonged intracellular retention of the anti-cancer drug mitoxantrone has been linked to its cytotoxicity by several investigators. The persistent association of mitoxantrone with intermediate filament cytokeratin proteins was likewise observed, and its relationship to drug cytotoxicity was investigated.

To test whether this association was indicative of mitoxantrone damage to cytokeratin, human WiDr/S colon carcinoma cells or mitoxantrone-resistant WiDr/R cells were treated with mitoxantrone and isolated cytokeratins were examined. Although an affinity of mitoxantrone for cytokeratin proteins could be demonstrated, no drug damage was apparent using a two-dimensional gel electrophoretic analysis. Neither did mitoxantrone affect the assembly or disassembly of tetrameric cytokeratin complexes from or to monomers. Chronic exposure of WiDr/R cells to mitoxantrone does not affect cytokeratin structure or dynamics as determined by electrophoretic analyses.

To test whether drug binding to cytokeratin might prevent drug damage at other sites, murine fibroblasts transfected with sequences encoding cytokeratins were subjected to drug cytotoxicity assays. Transfectants were not only resistant to mitoxantrone, but also to doxorubicin and the unrelated drugs methotrexate, melphalan, vincristine

and colcemid relative to untransfected and mock-transfected fibroblasts. Transfectants were especially resistant to the cytotoxic effects of anti-tubulin drugs, possibly due to stabilizing interactions between the ectopic cytokeratins and the endogenous microtubules. Drug resistance can not be attributed to differences in growth rates, cell cycle distribution, drug accumulation or drug efflux. The intranuclear distribution of doxorubicin is different in transfected than parental cells, which may affect the resistance phenotype.

The data suggest that cytokeratins may function to protect cells from drug damage, which could be a novel explanation for intrinsic drug resistance in some tumors.

STATEMENT OF THE PROBLEM

The prolonged retention of the anti-cancer drug mitoxantrone by cultured cell lines (1, 2) and its slow elimination from body tissues in patients (3, 4, 5) suggest that mitoxantrone binds extensively to cell and tissue components. Whether drug binding necessarily represents drug damage is unknown. However, the identification of new drug targets holds the potential to reveal new insights into pharmacological mechanisms of action of and resistance to mitoxantrone.

We have observed the apparent binding of mitoxantrone to cytokeratin intermediate filament proteins. The first set of experiments described herein were designed to determine whether cytokeratin might be a novel target for mitoxantrone-induced damage. Because both cytokeratin structure and dynamics are required for proper function, the damage sought included mitoxantrone-induced changes in structure and assembly dynamics.

The second set of experiments was based on a revised model suggesting that cytokeratins confer drug resistance, and that mitoxantrone binding might be a mechanism by which the cell is protected from the cytotoxic effects of the drug.

CHAPTER 1. ANTI-NEOPLASTIC DRUGS

Cancer is a family of diseases in which the control of cellular proliferation is deregulated. No single cause or cure has been identified, but chemotherapies are designed with a common rationale that takes into account the accelerated growth rates of tumor versus normal cells. Anti-neoplastic agents are designed to eliminate rapidly dividing cells by inhibiting cell division, but are limited by their inability to distinguish proliferating tumor cells from normal cycling cells, and by their lack of efficacy against non-cycling tumor cells. Despite these limitations, nearly all anti-cancer drugs target replicating DNA, DNA precursors, DNA synthetic enzymes and/or mitotic proteins.

The broadest category of anti-cancer drugs encompasses the drugs that target DNA. These drugs generally cause direct DNA damage by binding to and causing structural distortions of the helix, creating numerous or irreparable DNA strand breaks, cross-linking bases between or within strands, or stabilizing DNA-protein complexes (reviewed in 6).

Drugs that target DNA precursors frequently compete with endogenous substrates in reactions required for DNA synthesis, or become incorporated into non-functional biomolecules. Analogs of purines and pyrimidines or their metabolites can also inhibit DNA polymerase (7, 8).

The cytoskeletal microtubule proteins are among the few anti-cancer drug targets not involved in the synthesis or replication of DNA. Microtubules form spindle fibers that enable the segregation of genetic material to daughter cells (9). Drugs like the *Vinca* alkaloids inhibit the formation of new spindle fibers and disassemble preexisting microtubules (10).

None of the drugs currently available for the treatment of cancers interacts exclusively with its intended cellular target. For example, anthracyclines and related compounds not only inhibit DNA synthesis, but also affect membrane fluidity (11), protein synthesis (12) and ion transport (13). It is therefore probable that anti-neoplastic agents cause damage at multiple intracellular sites, some of which remain to be discovered or described.

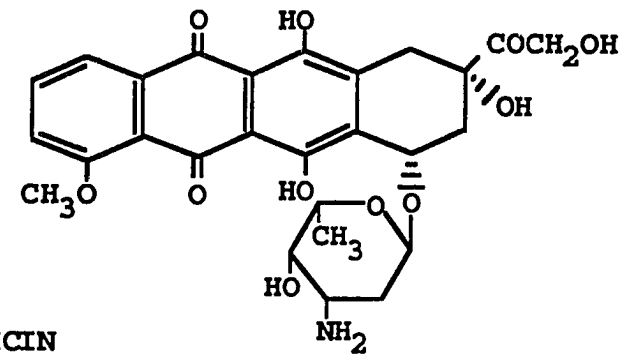
CHAPTER 2. MITOXANTRONE

1. The design of mitoxantrone

Mitoxantrone falls into the category of drugs that target DNA. Its development was based on the structure-activity relationships of intercalating agents, which are characterized by planar ring systems (14). Intercalators mimic the behavior of DNA base-pairs by stacking between nitrogenous bases within the helix (15, 16).

Mitoxantrone's most recent predecessor is doxorubicin, a "natural product" intercalating drug that is widely used as a broad spectrum anti-tumor agent (6, Figure 1). Though relatively effective, the usage of doxorubicin is limited by its severe cardiotoxicity (17, 18). The undesirable cardiotoxic side effects of doxorubicin have been attributed to its amino sugar functional (15), whereas its clinical anti-proliferative effects probably involve its conjugated ring system (14, 18). Mitoxantrone was therefore designed so as to retain the latter ring system and substitute aminoalkyl side chains for the amino sugar ring (19).

It was hypothesized that the biological activity of both mitoxantrone and doxorubicin depended on their abilities to bind DNA via intercalation. However, data later revealed that mitoxantrone binds DNA only partly by intercalation, and partly by the electrostatic interactions



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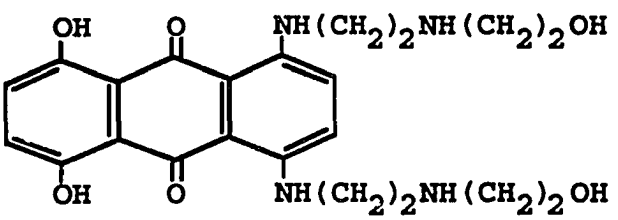


MITOXANTRONE

Figure 1. Doxorubicin and mitoxantrone are structural analogs.

of its side chains with the DNA backbone (20). Because the anti-proliferative activity of mitoxantrone seemed inconsistent with its relatively weak DNA intercalation, the potential mechanisms of action of the drug were investigated further.

2. Mechanisms of action of mitoxantrone

Some clues suggesting that other mechanisms of action might be involved in the biological activity of both mitoxantrone and doxorubicin were gleaned from the ability of these drugs to cause DNA strand breaks (16). Berlin and Haseltine hypothesized that reactive metabolites might cause this type of damage, and showed that doxorubicin is metabolized to a reactive semi-quinone free radical which can create DNA strand breaks (21). However, despite its similarity in structure to doxorubicin, mitoxantrone does not undergo a similar reduction reaction (22), a fact that sets it apart mechanistically from its predecessor.

Comparative studies of the efficacy of mitoxantrone and doxorubicin in cultured cells indicate other differences as well, with mitoxantrone frequently emerging as the more cytotoxic drug (23). Importantly, cell lines selected for resistance to doxorubicin are often sensitive to mitoxantrone-induced cytotoxicity (23). For example, doxorubicin-resistant LoVo/Dx colon adenocarcinoma cells are

sensitive to mitoxantrone (24), as are doxorubicin-resistant NCI-H69 human small cell lung cancer cells and EMT6/Ca/VJAC murine mammary tumor cells (25).

Other investigators have reported a cross-resistance to mitoxantrone in doxorubicin-resistant cell lines (26, 27, 28). This apparent contradiction suggests that mitoxantrone and doxorubicin work via more than one mechanism of action, and that at least some differences between the two drugs exist, despite their structural similarities.

A complete survey of the DNA damage caused by mitoxantrone and doxorubicin revealed that some of the drug-induced DNA strand breaks are associated with protein (29, 30). This protein has now been identified as a topoisomerase, one of the enzymes that function to relieve topological stresses during DNA replication and transcription (31, in 30).

Topoisomerases bind to DNA sites to form enzyme-substrate complexes, which can be separated into denatured enzyme and intact DNA upon treatment with sodium dodecyl sulfate (SDS) or alkali (32). During uninterrupted catalysis, topoisomerases will cut either one (topoisomerase I) or both (topoisomerase II) DNA strands, causing the formation of "cleavable complexes", so named because SDS or alkali denaturation yields cleaved DNA covalently attached to the enzyme at the sites of strand breakage (32, 33).

Each subunit of a type II topoisomerase homodimer is thought to cleave one strand of the DNA and become covalently associated with it at the 5′ ends of the break points. The enzyme can then pass the DNA strands through the cleavage gap, relieve the topological stress and re-anneal the substrate DNA.

The detection of mitoxantrone-induced topoisomerase-associated DNA strand breaks suggested that mitoxantrone may act via the inhibition of topoisomerase II (34, 35). In fact, mitoxantrone has been shown to bind to both DNA and topoisomerase II, misalign the cleaved DNA strands and irreversibly stabilize the cleavable complex (29, 30, 36).

3. Pre-clinical and clinical trials

The pre-clinical testing of mitoxantrone appeared very promising. Mice inoculated with leukemias or melanoma, and then treated with mitoxantrone survived longer than inoculated but untreated mice (37). Also, the frequency of long-term survivors of each tumor type was higher among the mitoxantrone-treated group (37). In a comparative test of the effects of mitoxantrone in Friend leukemia cells, L1210 leukemia cells and human lymphocytes, mitoxantrone reduced the number of viable tumor cells without detrimental effects on normal lymphocytes (38). The federal Food and Drug Administration therefore approved mitoxantrone for the

treatment of human acute myelogenous and lymphocytic leukemias in 1987 (39).

The encouraging test results against leukemias in mice prompted investigators to evaluate the effectiveness of mitoxantrone against solid tumors. Mitoxantrone was tested *in vitro* in clonogenic assays against ovarian cancer (40). The results suggested its superiority over other drugs, although subsequent clinical trials were somewhat disappointing (39).

However, the clinical trials of mitoxantrone for the treatment of leukemias, lymphoma and breast cancer have been relatively successful (39). As a single agent, mitoxantrone has a mean response rate of about 30% in acute non-lymphocytic and acute myelogenous leukemias, about 20% in acute lymphocytic leukemia, and about 25% in advanced breast cancer. In combination with other drugs, mean response rates are about 60% for leukemias and 33-68% for breast cancer (reviewed in 41).

When breast cancer patients treated with mitoxantrone were compared to those treated with doxorubicin, the response rates, remission times and times to treatment failure were not statistically different (39). However, mitoxantrone caused fewer severe side effects, the most limiting being myelosuppression and to a lesser extent, cardiotoxicity (3, 41).

4. Pharmacokinetics

Pharmacokinetic data collected from both mice and humans indicates that mitoxantrone is poorly absorbed when administered orally. When administered to humans as a single intravenous dose, the drug is rapidly distributed into the blood elements within 2.4-15 minutes, where approximately 78% of the mitoxantrone is bound by plasma proteins (4). The drug redistributes from the blood to the tissues within 17 minutes to 3 hours (4, 39).

A unique pharmacokinetic feature of mitoxantrone is its slow elimination half-life, which can be as long as 13 days (4, 5). This prolonged terminal half-life is due to extensive tissue binding. An autopsy 35 days after administration of mitoxantrone revealed as least 15% of the drug had been retained by body tissues (5).

The unusual retention of mitoxantrone has also been observed in a comparative study of mitoxantrone and m-amsacrine in human fibroblast cell lines (1). Cells treated with mitoxantrone were not only more susceptible to damage, but also did not recover as easily as the cells treated with m-amsacrine, even though both drugs formed similar levels of DNA-protein complexes (1). Fox and Smith have suggested that prolonged intracellular retention of mitoxantrone may explain these results, possibly by causing a long-term depression of DNA replication.

Similarly, Ellis et al. observed the extended cellular retention of mitoxantrone in hepatoma cells, which they also suggested may be related to its cytotoxicity (42). In their system, neither DNA-protein crosslinks nor inhibition of DNA synthesis could be correlated with the anti-proliferative activity of mitoxantrone, suggesting the existence of an alternative mechanism of drug action (42).

Cress et al. hypothesized that such a mechanism might involve drug-induced modification of intermediate filament cytoskeletal proteins in human colon carcinoma cells (43). A second group also reported data suggesting that mitoxantrone binds to cytoskeletal proteins (44).

The relationship of the persistent intracellular retention of mitoxantrone to its cytotoxicity is not clear, and requires the testing of new hypotheses. The possibility that the retention of mitoxantrone by intermediate filament proteins could be cytotoxic was tested in the present work, as described in Chapter 5.

CHAPTER 3. DRUG RESISTANCE

1. Relevance

A major limitation of any cancer chemotherapy is the selection of chemoresistant cells within a tumor, either by intrinsic resistance mechanisms or by mechanisms acquired by the cell in response to therapy. Intrinsic resistance involves endogenous protective mechanisms and/or molecules, and manifests itself clinically in patients who do not respond to chemotherapy. In contrast, patients with acquired resistance respond to their initial drug treatments, but repeated treatments select for sub-populations of tumor cells capable of surviving drug exposure. Moreover, patients frequently become cross-resistant to unrelated anti-cancer drugs as acquired resistance develops, a phenomenon known as multidrug resistance (mdr).

The molecular basis for drug resistance depends on natural protective mechanisms of the host, previous exposure to drugs, type of drug, genomic stability of the tumor cells and many other factors. Drug resistance has therefore become an active area of research. Drug-resistant tumors are modelled in the laboratory by selecting resistant tumor cells from a parent population based their ability to grow in the presence of drug. Levels of drug resistance over 100-fold that of parental cells can be attained in culture

by gradually increasing the concentration of the selecting agent.

2. Intrinsic resistance

Intrinsic resistance describes the collective responses of a cell or tumor that enables it to circumvent drug toxicity, and enhance survival potential. Multiple biochemical mechanisms that efficiently remove or inactivate cytotoxic drugs in normal cells probably confer intrinsic or *de novo* resistance to tumor cells (45, 46).

For example, unresectable and metastatic colon cancers tend to be resistant to chemotherapy (47). Several investigators have suggested a link between intrinsic resistance and drug metabolizing enzymes, especially the enzymes involved in the synthesis, conjugation and redox cycling of glutathione (47-50). Four comparative studies of normal versus malignant mucosa from patients with colorectal cancers reported higher levels of total glutathione and the acidic class of glutathione S-transferase in the malignant samples compared to normal mucosa (47-50).

Others have suggested a role for P-glycoprotein, a putative drug transport protein, in intrinsic resistance. The P-glycoprotein is normally expressed in colon, liver and other tissues, and may have evolved to equip cells with the capability of removing toxic environmental compounds that

penetrate the plasma membrane (51).

Other mechanisms of intrinsic or *de novo* drug resistance no doubt remain to be identified. All will have in common the ability to use the natural protective mechanisms existing in normal cells to overcome the cytotoxic effects of anti-cancer drugs.

3. Acquired Resistance

(a) Acquired resistance to mitoxantrone

Tumor cells can acquire resistance to any drug presently being used against cancer; mitoxantrone is no exception. Acquired resistance to mitoxantrone does not usually result in the commonly observed multidrug resistance phenotype characterized by the overexpression of P-glycoprotein, although mitoxantrone may be rendered ineffective by P-glycoprotein-mediated efflux.

Mitoxantrone-selected resistance mechanisms frequently involve other kinds of altered drug transport. A lower rate of mitoxantrone uptake in resistant human WiDr/R colon carcinoma versus parental cells has been reported (52), and a unique vesicular transport mechanism has been described in a mitoxantrone-resistant EPG85-257NOV gastric carcinoma variant (53). Ironically, extrusion of drug-containing vesicles has also been proposed as a mechanism of action for the P-glycoprotein, suggesting a possible explanation for

the recognition of mitoxantrone as a P-glycoprotein substrate (54).

The existence of a mitoxantrone-selected efflux pump distinct from P-glycoprotein has been proposed by Taylor et al (55, 145). Their efflux and energy-consumption data from a mitoxantrone-resistant breast cancer cell line are consistent with such a hypothesis, although no such pump has yet been identified.

The proposed pump could be related to the recently described multidrug resistance-related protein (MRP, 56). MRP is an ATP-binding protein that is overexpressed in doxorubicin-resistant H69 human small cell lung carcinoma cells (56). Experiments transfecting the gene or CDNA encoding MRP into sensitive cells have not yet been performed to verify its role in drug resistance.

Acquired resistance to mitoxantrone does not necessarily result in altered uptake or efflux processes. Some cultured cells selected for resistance to mitoxantrone display an "atypical multidrug resistance" phenotype, characterized by cross-resistance to many natural product drugs, sensitivity to *Vinca* alkaloids, unchanged drug accumulation and efflux, and lack of overexpression of P-glycoprotein (57, 58). Drug resistance in these cell lines is attributed to a decrease in the activity or quantity of topoisomerase II, the presumed drug target (reviewed in 58).

(b) P-glycoprotein

The efficacy of mitoxantrone can be diminished by P-glycoprotein, although selecting resistant cells with mitoxantrone does not generally select for cells overexpressing this protein (53, 55, 59, 60). (The one notable exception is the unique selection of CHO AA8 cells with mitoxantrone described by Muller et al. (61)).

The P-glycoprotein apparently acts as a drug transporter, based on observations in resistant cells of decreased drug accumulation, increased drug efflux, higher mdr1 messenger RNA levels and increased expression of P-glycoprotein on cell surfaces compared to sensitive cells (reviewed in 58). A high degree of sequence homology between the mdr1 cDNA and the coding sequences of several bacterial transport proteins strengthens this hypothesis (62, 63). That P-glycoprotein can confer multidrug resistance has been experimentally confirmed by transfection of the mdr1 cDNA into both sensitive cells (64, 65) and intact animals (66).

The mechanism by which P-glycoprotein exports mitoxantrone and other drugs is not clear. Its deduced amino acid sequence suggests a symmetrical structure in which 12 hydrophobic domains span the plasma membrane, encompassing a type of channel or pore through which drug efflux can occur (62). The extracellular domains are

predicted to be very short, but the two intracellular domains are relatively large, each possessing an ATP-binding site (62, 67). Hydrolysis of ATP probably generates the energy required for drug efflux (68).

The mechanism of substrate selection for transport is also largely unknown. Substrates for P-glycoprotein have few structural features in common, but are generally "natural product" drugs which have lipophilic moieties and charged nitrogen groups at physiological pH (in 69). As a synthetic analog of a natural product drug, mitoxantrone is a bit of an exception, although it does possess the requisite functional groups.

The existence of several isoforms of P-glycoprotein have been postulated to account for its apparent lack of substrate specificity (70, 71). Choi et al. separately selected human KB epidermoid carcinoma cells with doxorubicin or colchicine, and demonstrated a genetic difference at a single DNA base that apparently accounts for the preferred transport of the selection agent (72). Others discovered at least two transcription initiation sites, also suggestive of multiple isoforms of P-glycoprotein (73). Finally, variants of P-glycoprotein might also occur via post-translational modifications including glycosylation and phosphorylation, although the former modification appears to be unrelated to multidrug resistance (74).

The question of substrate specificity is not yet resolved. Several interesting models in addition to the aforementioned modifications to the transport protein have been postulated. Higgins et al. has likened P-glycoprotein to a "flippase" (75). They propose that drug gains access to the transporter directly via the lipid bilayer, as opposed to through a hydrophilic pore. In their model, the drug is "flipped" from the inner leaflet of the bilayer to the outer leaflet, or alternatively, to the extracellular medium.

In keeping with bacterial transport proteins, Gerlach et al. speculated on the existence of a carrier protein which would directly bind drug and result in the export of a drug-protein complex (67). By non-specifically binding drugs, such a protein could act as a scavenger to effectively remove drug from cellular targets.

Along the same lines, it has been proposed that the putative drug carrier is glutathione, and that drugs removed are actually glutathione conjugates (76). In this way different members of the large family of glutathione S-transferases may recognize specific drug epitopes, but the net result would be to form drug-glutathione complexes which could be removed via a common glutathione transporter (76). This model is especially intriguing in light of the normal expression pattern of P-glycoprotein in tissues such as

liver, which also contain high levels of glutathione.

(c) Intracellular drug distribution

The intracellular distribution or compartmentalization of drug is critical to its efficacy. Both intrinsic and acquired drug resistance frequently depend on sequestering drug from its intended target.

A low but toxic concentration of mitoxantrone was shown to be excluded from nuclear compartments and sequestered in the cytoplasm of human SV40-transformed fibroblasts (77). DNA damage in these cells was minimal, suggesting that the ability to prevent mitoxantrone from reaching nuclear targets is an important factor in drug resistance.

Using video fluorescence microscopy, Hindenburg et al. demonstrated a difference in the intracellular distribution of a mitoxantrone analog (daunorubicin) between parental HL-60 human leukemia cells and a resistant subline, HL-60/AR (78). In resistant cells the distribution of daunorubicin shifts from the Golgi apparatus to the lysosomes and mitochondria (79). Chemomodifiers known to block daunorubicin efflux and reverse drug resistance also prevent its distribution to these compartments.

All drug resistance mechanisms prevent toxic agents from taking part in reactions detrimental to the cell. Therefore, mechanisms of drug resistance depend on mechanisms of drug action. The identification of novel drug

targets can yield an improved understanding of both the biological activity of the drug and the mechanisms by which cells circumvent cytotoxicity.

CHAPTER 4. CYTOKERATINS

Cytokeratins are members of the intermediate filament protein family, a group of proteins that comprises one of the major cytoskeletal networks in mammalian cells. Compared to the actin and tubulin subunits of the other cytoskeletal networks, the intermediate filament proteins are a diverse group, subdivided into at least six classes (80). Cytokeratins belong to two of these classes and are the most heterogeneous group of the family. Type I cytokeratins are classified by their homology to type I wool keratins (81). They range in size from about 40 to 57 kilodaltons and in isoelectric pH from about 4.9 to 5.7 (82). Type II cytokeratins are larger and more basic, ranging in size from approximately 52 to 70 kilodaltons and in isoelectric pH from 5.4 to 7.8 (82). Type I and type II cytokeratins are approximately 30% homologous to each other and to non-keratin intermediate filament proteins (82).

1. The structure of cytokeratins

(a) Monomers

Intermediate filament monomers are characterized by structural features common to all members (83). Both gene and protein secondary structures are conserved, despite the variability in their primary sequences (84). Therefore, intermediate filament proteins are likely to resemble each

other in their interactions with both cellular components and exogenous compounds.

The most highly conserved structural feature of all intermediate filament proteins is the central α-helical rod domain (82, 83; Figure 2). The central domain typically consists of about 310 amino acids, except in nuclear lamins, which have an additional 50 amino acids extending the central rod (85, 86). The rod is composed of 4 helical sections delineated by short nonhelical linker regions (82). Within these helical subdomains are repeating amino acid heptads which bear hydrophobic residues in the 1st and 4th positions (82, 83). The heptad repeats create a hydrophobic seam between type I and type II monomers which stabilizes larger filament structures (83).

The nonhelical cytokeratin end domains are less well conserved than the central rod, but are nonetheless organized into subdomains (87). The most highly conserved subdomains are the globular H1 and H2 domains located immediately adjacent to the rod (see Figure 2). Type I cytokeratins have H1 domains, but lack a conserved H2 domain. Both type I and type II cytokeratins contain domains with variable sequence and structure (V domains), which are located outside the central rod domain and adjacent to the H domains (87). The V domains account for the diversity of the twenty known cytokeratin proteins, and

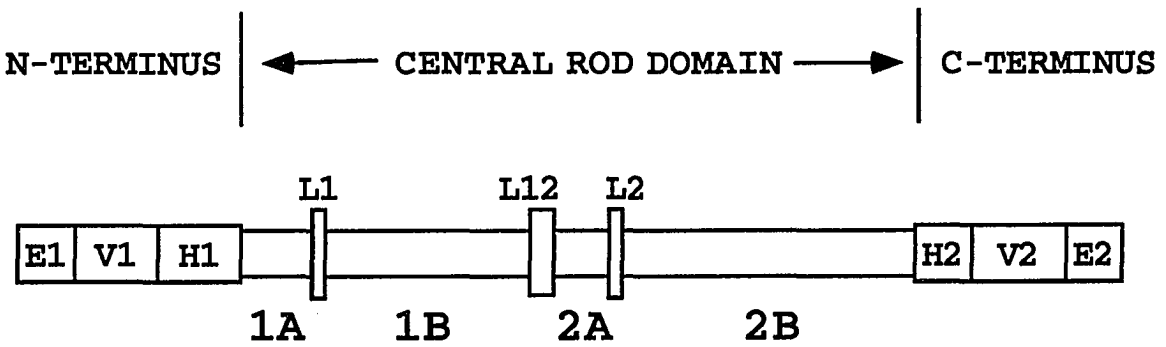


Figure 2. Cytokeratin monomers are divided into conserved and variable domains. The conserved central rod domain of this type II monomer is composed of α-helical subdomains (1A, 1B, 2A, 2B) joined by nonhelical linker regions (L1, L12, L3). The amino and carboxy termini are subdivided into basic (E), variable (V) and conserved (H) domains (119, modified.)

tend to be rich in glycine and/or serine residues (87). The most terminal subdomains (E domains) are basic regions, also of variable sequence (87).

Type I and type II cytokeratin monomers form obligate pairs from which the heterodimer and heterotetramer subunits are formed and eventually elongated to filaments (88, 89, 90). Cytokeratins are unique in this regard; type III (vimentin, desmin and glial fibrillary protein), and type IV (neurofilaments and peripherin) intermediate filaments form homopolymers (91). Type V intermediate filaments, the nuclear lamins, create a unique nuclear network (85).

The expression of intermediate filament proteins is regulated by cell type and stage of development (92, reviewed in 93). Cytokeratins are only expressed in epithelial cells, and only in specific type I/type II pairs. They are expressed early in development, frequently being replaced by other intermediate filament protein as development proceeds.

(b) Dimers and tetramers

Several assembly steps precede filament formation, beginning with the obligate pairing of type I and type II monomers (94). The pairing enables the formation of a stable subunit; a single monomer without its partner is degraded (95, 96). By introducing cysteine mutations into cytokeratins 8 and 18 and artificially crosslinking them by

oxidation, both homodimers and heterodimers can be formed, but only the heterodimers are competent for filament assembly (97).

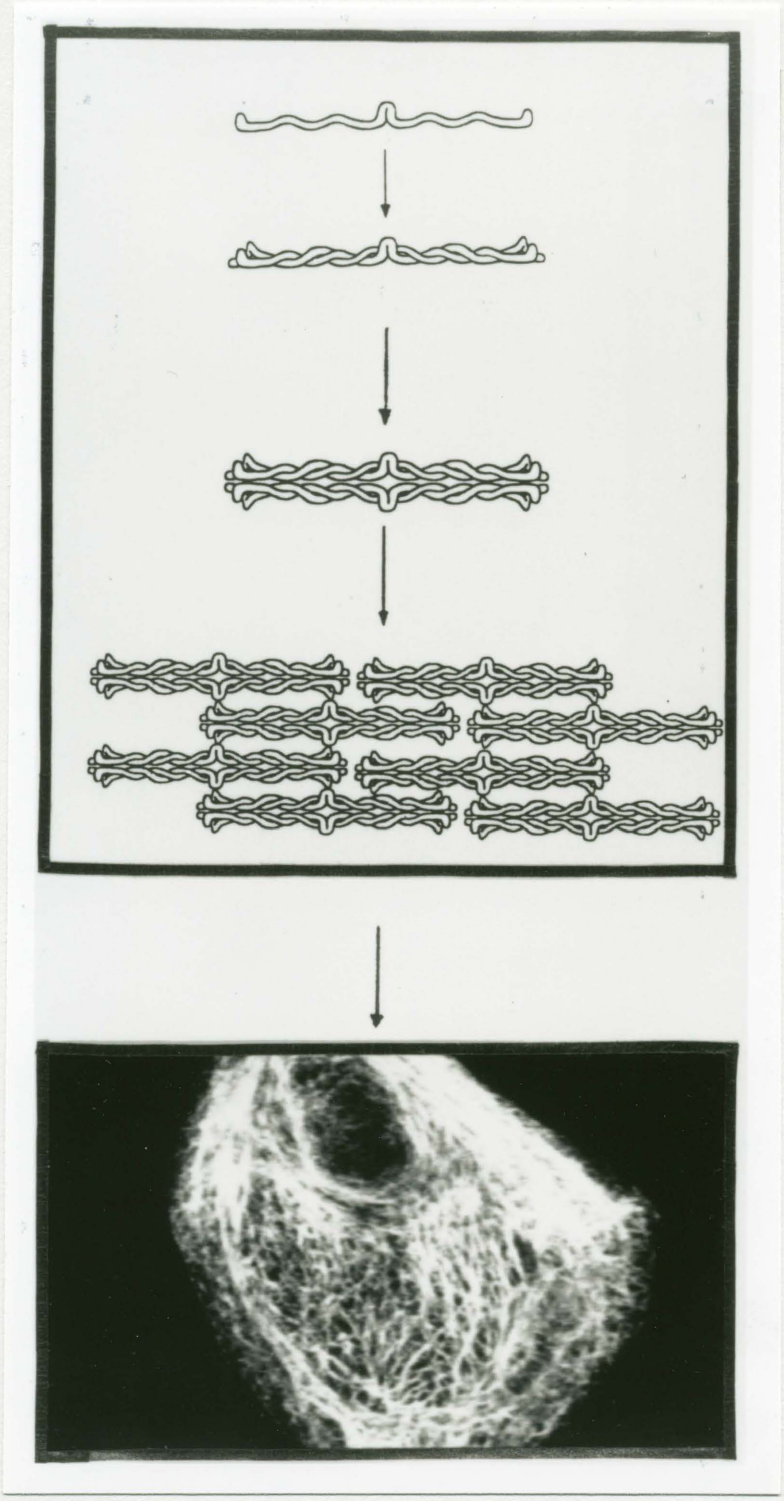
The resultant dimer is composed of the two monomers in an anti-parallel alignment (98, Figure 3). Whereas some reports in the literature suggest that the alignment is in register (89, 99, 100), others suggest that it is staggered (101, 102, 103). In solution, the staggered alignment is thermodynamically favored (104), but both alignments may be possible (98, 104). Although more stable than lone monomers, heterodimers also spontaneously interact to yield heterotetramers (89, 91).

(c) Filaments and networks

An intact central rod domain is critical to proper filament formation. Deletion mutations within end of the central helical rod disrupt the endogenous cytokeratin networks in both kangaroo rat PtK2 cells and SCC-13 human squamous carcinoma cells (105, 106). Large deletions at the amino terminus of the rod cause endogenous filaments to collapse around cell nuclei, and preclude the reestablishment of normal networks (105). Deletions at the carboxy terminus of the rod are less severe and are reversible (106).

Experiments suggest that different levels of structural complexity require interactions between different cytokeratin

Figure 3. Cytokeratin filaments are assembled from heterodimers. One type I and one type II monomer pair to form a cytokeratin heterodimer. Two heterodimers form heterotetramers, which in turn build protofilaments, filaments and networks.



domains (107). The central rod domain is likely to be involved in the initial pairing of type I and type II monomers. Higher order structures also require correct type I and type II pairing, which depends on sequences within specific helical subdomains (108). Normal filament formation also requires at least one amino terminal and one carboxy terminal domain per pair monomers, implying that head to tail interactions are essential for elongation (107).

The role of head to tail interactions in filament formation may depend on cell-specific factors. Other groups have paired constructs of type I and II cytokeratins lacking carboxy termini ("tail-less" constructs), or tail-less cytokeratin 8 with naturally tail-less cytokeratin 19 and observed apparently normal filament formation (109, 110, 111). The lack of a tail, however, was correlated with the abnormal localization of cytokeratin in the nucleus in one study (111) and with decreased filament stability in another (109).

Steps in filament elongation have not been thoroughly characterized. Tetrameric and larger oligomeric complexes are extended to produce 2-3 nm protofilaments (112). Two protofilaments form a 4.5 nm protofibril, and four protofibrils coil around each other to form the 8-10 nm intermediate filament (112).

The process by which larger subunits align is unknown. A twenty-residue peptide that mimics an intermediate filament consensus sequence (KMALDIEIATYRKLLEGEESRI) at the COOH end of the central rod has been used to probe filament formation (113). The addition of 10 to 20-fold excess of this peptide to pre-formed filaments causes them to unravel; a 50 to 100-fold excess causes complete disassembly (113). Point mutations changing the central tyrosine to alanine and the terminal leucine to glycine cause the formation of *in vitro* aggregates under conditions normally conducive to filament formation (113). The domain mimicked by this peptide probably helps align subunits during filament formation, although its exact role has yet to be determined.

The branching of filaments and the formation of networks are not well characterized. An intermediate filament network organizing center in the peri-nuclear region has been proposed (114). However, within 15 minutes of microinjection of monomers, biotinylated keratins can be observed in the cytoplasm, suggesting that an organizing center may be unnecessary (115).

2. Filament assembly and disassembly

Cytokeratin filaments are dynamic cellular structures, continuously exchanging subunits at several sites along their lengths (115). Their most dramatic restructuring

occurs just prior to cell division in a manner specific to cell type. Horwitz et al. observed the collapse of cytokeratin filaments into discrete compartments at the onset of mitosis in three distinct cell lines, but only a partial disassembly of filaments in PtK2 cells (116), a result later confirmed by Aubin et al. (117). It is possible that the compartmentalization of cytokeratin in certain cell types keeps it from interfering with spindle function, and organizes it for distribution to daughter cells, as postulated by Knapp et al. (118).

A survey of several cell lines indicates that there are degrees of cytokeratin disassembly from slight to extensive (119). Cytokeratin can reorganize to smaller filaments (120) or disassemble to oligomeric structures (121) in various systems. Franke et al. observed the unravelling of intermediate filaments into protofilamentous threads, and described the clustering of intermediate filament proteins in dense granules (122).

The cell cycle-dependent reorganization of intermediate filaments is regulated via posttranslational modifications (123). Vimentin, desmin and nuclear lamins are hyperphosphorylated at specific sites by the $p34^{cdc2}$ kinase, which stimulates cells to both disassemble their intermediate filament networks and to enter mitosis (124-127).

The reorganization of cytokeratins is probably also signalled by phosphorylation. Growth-stimulated cultured hepatocytes from suckling rats phosphorylate cytokeratin prior to filament rearrangement, entry into S phase and mitosis (128). In Xenopus oocytes, the disassembly of the cortical cytokeratin filament system into oligomers during meiotic maturation is in part controlled by the hyperphosphorylation of the type II cytokeratin (121).

Chou and Omary have demonstrated that cytokeratins 8 and 18 are phosphorylated on serine residues in HT29 cells, possibly through the activity of protein kinase C (129). In cells arrested in metaphase, the phosphorylation of the type II cytokeratin (cytokeratin 8) is enhanced, as is the glycosylation of cytokeratin 18 (130).

Because these changes are cell cycle dependent, agents that disrupt the cell cycle also have the potential to perturb cytokeratin disassembly or reorganization. Therefore cytokeratins may be novel anti-cancer drug targets.

3. The functions of cytokeratins

The functions of cytokeratins are primarily structural and mechanical (in 131). Their structural roles are dictated by tissue type and may transcend cellular function. Frequently, the collective organization of intermediate

filaments within cells serves to strengthen the entire tissue (in 132). This is most easily observed when filament formation is perturbed. Disruption of cytokeratin filament formation in basal epidermal cells, for example, is the probable cause of an abnormal blistering of the skin in humans, a syndrome known as epidermolysis bullosa simplex (133, 134). This blistering, a direct result of basal cell lysis, is also exhibited by transgenic mice with mutant basal epidermal keratin genes (133). The same mutations perturb *in vitro* filament assembly and *in vivo* network formation (133, 134).

Mutations in cytokeratin genes in differentiating skin cells cause the related disease, epidermolytic hyperkeratosis (EHK, 135, 136). Blistering and thickening also occur due to defective filament formation (135). One EHK mutation has been identified in the conserved H1 subdomain of cytokeratin 1 (136). A role of this subdomain in filament formation has been deduced based on the ability of the wild type, but not mutant H1 peptide to efficiently disassemble pre-formed keratin filaments *in vitro* (136). EHK mutations have also been identified in K10, and probably also compromise filament elongation (135).

In addition to structural functions, cytoplasmic intermediate filaments may serve as intracellular signal transducers (in 131). Their distribution throughout the

cytoplasm and apparent connections to both the plasma membrane and the nucleus are ideally situated to transmit information throughout the cell.

Recent evidence for the mechanical transduction of extracellular information to intracellular receivers has been cleverly generated by Wang et al. Cell surface proteins called integrins bind both to extracellular matrix components and to intracellular adaptor proteins that link them to the cytoskeletal networks (137). Using coated magnetic beads designed to bind integrins, Wang et al. showed that a magnetic force capable of turning an integrin-bound bead increases the mechanical tension on all three cytoskeletal networks. The extracellular magnetic signal was mechanically transduced through the cell by way of the cytoskeletal networks (137).

Additional functions for intermediate filaments have been sought. The ectopic expression of desmin in transgenic mouse cells that normally express vimentin was without an obvious effect (138), as was the global expression of neurofilament L in a variety of mouse cell types (in 132). An attempt to disrupt and examine putative cytokeratin functions by microinjecting antibodies against cytokeratin into mouse embryos successfully inhibited the formation of cytokeratin filaments, but apparently did not affect the formation of an epithelial trophectoderm (139). However,

similar experiments using fertilized Xenopus eggs did result in gastrulation defects, due to a collective lack of cytokeratin filaments (140).

4. Drug damage to cytokeratin

Because the primary functions of cytokeratins appear to be structural and mechanical, damage to cytokeratin is defined by structural aberrations. Agents that damage cytokeratin perturb or stabilize the network or filaments, presumably by interfering with normal interactions between monomers or larger subunits.

As a general rule, cytokeratin filaments are most readily damaged by agents that disrupt protein-protein interactions. For example, 2-methyl-1,4-naphthoquinone (menadione) oxidizes sulfhydryl groups and causes a peri-nuclear condensation of the cytokeratin networks (141). The disruption of cytokeratin interactions with other cytoskeletal proteins by the simultaneous administration of anti-microtubule and anti-microfilament drugs causes the concomitant collapse of cytokeratin networks (142). The individually administered drugs have no effect on cytokeratin networks (142).

Cytokeratins sometimes reorganize without collapse in response to drugs. Dexamethasone induces the synthesis and reorganization of cytokeratin concomitant with growth

inhibition in rat thymic epithelial cells in primary culture (143). Similarly, teleocidin enhances cytokeratin assembly, alters cell morphology and decreases the proliferation of human hepatoma cells (144). The exact relationship between the enhanced synthesis and/or assembly of cytokeratin and growth inhibition in these systems is unclear.

Cytokeratins are modified by the anti-neoplastic agent mitoxantrone (2, 43), which remains associated with cytokeratins despite the relatively harsh conditions employed during the isolation of these insoluble proteins from WiDr cells. The co-isolation of mitoxantrone with cytokeratin suggested a potential link to the prolonged intracellular retention of the drug, and possibly to its cytotoxicity. The hypothesis formulated to explain these observations is: ***Cytokeratin is a novel mitoxantrone target.*** It was tested using WiDr human colon carcinoma cell lines that express the simple epithelial cytokeratins 8, 18 and 19, as described in Chapter 5.

As data were generated and analyzed, the hypothesis was revised, as described in Chapter 6. The revised hypothesis is: ***By binding mitoxantrone, cytokeratin protects the cell from drug damage.*** This hypothesis suggests that the binding of mitoxantrone to cytokeratin diverts the drug from its true target sites. It was tested using murine L cells and L cell transfectants.

CHAPTER 5. METHODS

1. Hypothesis 1: Cytokeratin is a novel mitoxantrone target. Experiments using WiDr cell lines.

(a) Cell culture conditions

The WiDr/S human colon carcinoma cell line was originally obtained from American Type Culture Collection. A mitoxantrone-resistant variant (WiDr/R) was established by continuously exposing WiDr/S cells to gradually increasing concentrations of mitoxantrone as described previously (52, 145). Both cell lines were maintained at 37° in exponential growth in RPMI 1640 medium (Gibco Inc., Grand Island, NY) containing 10% fetal bovine serum (FBS, Whittaker) and 1% penicillin-streptomycin (Gibco) in a humidified incubator under 95% air/5% CO_2. The media used to maintain WiDr/R cells was supplemented with 50 ng/ml mitoxantrone (Lederle Laboratories). All cells were grown for at least 2 days after passage prior to experiments.

For radiolabelling cellular proteins, 5 μCi/ml [^{35}S]-methionine (Escherichia coli hydrolysate labelling agent, ICN) or 1 μCi/ml [^{3}H]-leucine was added to RPMI prepared with 10% of the supplier's recommended amount of unlabelled methionine or leucine. Cells were incubated in labelling media overnight.

WiDr/S cells were treated with 1, 10 or 50 μM

mitoxantrone for 1 hour as indicated in appropriate figure legends. In some cases, drug was removed by aspiration, cells were washed with phosphate-buffered saline (PBS), and fresh medium was added to the monolayer for additional incubation times.

WiDr/R cells were treated with equivalent intracellular doses of 1.25, 12.5 or 62.5 μM mitoxantrone. Viability under these conditions was determined by the MTT dye assay, as described below.

All chemicals were reagent grade or purer and purchased from standard laboratory sources unless otherwise indicated.

(b) Drug survival assays

The sensitivity of both WiDr cell lines to mitoxantrone was assayed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) dye assay (146).

Cells in exponential growth phase were trypsinized and plated in 180 μl media per well in 96-well plates. A pilot study of untreated cells determined the number of cells required to metabolize the yellow MTT dye to a purple product in order to yield absorbance values of between 1 and 2.

For viability experiments, cells were incubated in the plates for 3 or 4 days. At that time, twenty μl of a ten-fold stock of mitoxantrone was added at various doses to the plated cells. Controls using vehicle only were performed to

assure that toxicity was due to drug exposure. After 1 hour drug was removed. In some experiments, 50 μl of 2 mg/ml MTT in PBS was immediately added to each well, and plates incubated an additional 4 hours. In others, cells were washed free of drug and incubated an additional 24 hours before the addition of the dye. The dye was then removed by aspiration and 100 μl of dimethyl sulfoxide was added to each well. Plates were shaken for 5 minutes and the absorbance of each well at 540 nm was immediately recorded.

Absorbance values were normalized to the values obtained from wells incubated without drug. To compensate for colored drugs which interfered with the assay at high doses, extra wells containing medium and drug (but no cells) were used to determine the amount of absorbance due to drug alone.

(c) Cytokeratin preparations

Exponentially growing WiDr/S or WiDr/R cells were washed with PBS, then harvested by incubation in PBS plus 0.5 mM EGTA at room temperature. Enriched cytokeratin preparations were obtained by extracting cells once with 10 mM Tris-HCl, 140 mM NaCl, 0.5 % Triton X-100, 5 mM EDTA, then again with the same buffer plus 1.5 M KCl. Some extractions buffers also contained 1 mM EGTA, 20 μM sodium vanadate and 1 mM sodium fluoride to inhibit phosphatases. The insoluble pellet was washed in PBS before experimental

treatment. Cytokeratins were identified by two-dimensional gel electrophoresis as the major proteins in the pellet.

(d) Mitoxantrone binding

All mitoxantrone binding experiments were designed and executed by Robin Roberts. Statistical analysis of these data was performed by John Kittelson, and biological analysis by Patricia Bauman.

WiDr/S and WiDr/R cells metabolically labelled with [^{3}H]-leucine were treated for 1 hour at 37° C with 1, 10 or 50 μM [^{14}C]-mitoxantrone. After treatment, cells were washed, removed from tissue culture dishes by scraping, washed again and solubilized in tissue solubilizer. Alternatively, cells were washed to remove excess drug and incubated an additional 24 hours before solubilization.

Cells treated identically with [^{3}H]-leucine and [^{14}C]-mitoxantrone were also collected in order to isolate cytokeratin. Solubilized cytokeratin was shaken, neutralized and counted for both [^{14}C]-mitoxantrone and [^{3}H]-leucine as described.

Cells treated with [^{3}H]-leucine only were collected to determine the protein concentrations of samples by BCA assay (Pierce) and the incorporation of [^{3}H]-leucine per μg protein by scintillation counting.

(e) Electrophoresis and blotting

Cytokeratins from mitoxantrone or vehicle treated cells were separated on 10 or 12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels according to the method of Laemmli (147). Two dimensional gels were performed by separating samples on isoelectric focussing gels in the first dimension (148) and SDS-PAGE gels in the second dimension (147). Prior to electrophoresis, the protein concentration of whole cell sonicates was determined using the BCA assay (unless otherwise indicated). Incorporation of [^{35}S]-methionine per μg protein was determined by counting an aliquot of the same radioactive cell sonicate. Equal counts or equal amounts of protein were applied to electrophoretic gels for direct comparisons.

Gels were electroblotted to nitrocellulose at 60 volts (200 mamps) overnight. Some blots were reacted with the anti-cytokeratin monoclonal antibody 10.11 (151) to confirm the identities of specific spots or bands. These were visualized on autoradiographic film using either a chemiluminescent horseradish peroxidase-conjugated secondary antibody and the ECL detection kit from Amersham. Alternatively, [^{35}S]-labelled proteins were visualized directly on autoradiographic film, and were quantitated using a Bio-Rad model 620 video densitometer.

(f) Cytokeratin assembly and disassembly assays

Cellular protein was metabolically labelled overnight with [^{35}S]-methionine before exposing WiDr/S or WiDr/R cells to mitoxantrone. After exposure, cells were washed and cytokeratin was isolated as described. The solubility assay of Coulombe and Fuchs (94) was modified and used to determine urea concentrations at which cytokeratin monomers would assemble to tetramers or, conversely, in which cytokeratin tetramers would disassemble to monomers.

Cytokeratin monomers (in 9M urea) or complexes (in 4M urea) were dialyzed overnight against 50 mM phosphate buffers containing various concentrations of urea. Products of dialysis were crosslinked with glutaraldehyde (final concentration 0.01%), and equal amounts of protein were applied to 5-12.5% SDS-polyacrylamide gradient gels.

A direct solubility assay was also employed to assess the stability of cytokeratin complexes. WiDr/S cells labelled with [^{35}S]-methionine were subsequently treated for 1 hour with 50 μM mitoxantrone or vehicle. Cytokeratin pellets were isolated and extracted twice in urea buffers (4 M to 9 M urea). Radioactivity from soluble and insoluble fractions was quantitated. The percentage of soluble radioactivity in treated cells at specific urea concentrations was compared to that of untreated cells at

the same urea concentrations.

2. Hypothesis 2: By binding mitoxantrone, cytokeratin protects the cell from drug damage. Experiments using L cell lines.

(a) Cell culture conditions

Parental murine L cells and transfected LK8+18 cells were obtained from Dr. Robert Oshima at La Jolla Cancer Research Foundation, La Jolla California (95). The parental cells were cultured in Dulbecco's minimal essential medium (DMEM), supplemented with 10% FBS and 30 μg/ml gentamycin. The transfectants were maintained in selection medium, which consists of the supplemented DMEM plus 1.6 mM xanthine, 30 μg/ml mycophenolic acid, and 400 μg active G418/ml. (Xanthine was dissolved in 1N NaOH, and the medium was neutralized after the addition of xanthine with an equal volume of 1N HCl.)

Plasmid vectors pGEM-3 (Promega) and pSV_2neo (149) were amplified and purified by standard methods (see appendix; Bio-Rad technical update FOCUS, volume 7, number 2) for the creation of mock transfectants (LPBMOC cells). Transfection of pGEM-3 and pSV_2neo were performed simultaneously (150), using a ratio of 10:1 pGEM-3:pSV_2neo, as described by Kulesh and Oshima (95). LPBMOC cells were maintained in DMEM,

supplemented with 10% FBS, 30 μg/ml gentamycin and 400 μg active G418/ml. (Xanthine was not required for the maintenance of LPBMOC cells.)

All cells were maintained in a humidified incubator under 95% air/5% CO_2 at 37°C for at least 2 days after passage prior to experiments. Growth rates were obtained by seeding 1 x 10^5 cells in several individual 35 mm dishes and counting them daily.

(b) Fluorescence-activated cell sorting

To prepare cells for flow cytometric cell cycle analysis, cells were trypsinized and resuspended in DMEM. As resuspended cells were being gently vortexed, two volumes of a 3:1 solution of methanol:acetic acid were added to fix cells prior to analysis. The suspension was then washed free of acid, treated with 0.1 mg/ml DNase-free RNase A and stained with 100 μg/ml propidium iodide. Stained samples were refrigerated in the dark until use. Cell cycle analysis was performed as described (2).

(c) Drug resistance assays

Resistance to mitoxantrone in the L cell lines was assayed by the MTT dye assay, as described for the WiDr cell lines, with some modifications.

Cells were plated in 180 μl media per well in 96-well plates. Twenty μl of a ten-fold drug stock was added at

various doses to freshly plated cells. Controls using vehicle only were performed to assure that toxicity was due to drug exposure. The plates were then incubated for 4 days at 37^0. On the fourth day MTT dye was added and plates were processed as described.

(d) Indirect immunofluorescence

Cells were grown an #1.5 cover slips in 35 mm dishes for at least 2 days prior to fixing with ice cold methanol for 2-5 minutes. Methanol was removed and cover slips air dried. Cells were then permeabilized with acetone for less than one minute. After removal of acetone, cells were washed twice with PBS containing 1% FBS and 1 mM azide. A monoclonal anti-cytokeratin antibody recognizing cytokeratins 8 and 18 (antibody 10.11, 151) was added to the dishes at a dilution of 1:800 for 30 minutes at room temperature. Cells were washed twice with PBS as previously, before incubation with a 1:100 dilution of rabbit anti-mouse IgG conjugated to fluorescein. Confocal microscopy was performed using a Zeiss confocal laser scanning microscope with an Ar laser, a scan time of 8 seconds and a line average of 18 times, with the assistance of Dr. Anne Cress. Images of both L and LK8+18 cells were recorded on ASA 400 film using the same brightness and contrast parameters.

(e) Cytokeratin preparations

Cytokeratins were prepared from LK8+18 cells in 20 mM Tris-HCL, pH 7.4 containing 0.6 M KCl and 1% Triton X-100. Extractions were centrifuged at 10,000 rpm to pellet insoluble material. Similar extractions were performed using L and LPBMOC cells. Insoluble proteins were dissolved in 4x cracking buffer (see appendix) and separated on 10% polyacrylamide gels as described.

(f) [^{14}C]-Mitoxantrone accumulation and efflux

The accumulation and efflux of [^{14}C]-mitoxantrone were simultaneously analyzed in the L and LK8+18 cells. One day prior to the assay, cells were plated directly into glass scintillation vials at a density of 1 x 10^6 cells per vial and allowed to adhere overnight. At various times [^{14}C]-mitoxantrone was added to triplicate vials to a final concentration of 10 μM and allowed to incubate with the cells for 1 hour.

To determine the amount of drug accumulated intracellularly in 1 hour, the incubation was terminated by aspirating the monolayer and washing twice with ice cold PBS. Cells were digested by shaking for 1-2 hours in the presence of 500 μl of 0.2 N NaOH. Prior to the addition of scintillation fluid, 100 μl of 1 N HCl was added to neutralize each vial. Vials were shaken by hand and

incubated in the cold room for at least 1 hour prior to measuring radioactivity.

To determine drug efflux rates, drug was replaced by fresh media at the end of the initial incubation, and cells were allowed to incubate for additional designated times. The medium was then removed and the cells washed twice with ice cold PBS, digested with 0.2 N NaOH and neutralized as described. Radioactivity was measured as by scintillation counting.

(g) Cobalt irradiation and colony forming assays

Cells in logarithmic growth were subject to γ-irradiation by ^{60}Co at doses ranging from 0 to 10 Gray. Immediately following irradiation, cells were trypsinized and counted. Irradiated cells were plated at cell densities of 200 or 500 cells per 60 mm tissue culture dishes in 5 ml growth medium, and incubated at 37°C for 14 days. The resulting colonies were fixed with 5 ml of a 3:1 solution of methanol:acetic acid, then stained with crystal violet and counted manually. For each dose, 2 sets of triplicate counts were obtained. The means of the 2 sets were added together and divided by 2 to determine an experimental mean value.

(h) Direct fluorescence and confocal microscopy

Cells were incubated in 1 μM doxorubicin for 1 hour.

At the end of the hour, excess drug was removed by decanting and cells were immediately fixed with 7.5% glutaraldehyde without washing. After 5 minutes, the glutaraldehyde was removed, the cells were rinsed with PBS and the doxorubicin directly visualized by confocal microscopy as previously described, using a HeNe laser and a scan time of 8 seconds.

CHAPTER 6. RESULTS AND DISCUSSION OF HYPOTHESIS 1: *CYTOKERATIN IS A NOVEL MITOXANTRONE TARGET.*

Previous studies identified cytokeratin as a cellular component that associates with mitoxantrone, based on the following observations. First, the intense blue color of mitoxantrone can easily be seen in isolated cytokeratin samples after sequential salt and detergent extractions (Figure 4). Second, [^{14}C]-mitoxantrone is detectable in the cytoskeletal compartment of WiDr/S cells as determined by measuring radioactivity in various subcellular fractions (2, 42). Finally, cytokeratin proteins can co-migrate with [^{14}C]-mitoxantrone on two-dimensional electrophoretic reducing gels (42).

The unusual persistence of the drug with cytokeratin suggested a potential link to the retention of mitoxantrone in patient tissues and cultured cell lines. Prolonged intracellular retention has been proposed to cause long-term depression of DNA replication (1), and could be a major factor in mitoxantrone cytotoxicity. As a first step in determining whether cytokeratin is a novel drug target, the association of mitoxantrone with cytokeratin was investigated further.

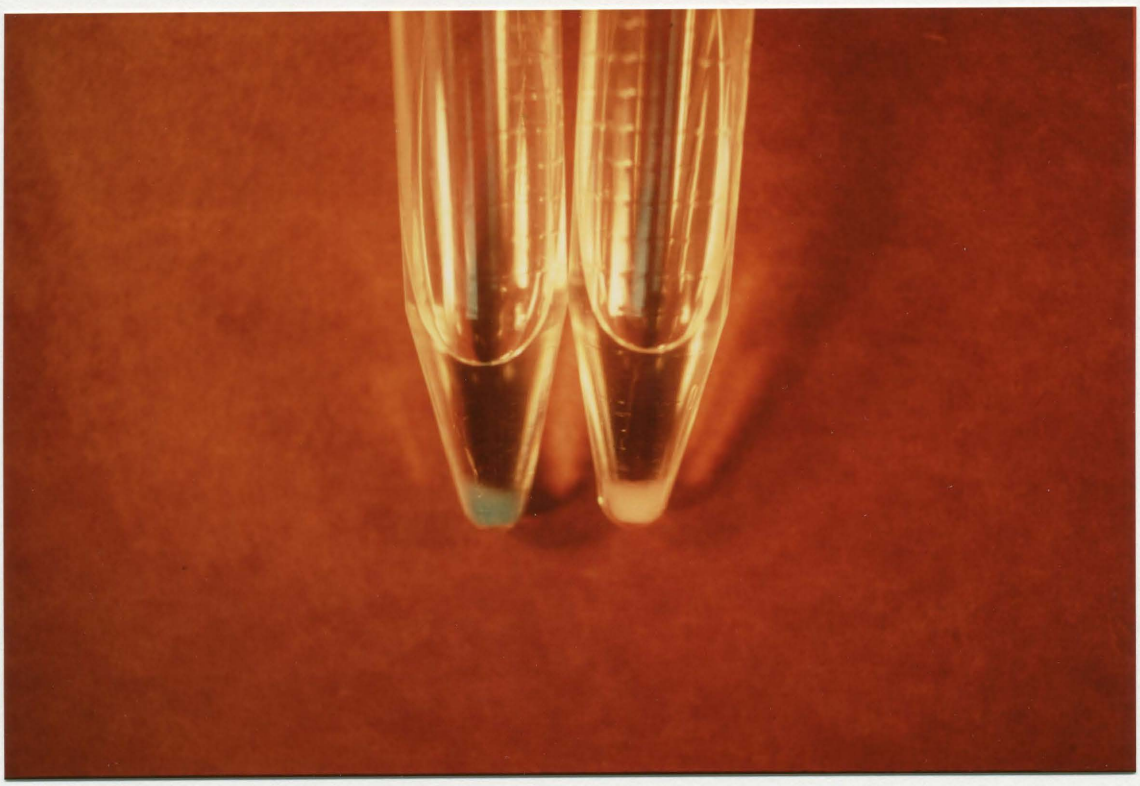


Figure 4. Mitoxantrone associates with cytokeratin protein. WiDr/S cells were treated with 50 μM mitoxantrone (left) or vehicle (right) for 1 hour prior to cytokeratin isolation. Cytokeratins are shown as insoluble pellets in PBS.

2. Results

(a) Mitoxantrone has an affinity for cytokeratin.

Preliminary observations suggested that WiDr cells could withstand short exposures to relatively high doses of mitoxantrone. To generate dose-response curves and confirm cell viability after exposure to mitoxantrone, the MTT cytotoxicity assay was employed (Figure 5).

Approximately 82% of WiDr/S cells remain viable after a 1 hour exposure to 50 μM mitoxantrone. When the cells were given an additional 24 hour incubation time after exposure to 50 μM mitoxantrone, viability is about 60%. It was thus possible to use a relatively high dose (50 μM) of [^{14}C]-mitoxantrone to treat Widr/S cells, ensuring that a measurable quantity of drug remained associated with cytokeratin throughout the isolation procedure.

The results of the binding experiments suggest that mitoxantrone has an affinity for cytokeratin. When WiDr/S cells are harvested immediately after drug treatment (time 0, Figure 6), approximately 13.5 ng mitoxantrone binds per μg cytokeratin protein, compared to approximately 9.4 ng mitoxantrone that binds per μg cell lysate protein. This affinity persists for an additional 24 hours after drug treatment, despite the fact that WiDr/S cells have an apparent mechanism for removing drug from intracellular

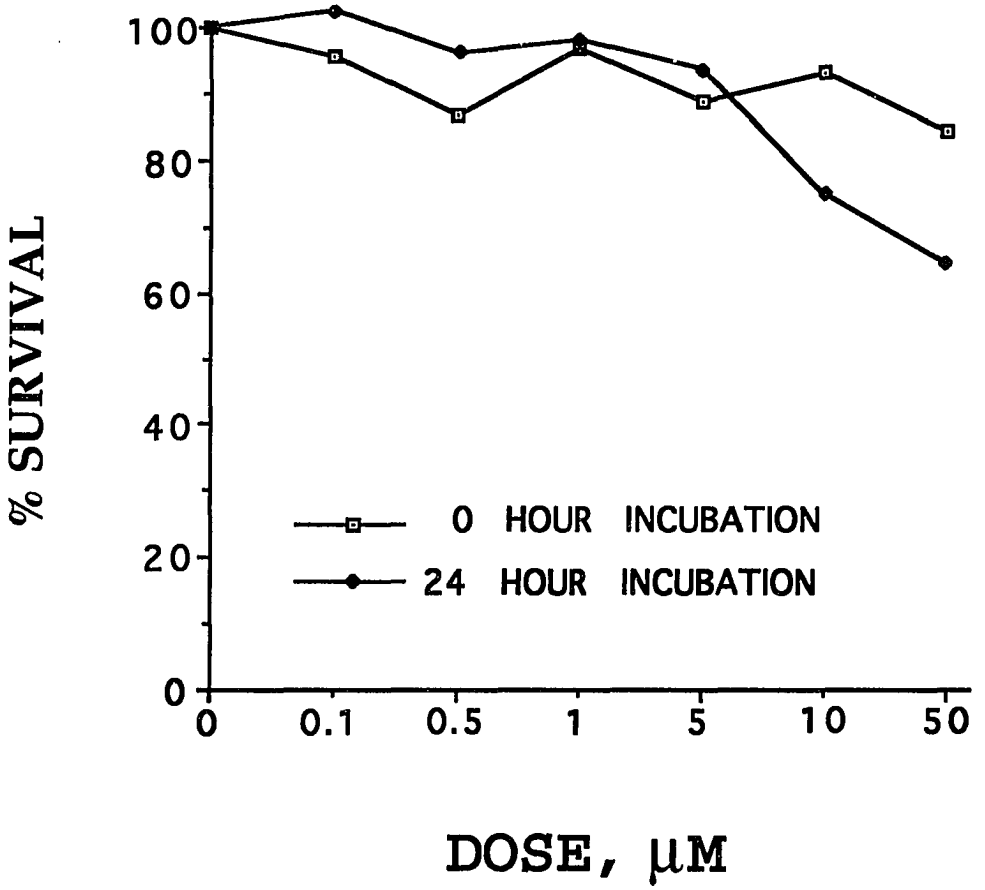


Figure 5. WiDr/S cells are viable after treatment with 50 μM mitoxantrone. WiDr/S cells were plated for MTT assays as described. Open symbols represent cells that received dye immediately after drug exposure. Closed symbols represent cells that were washed after drug treatment and incubated an additional 24 hours before the addition of MTT dye.

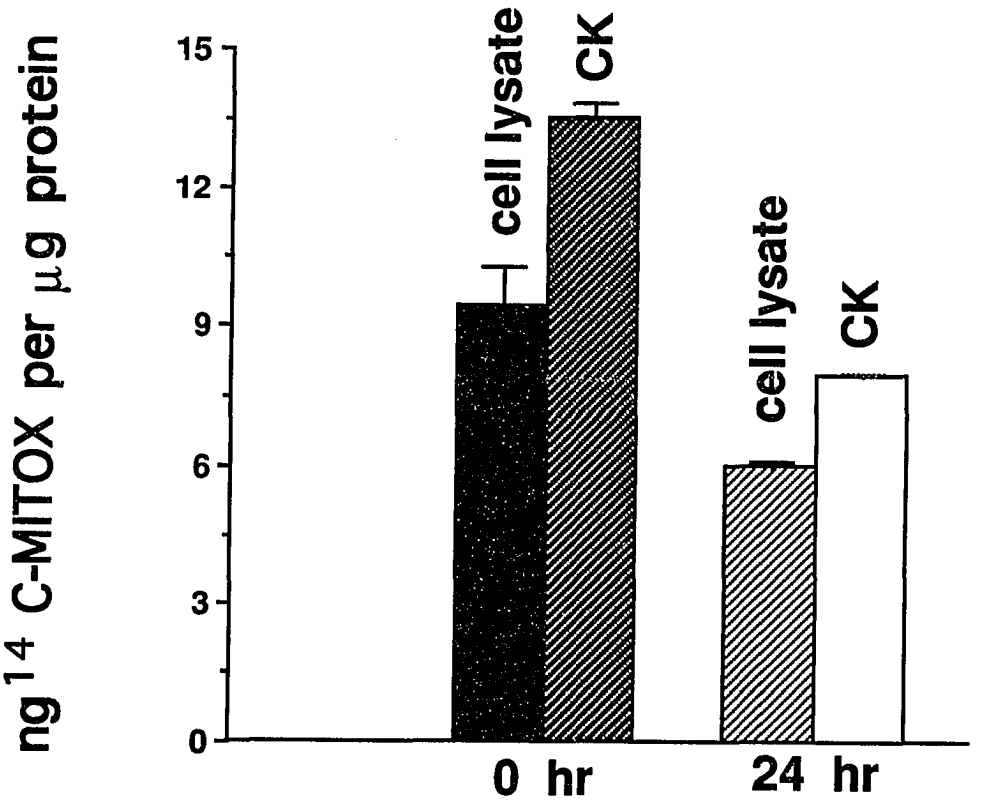


Figure 6. [^{14}C]-Mitoxantrone has an affinity for cytokeratin. WiDr/S cells were treated with 50 µM [^{14}C]- mitoxantrone for 1 hour. Time 0 indicates that protein was harvested immediately after drug exposure, and time 24 indicates that cells were washed and incubated an additional 24 hours after exposure before harvesting. Bars labelled "cell lysate" represent the mean amount of [^{14}C]-mitoxantrone that binds per µg protein from whole cell lysates. Bars labelled "CK" represent the amount of [^{14}C]-mitoxantrone that binds per µg cytokeratin. (N=6 samples +/- standard error.)

compartments over time.

However, the decrease in the amount of [^{14}C]-mitoxantrone bound to cytokeratin over 24 hours is greater than the corresponding decrease in the amount of drug bound to cell lysate protein. The affinity of mitoxantrone for cytokeratin thus appears to have diminished over 24 hours, suggesting that either mitoxantrone is binding to low affinity sites along cytokeratin filaments, or that mitoxantrone is being specifically removed from the cytokeratin.

(b) Mitoxantrone does not damage cytokeratin monomers.

Cytokeratins provide structural support for the cell, and an organizational scaffold for cell organelles (152, 153). The biological consequences of the binding of mitoxantrone to cytokeratin were investigated by seeking mitoxantrone-induced structural damage to cytokeratin proteins. Structural damage includes, but is not limited to, changes in size due to adduct formation or drug-induced degradation, changes in electrophoretic migration due to alterations in folding, or changes in post-translational modifications, possibly due to the masking of specific modification sites by the drug.

Electrophoretic gel patterns of isolated cytokeratins from treated and untreated cells were analyzed for drug-

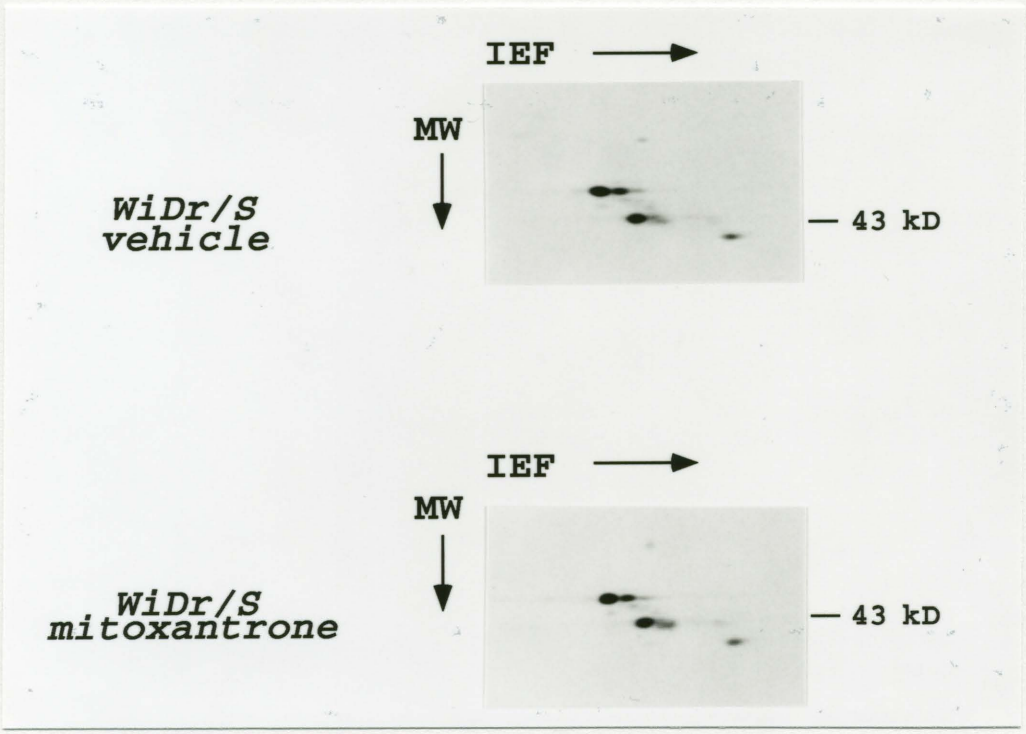


Figure 7. Mitoxantrone does not alter the pattern of cytokeratin proteins on two-dimensional electrophoretic gels. WiDr/S cells were treated with 1 μM mitoxantrone for 1 hour, followed by a 24 hour incubation in fresh medium. Isolated cytokeratins were separated by isoelectric focussing (left to right) and SDS-PAGE (top to bottom). The molecular weight of cytokeratin 18 is indicated at the left.

induced structural changes.

Three cytokeratin proteins are consistently isolated from WiDr/S cells. These are the simple epithelial cytokeratins 8, 18 and 19 (Figure 7). Cytokeratin 8 is of the neutral/basic class (type II) and has a molecular weight of about 50 kilodaltons. It is a phosphorylated protein, and migrates as three distinct isoforms depending on its phosphorylation state (see Figure 7). Cytokeratin 18 (type I) is approximately 43 kilodaltons, is also phosphorylated, and migrates as two isoforms. Cytokeratin 19 (also type I) is unique in that it is the only cytokeratin to naturally lack the intermediate filament carboxy terminal "tail" domain (110). It therefore has the smallest molecular weight of the three (about 40 kilodaltons) and the most acidic isoelectric pH (5.2).

When WiDr/S cells are treated with up to 50 μM mitoxantrone, no reproducible changes in the quantities, sizes or patterns of migration of cytokeratins 8, 18 or 19 were observed. The most acidic isoform of cytokeratin 8 appeared somewhat sporadically on the gels, but its appearance was independent of mitoxantrone treatment. Therefore, mitoxantrone does not cause structural damage to cytokeratin monomers, as defined by specific alterations in electrophoretic patterns.

(c) Mitoxantrone does not affect cytokeratin assembly or disassembly.

Mitoxantrone causes many types of cell damage. It inhibits nucleic acid synthesis, creates topoisomerase-mediated DNA strand breaks, stabilizes microtubules and disrupts cell cycle (reviewed in 5, 39, 154). Several of these events may be causally linked.

Agents, such as acrylamide or nickel, that damage cytokeratins tend to alter the cytokeratin network phenotype (reviewed in 132). It was hypothesized that mitoxantrone may stabilize cytokeratin networks, preventing their cell cycle-dependent reorganization. The stabilization of cytoskeletal proteins could then be a causal factor in mitoxantrone-induced G_2 arrest.

Modified solubility assays were used to assess the stability of cytokeratin heteropolymers from mitoxantrone or vehicle treated WiDr/S cells. When cytokeratin complexes from treated cells were subjected to increasing urea concentrations, they partially or fully disassembled to monomers at the all urea concentrations tested, as determined by electrophoretic migration (Figure 8). A greater proportion of cytokeratin complexes disassembled to monomers as the urea concentration was increased, but in no case did treatment with mitoxantrone stabilize cytokeratin

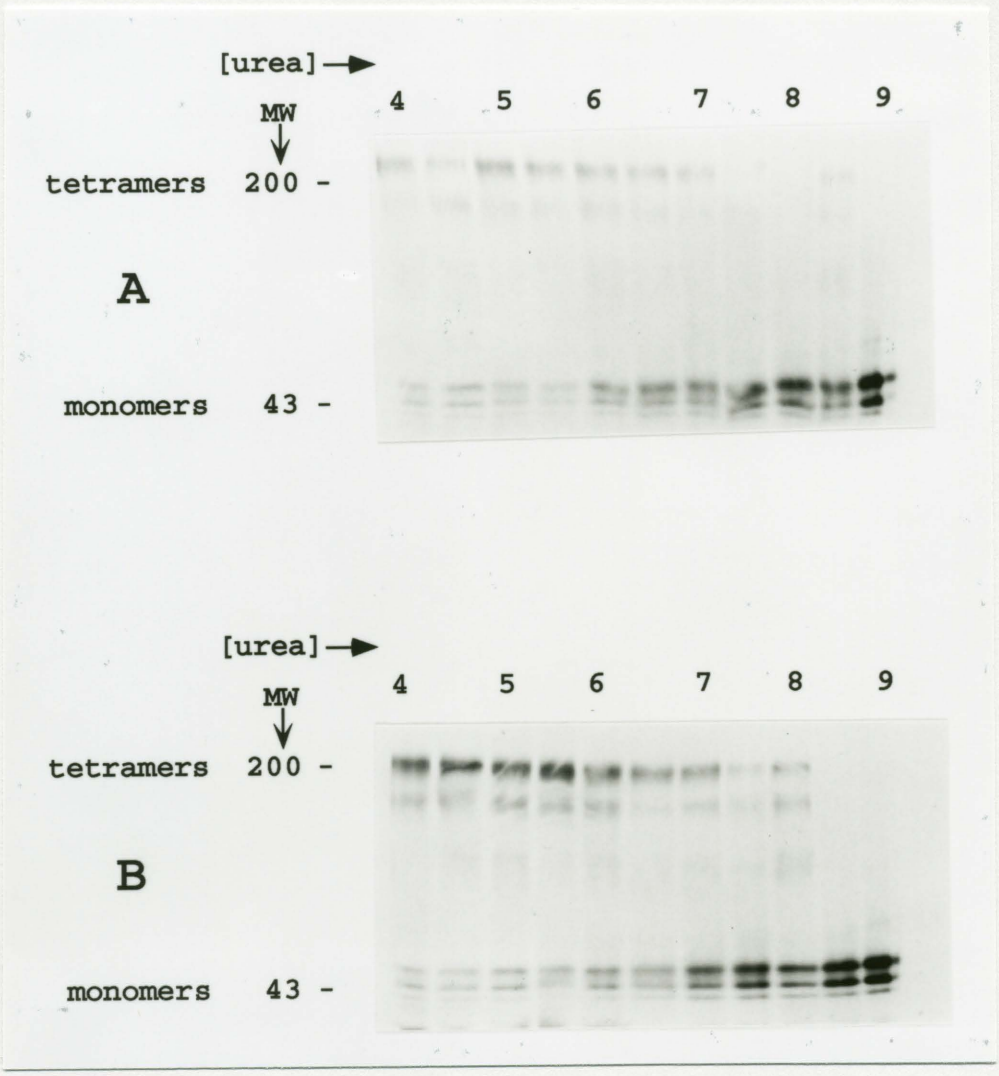


Figure 8. Mitoxantrone does not stabilize cytokeratin complexes. WiDr/S cells were treated with vehicle (A) or 1 μM mitoxantrone (B) for 1 hour, followed by a 72 hour incubation in the absence of drug. These conditions are sufficient to arrest 50-80% of the cells in G_2/M phase of cell cycle (not shown). The urea concentrations are indicated at the top of each lane, and increase by 1/2 Molar increments. Tetrameric complexes and monomers are indicated.

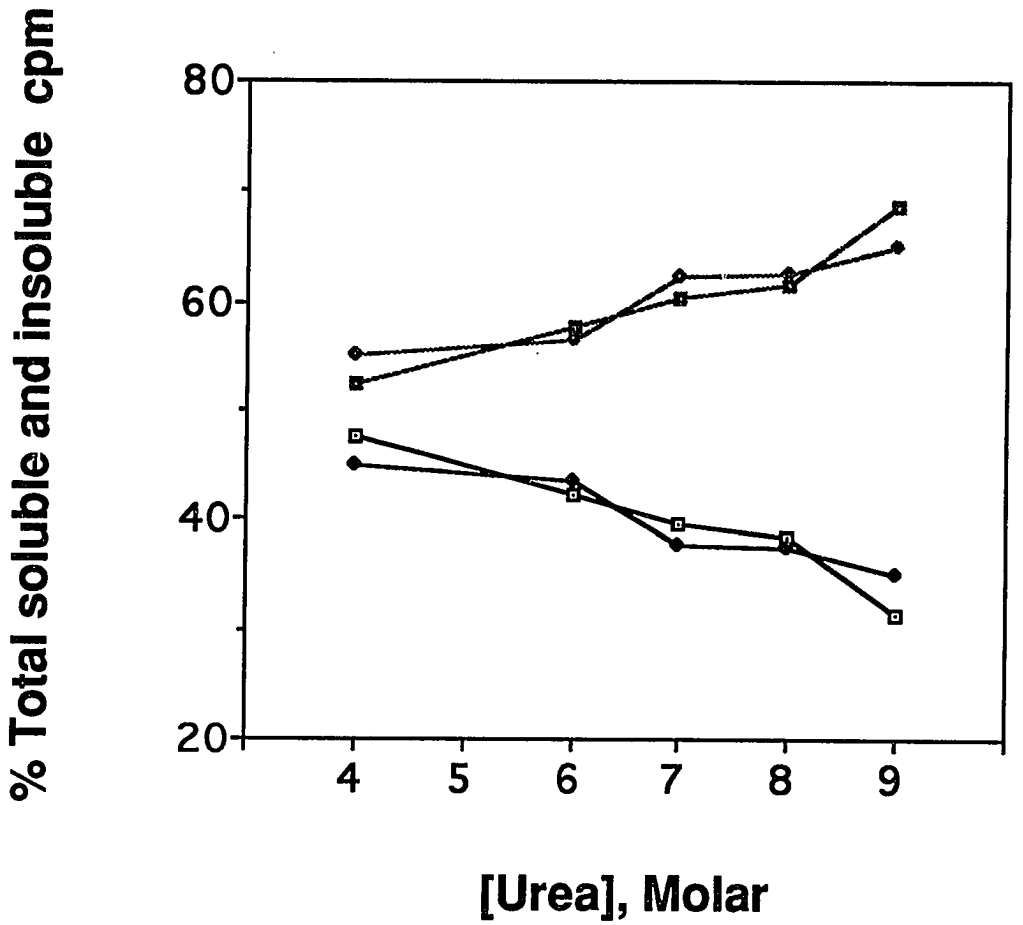


Figure 9. Cytokeratin solubility is not affected by mitoxantrone in a direct solubility assay. WiDr/S were treated with 50 μM mitoxantrone or vehicle. Isolated cytokeratins were extracted with various concentrations of urea, and separated into soluble and insoluble fractions. Squares indicate that WiDr/S cells were treated with vehicle only. Heavy squares indicate soluble counts and light squares indicate insoluble. Diamonds indicate that WiDr/S cells were treated with mitoxantrone. Open diamonds indicate soluble counts and closed diamonds indicate insoluble counts.

complexes relative to controls.

Disassembly was also assessed by direct quantitation of soluble and insoluble [^{35}S]-labelled cytokeratin protein from mitoxantrone or vehicle treated cells (Figure 9). Drug treatment did not affect cytokeratin solubility at any urea concentration tested.

The reassembly of disassembled cytokeratins from mitoxantrone or vehicle treated WiDr/S cells was also examined. Tetrameric complexes assembled as the urea concentration dropped from 9 M to 6.5 M, regardless of prior drug treatment, with small amounts of tetramers occasionally detectable at higher urea concentrations (Figure 10). The treatment of WiDr/S cells with mitoxantrone did not damage cytokeratin by preventing the assembly of cytokeratin monomers to tetramers.

(d) Chronic exposure to mitoxantrone does not damage cytokeratin.

Because of the slow removal of mitoxantrone from biological systems, it was hypothesized that chronic exposure to, and persistent binding of mitoxantrone might damage cytokeratins over time. This hypothesis was tested in WiDr/R cells which have been selected for resistance to mitoxantrone and are continuously maintained in low levels of the drug.

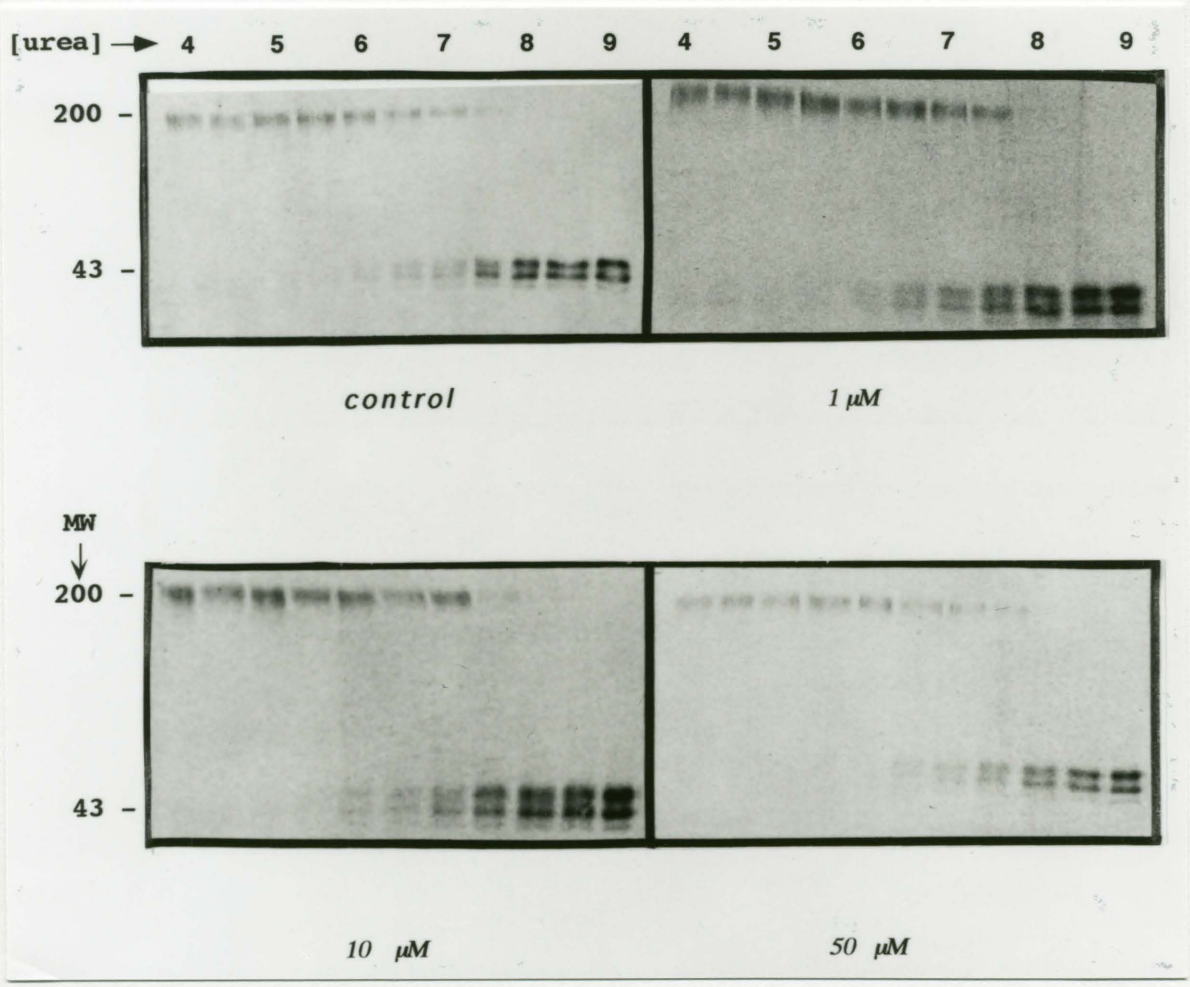


Figure 10. Mitoxantrone does not inhibit cytokeratin reassembly. WiDr/S cells were exposed to the indicated doses of mitoxantrone. Cytokeratins were isolated and solubilized in 9 M urea, then dialyzed against decreasing concentrations of urea. The concentrations of urea are indicated at the tops of each gel. The migration of molecular weight standards are shown at the left. Tetramers first appear at 7.5M urea; monomers can be detected at concentrations up to 6M urea.

Cytokeratins from WiDr/R cells were analyzed using the drug binding, two-dimensional gel electrophoresis and urea solubility assays described.

Mitoxantrone has approximately the same affinity for cytokeratin in both cell lines, although more drug accumulates and is retained by WiDr/S cells than WiDr/R cells. Other investigators have proposed decreased drug uptake (154) and increased drug efflux (145) as possible explanations for the difference in accumulation.

WiDr/R cells produce the same types and amounts of cytokeratins as WiDr/S cells. The gel patterns of cytokeratins 8, 18 or 19 from the two cell lines were indistinguishable. Cytokeratin complexes from WiDr/R cells also assemble from, and disassemble to monomers exactly as they do from WiDr/S cells. Taken together the data suggest that chronic exposure to mitoxantrone does not damage cytokeratin in WiDr/R cells.

2. Discussion

The question of whether a protein sustains drug damage is most easily answered by determining how the structure and function of that protein is altered in response to drug. The structural complexity of cytokeratin offers a range of drug targets from a single monomer to a highly branched network. Functionally, cytokeratin is a dynamic skeletal

web supporting and organizing intracellular contents (152, 153). Disruption of function must therefore include not only structural and morphological changes, but also perturbations of the assembly or disassembly of the network.

Many forms of structural damage to cytokeratin are possible. For example, a truncation of the cytokeratin 14 cDNA within its fourth helical subdomain causes the translation of an incomplete monomer, and the collapse of an entire intermediate filament network upon its transfection into PtK2 cells (105). Truncated filaments, thickened filament bundles, fragmented or unravelled filaments, or non-filamentous aggregates of cytokeratin protein are also indicative of damage to cytokeratin networks. None of these gross morphological alterations in network phenotype were observed in either WiDr/S or WiDr/R cells treated with mitoxantrone (R. Roberts, unpublished data).

It remains possible that mitoxantrone causes subtle structural damage to cytokeratin filaments which is not detectable using the methods described herein. When cytokeratins 8 and 18 are altered by site-directed mutagenesis at specific residues, a slight shortening of the mutant filaments can be detected by electron microscopy, but not by tetramer formation in gel assembly assays similar to the those employed in this work (155).

Both cytokeratin structure and function rely on the

appropriate dynamic balance between the synthesis, degradation and reorganization of the filaments and networks, especially as a cell progresses through its reproductive cycle. In other intermediate filament networks, rearrangements are regulated by specific post-translational modifications (123, 124, 126). Kinases catalyze the phosphorylation of specific residues of vimentin, desmin and nuclear lamins, which regulates the disassembly of these networks at the onset of mitosis (123-127). The inhibition of phosphorylation signaling pathways can disrupt the co-ordination of network disassembly with later mitotic events (126).

The phosphorylation of cytokeratins probably also plays a role in regulating the localized network rearrangements preceding and following mitosis. Cytokeratin 8, for example, is more highly phosphorylated during cell division than interphase (130). The hyperphosphorylation of other type II cytokeratins has been correlated to both filament rearrangement and exit from quiescence in epidermal growth factor-stimulated cultured suckling rat hepatocytes (128). Furthermore, the hyperphosphorylation of a type II cytokeratin may signal filament severing and the visual disappearance of the cytokeratin network during meiotic progression in Xenopus oocytes (121).

Based on the structural homologies between intermediate

filaments and the observations described above, it is reasonable to speculate that the phosphorylation of cytokeratin is functionally linked to mitotic network rearrangements.

Therefore, drug-induced alterations in the phosphorylation states of cytokeratins can be considered drug damage. However, the two dimensional electrophoretic gel analysis did not indicate any drug-induced changes in the overall surface charge of cytokeratins 8, 18 or 19 that would imply changes in phosphorylation state.

The chronic exposure of WiDr cells to mitoxantrone results in the selection of a sub-population resistant to the effects of the drug (52). The combination of continuous exposure to mitoxantrone and tenacious retention of the drug in the cell has potentially damaging consequences, especially at sites of drug binding. Because an affinity of mitoxantrone for cytokeratin was demonstrated, cytokeratins from WiDr/R cells chronically exposed to mitoxantrone were compared to cytokeratins isolated from WiDr/S cells.

The affinity of mitoxantrone for cytokeratin was the same in both cell lines, regardless of the difference in drug accumulations observed. The accumulation difference has been attributed to decreased drug uptake (52) and increased drug efflux (145) in WiDr/S cells relative to

WiDr/R cells. It is also possible that the slow elimination of mitoxantrone from intracellular sites precluded its clearance from binding sites in WiDr/R cells, despite the absence of the drug in the culture medium for at least 2 days prior to experiments. A lower intracellular level of [^{14}C]-mitoxantrone in WiDr/R cells could then be explained by fewer unoccupied mitoxantrone binding sites available for radiolabelled drug to bind.

Neither the two-dimensional gel patterns of cytokeratins and nor the assembly/disassembly of cytokeratin complexes depend on the cell line from which cytokeratin is isolated, suggesting that their chronic exposure to mitoxantrone does not damage cytokeratin.

Two major conclusions can be drawn from these studies. The first is that cytokeratin is not damaged by mitoxantrone in WiDr cells. The binding of mitoxantrone to cytokeratins is not sufficient to damage these proteins either by acute or chronic dosing regimens.

The second conclusion is that cytokeratin is not altered during the selection of mitoxantrone resistant WiDr/R cells, and plays no role in the acquired resistance to this drug.

These data do not support the hypothesis of mitoxantrone-induced cytokeratin damage, but are consistent

with the alternative hypothesis, which suggests that drug binding to cytokeratin may protect the cell.

CHAPTER 7. RESULTS AND DISCUSSION OF HYPOTHESIS 2: *BY BINDING MITOXANTRONE, CYTOKERATIN PROTECTS THE CELL FROM DRUG DAMAGE.*

The data presented herein suggest that cytokeratin is not damaged by mitoxantrone and is not involved in acquired resistance to mitoxantrone in WiDr/R cells. The alternative hypothesis, that the binding of mitoxantrone by cytokeratin protects true cellular targets, was tested in a set of murine fibroblast cell lines. The set is composed of the parental L cells, which expresses vimentin but not cytokeratin; the LK8+18 cells, which have been stably transfected with sequences encoding cytokeratins 8 and 18 and express both vimentin and cytokeratins; and mock-transfected L cells (LPBMOC), which express only vimentin.

1. Results

(a) L, LK8+18 and LPBMOC cells have similar growth characteristics.

The growth kinetics of the three cell lines were obtained from growth curves as described. A statistical comparison of the slopes of the linear portions of the growth curves failed to reveal any significant difference in growth rate between transfected and untransfected cells (Figure 11). The population distributions in phases of cell

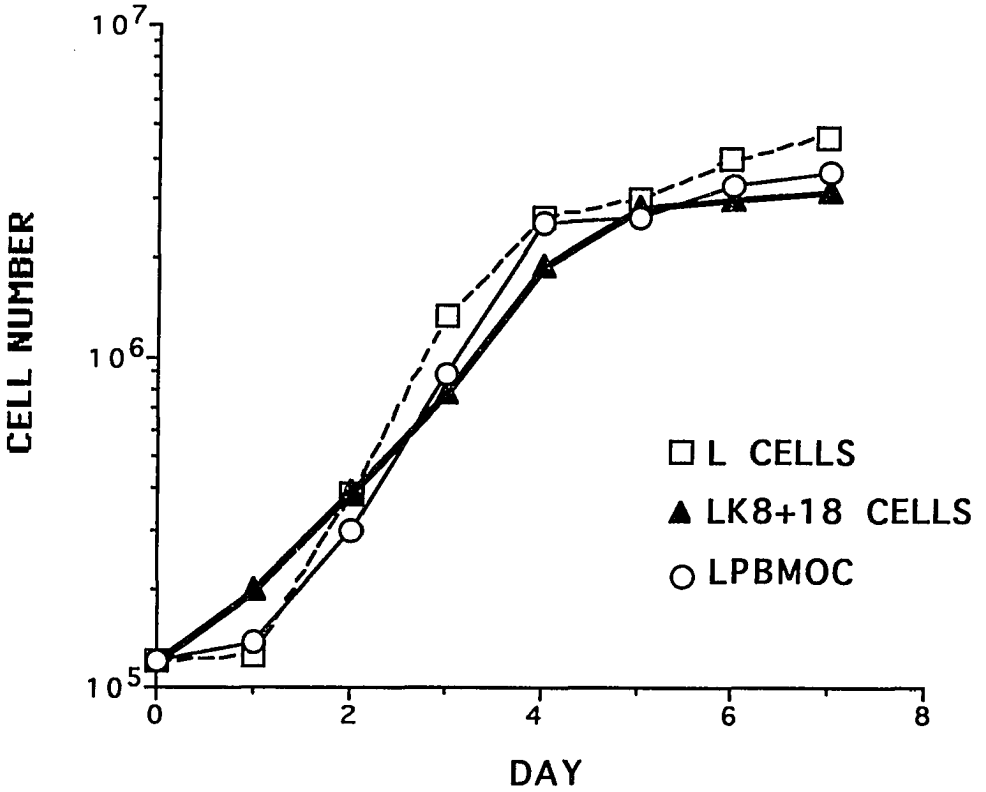


Figure 11. L, LK8+18 and LPBMOC have similar growth rates. L, LK8+18 and LPBMOC were plated at 1.2 x 10^5 cells per dish and grown for the specified number of days before counting. Open squares represent the mean number of L cells (n=2), open circles represent the mean number of LPBMOC cells (n=3), and closed squares represent the mean number of LK8+18 cells (n=3).

cycle are also similar. From 44-49% of log phase cells in each population are in G_1 phase, about 36% in S phase, and from 15-20% in G_2/M phase (Figure 12).

(b) LK8+18 cells produce and assemble cytokeratin.

The synthesis of cytokeratin proteins by LK8+18 cells could be demonstrated by Western blot analysis (Figure 13D). Antibody 10.11 reacted with two proteins of molecular weights 50 and 43 kilodaltons that were isolated from LK8+18 cells but not parental L cells.

The cytokeratin proteins are assembled into filamentous networks, as can be seen by indirect immunofluorescent staining (Figure 13), although the networks are generally less extensive than those of WiDr cells (not shown).

(c) Cytokeratins confer drug resistance.

MTT dye assays were used to test whether cytokeratin could protect LK8+18 cells against the cytotoxic effects of mitoxantrone. When L cells are treated with a range of mitoxantrone doses, a 50% decrease in absorbance compared to untreated cells (IC50) occurs at a mean concentration of 0.07 μM (Table 1). In contrast, the IC50 of mitoxantrone in LK8+18 cells is 0.54 μM, a concentration 7.6 times higher than the IC50 in L cells. LPBMOC cells resemble L cells, with a mean IC50 of 0.05 μM.

To test whether or not resistance in LK8+18 cells was

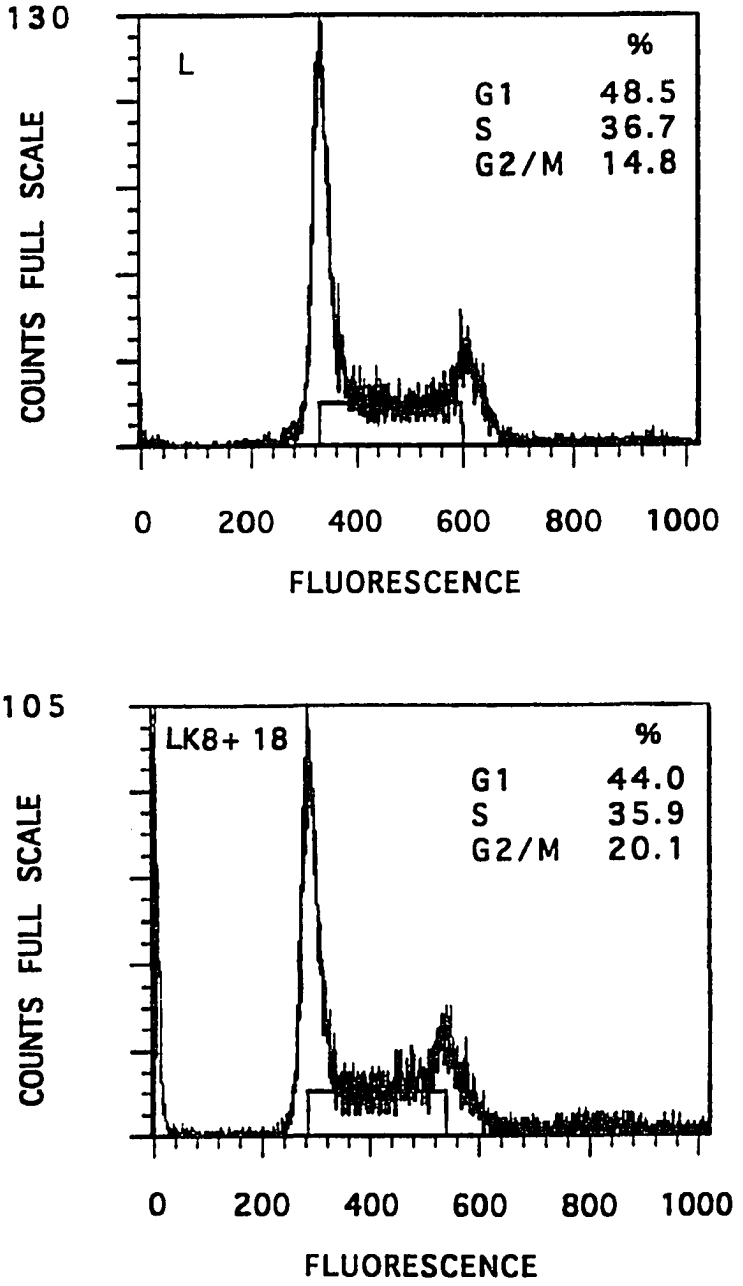


Figure 12. L and LK8+18 cells have similar cell cycle distributions. L and LK8+18 cells were fixed in 3:1 methanol:acetic acid and treated with propidium iodide for DNA content analysis by fluorescence activated cell sorting.

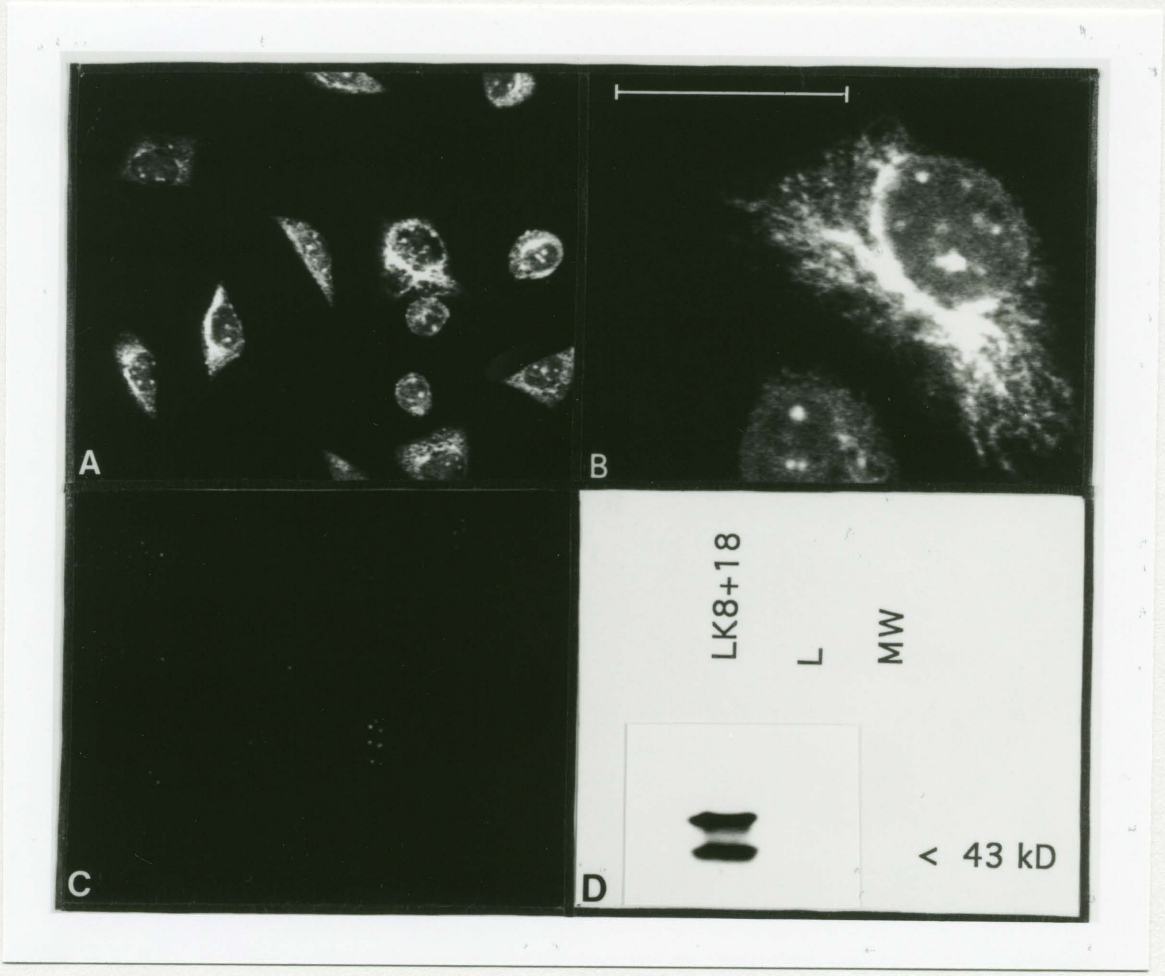


Figure 13. LK8+18 cells synthesize and assemble cytokeratin monomers. *(A)* Immunofluorescent staining of cytokeratins 8 and 18 in a population of LK8+18 cells. Cells were fixed with methanol and probed with antibody 10.11 followed by a FITC-conjugated goat anti-mouse secondary antibody. *(B)* Enlarged view of cytokeratin filaments in an LK8+18 cell. Bar represents 25 μm. *(C)* Lack of immunofluorescent staining in parental L cells. *(D)* Identification of cytokeratins 8 and 18 in LK8+18 but not L cells by Western blot analysis with antibody 10.11.

	MEAN IC50, μM (Fold resistance)						
Cells	MITOX	DOX	MTX	MEL	VIN	COL	SF (2Gy)
L	0.07 (1)	0.13 (1)	0.00133 (1)	6.5 (1)	0.20 (1)	179.8 (1)	75.6 (1)
LK8+18	0.54 (7.6)	0.68 (5.2)	0.00651 (4.9)	42.0 (6.5)	90.8 (454)	3084.0 (17.2)	57.4 (0.76)
LPBMOC	0.05 (0.7)	0.03 (0.2)	0.00134 (1.0)	11.2 (1.7)	0.03 (0.2)	119.9 (0.7)	69.7 (0.92)

Table 1. Cytokeratin confers drug resistance, but not radiation resistance in LK8+18 cells. IC50 (μM) drug concentrations were determined by MTT assay for mitoxantrone (MITOX), doxorubicin (DOX), methotrexate (MTX), melphalan (MEL), vincristine (VIN) and colcemid (COL). Values are calculated from three separate experiments, each with 8 replicate wells. Radiation resistance was determined by colony forming assays. Data reported are surviving fraction (SF) at 2 Gray (200 rads) and are calculated from experimental mean values from 2 separate experiments per cell line.

specific to mitoxantrone, doxorubicin was substituted for mitoxantrone and the experiments repeated. The LK8+18 cells are about 5 times more resistant to doxorubicin than L cells, with a mean IC50 of 0.68 μM in LK8+18 cells compared to 0.13 μM in L cells (Table 1).

Both mitoxantrone and doxorubicin bind DNA and inhibit topoisomerase II (6, 15, 156). Both probably cause DNA damage via formation of drug-dependent free radicals (21, 157). To test whether cytokeratin conferred resistance to only a specific class of drugs, experiments were repeated using the unrelated compounds methotrexate (a dihydrofolate reductase inhibitor) and melphalan (a DNA alkylator). The LK8+18 cells are about 5-fold resistant to methotrexate and about 6.5-fold resistant to melphalan compared to L and LPBMOC cells (Table 1).

The LK8+18 cells are most resistant to vincristine and colcemid, both of which bind tubulin and destabilize microtubule networks (10). A dose of approximately 3 mM colcemid is required to obtain an IC50 in LK8+18 cells, which is 17 times more colcemid than was required in parental or mock-transfected cells (Table 1). The IC50 of vincristine is lower, but the LK8+18 cells are 454 times more resistant to vincristine than were the L cells.

Gamma irradiation damages DNA without any interaction with cytokeratin by bypassing drug uptake, transport and

distribution processes. To determine whether cytokeratin conferred resistance to radiation damage, both L and LK8+18 cells were irradiated with ^{60}Co and damage scored by colony formation. The fraction of LK8+18 cells surviving a dose of 2 Gray was slightly lower than the corresponding surviving fraction of L cells (Table 1, Figure 14). LK8+18 transfectants are not resistant to radiation-induced cytotoxicity, suggesting that the interaction of cytokeratins with anti-cancer drugs is plays a role in the drug resistance phenotype.

(d) Drug resistance is not due to altered drug accumulation or efflux.

Because drug resistance phenotypes are frequently associated with aberrant expression of transport proteins that function to decrease cellular drug accumulation or increase the rate of drug efflux, we characterized accumulation and efflux of [^{14}C]-mitoxantrone in L and LK8+18 cells (Figure 15). Intracellular drug accumulation was determined after a 1 hour exposure as described. The mean amount of intracellular [^{14}C]-mitoxantrone was 3.2 μmoles versus a mean intracellular accumulation of 3.3 μmoles in LK8+18 cells for two experiments. The two cell lines therefore accumulate equivalent amounts of drug.

Likewise, comparable amounts of intracellular

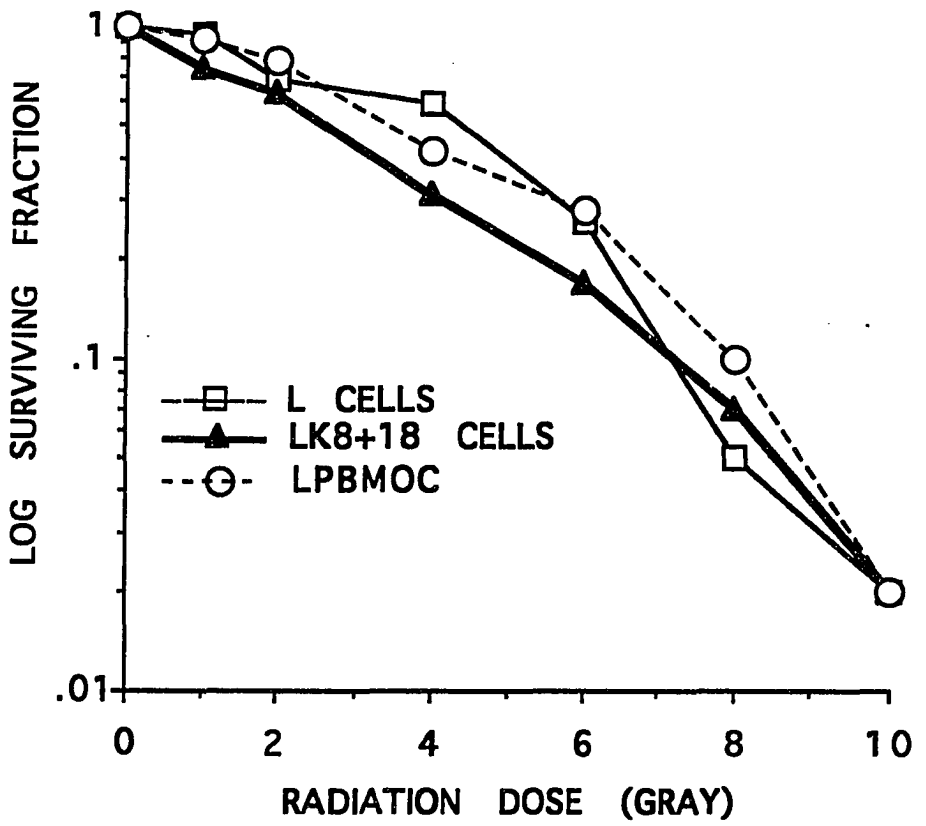


Figure 14. Cytokeratins do not confer radiation resistance. L, LK8+18 and LPBMOC cells were exposed to irradiation by ^{60}Co, then immediately trypsinized and plated as described. Surviving fractions were calculated by dividing the number of colonies obtained from cells treated with 2 Gray irradiation by the number of colonies obtained from untreated cells.

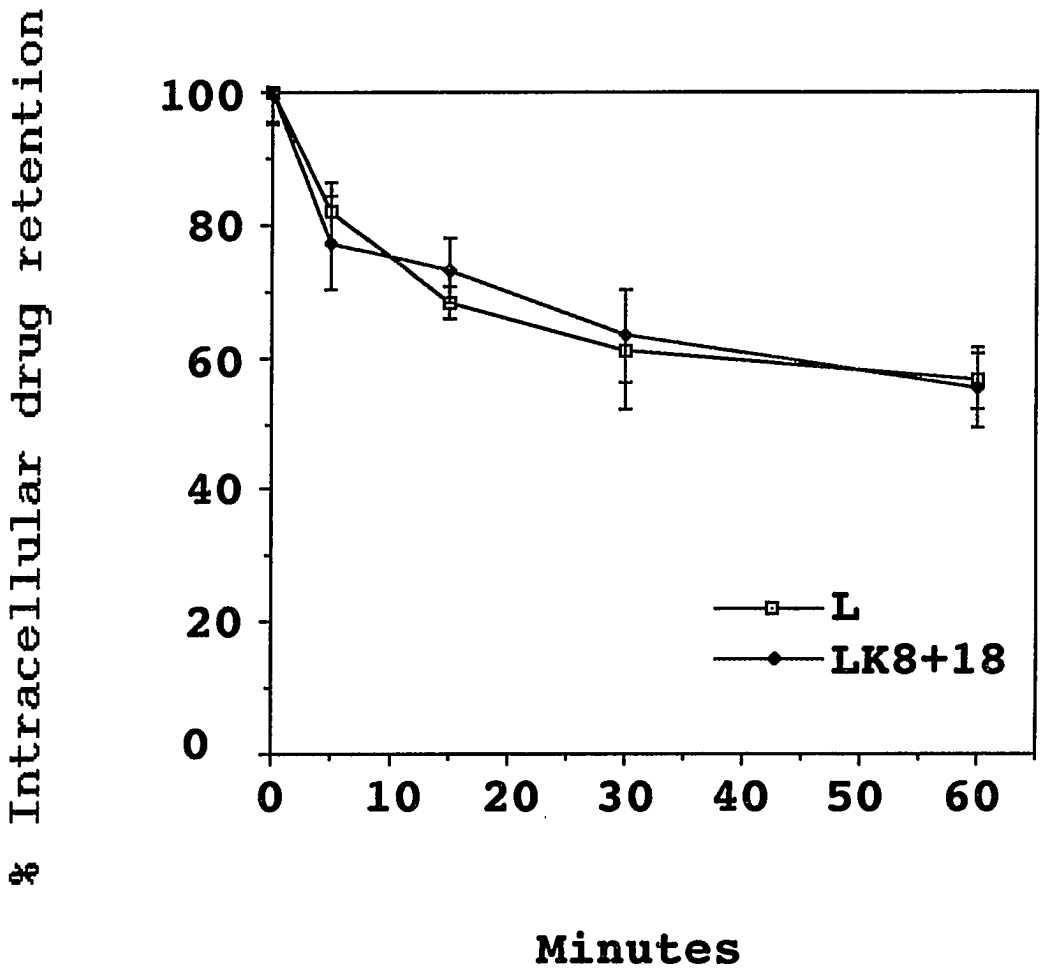


Figure 15. The accumulation and efflux of [^{14}C]-mitoxantrone in L and LK8+18 cells is similar. L and LK8+18 cells were exposed to 10 μM [^{14}C]-mitoxantrone for 1 hour. Cells were then washed, and radioactivity was measured by scintillation counting at various times after drug removal. Intracellular concentrations of drug at various efflux times were normalized to the highest amount of intracellular drug accumulated. The range of radioactivity was 4.3 x 10^4 to 1.5 x 10^5 dpm. Data points represent means +/- standard error.

mitoxantrone were detected at all efflux times in both L and LK8+18 cells (Figure 15). Hence, the rates of efflux in the two cell lines are also not significantly different.

(e) LK8+18 cells have a different intranuclear distribution of doxorubicin than L cells.

Collectively, the data suggest a model in which drug binding to cytokeratin protects LK8+18 cells by preventing the drug from reaching its true target sites. This model predicts that compared to L cells, the LK8+18 cells will retain a greater proportion of drug in their cytoplasmic compartments, presumably bound to cytokeratin.

The intracellular distribution of doxorubicin was investigated in L and LK8+18 cells by directly viewing its natural fluorescence in cells using confocal microscopy (Figure 15).

The most intense fluorescence in both cell lines localizes to cell nuclei, with some doxorubicin also accumulating in cytoplasmic areas. The LK8+18 cells tend to flatten more than L cells, giving the appearance of extended cytoplasmic areas in the cytoplasmic areas of the transfectant (Figure 16).

Within the nucleus of L cells, the distribution of doxorubicin is intense but diffuse. However, nuclear fluorescence in LK8+18 cells appears as a punctate pattern. These sub-nuclear fluorescent areas did not co-localize with

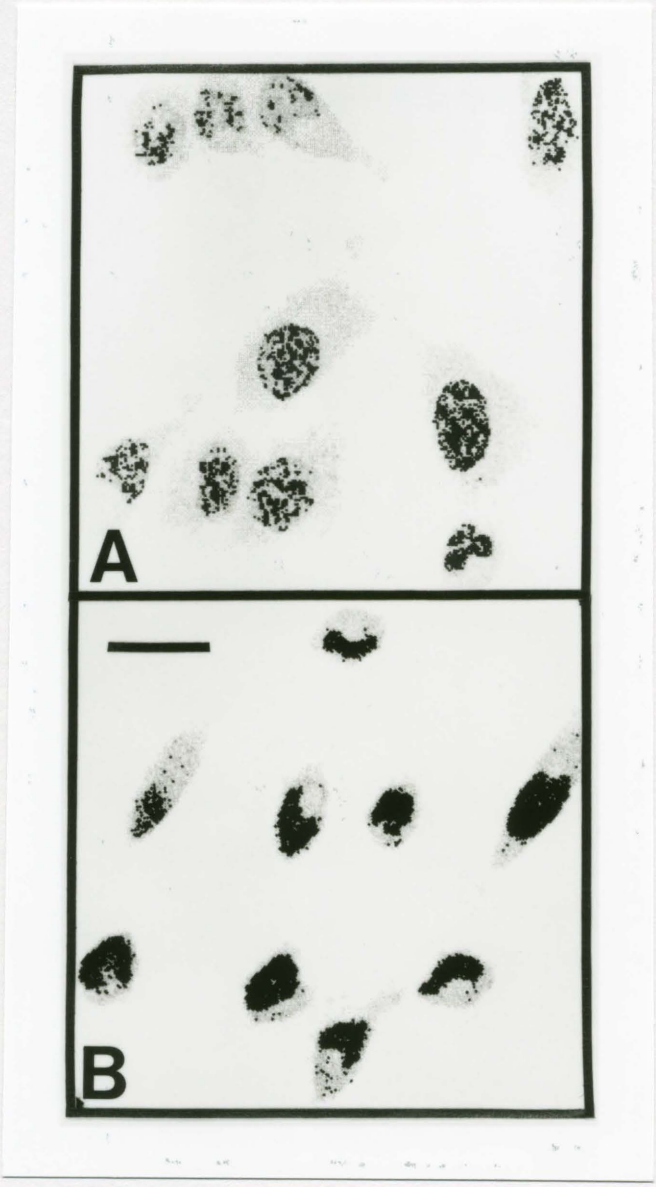


Figure 16. LK8+18 cells have a different intranuclear distribution of doxorubicin than L cells. Both L and LK8+18 cells were incubated for 1 hour with 1 μM doxorubicin and examined by confocal fluorescence microscopy. Panel A shows the distribution of doxorubicin in LK8+18 cells, and panel B shows the doxorubicin distribution in L cells. LPBMOC cells resemble L cells (not shown). Bar represents 25 μm.

nucleoli (not shown) and presently remain unidentified.

2. Discussion

Observations that the anti-cancer drug mitoxantrone binds to cytokeratin suggested the alternate hypotheses of (1) binding as a cause of drug damage and (2) binding as protection against drug damage. Mechanisms used by cells for protection lead to drug resistance phenotypes.

Transfection of cytokeratins 8 and 18 confers multiple drug resistance to LK8+18 cells compared to L cells, as measured by the MTT assay. LK8+18 cells are resistant to the mechanistically unrelated drugs mitoxantrone, doxorubicin, methotrexate, melphalan, vincristine and colcemid.

The greatest degree of resistance observed was to the anti-tubulin drugs. A possible explanation lies in the putative physical association of intermediate filament and microtubule networks, which was proposed to explain how a combination of microtubule and microfilament depolymerizing drugs could also collapse intermediate filaments networks (142). It may be that the introduction of an additional cytoskeletal network to LK8+18 cells stabilizes the endogenous microtubule network and precludes its depolymerization by vincristine and colcemid.

LK8+18 cells are resistant to several anti-cancer

drugs, yet their resistance can not be attributed to a difference in growth rate or cell cycle distribution compared to L cells. Nor can resistance be attributed to a non-specific response to DNA damage, since LK8+18 cells are not resistant to γ-irradiation. In fact, the transfected cells may be somewhat more sensitive to radiation damage than the parental cells, especially at low doses.

The data also demonstrate that drug resistance in LK8+18 cells is not due to decreased drug uptake or increased drug efflux since both cell lines accumulate equivalent amounts of [^{14}C]-mitoxantrone and remove it at similar rates. Resistance in LK8+18 cells is therefore not due to an overexpressed or activated drug transport protein.

The intracellular distribution of doxorubicin in the LK8+18 cells was similar to that of L cells in that doxorubicin tends to concentrate in the nuclei of both cell lines. But the intranuclear distribution of doxorubicin in LK8+18 cells is unique. Whereas nuclear fluorescence in L cells is diffuse, nuclear fluorescence in LK8+18 cells is punctate. The punctate pattern could indicate that some nuclear sites are protected from doxorubicin, as predicted by hypothesis 2. However, because L and LK8+18 cells accumulate equivalent amounts of drug, one would also predict that if less doxorubicin accumulates in LK8+18

nuclei, then more drug should accumulate in extra-nuclear compartments, a result that has not been clearly demonstrated.

The fact that the extra-nuclear compartment of LK8+18 cells appears somewhat larger than that of L cells also complicates the analysis. The flattened cytoplasms of LK8+18 cells could be due to structural alterations resulting from the ectopic expression of cytokeratin. It is possible that the expression of an additional intermediate filament network expands the cytoplasm, creating unique cytoplasmic drug binding sites.

Electron micrographs suggest that intermediate filaments are closely associated with several membrane-bound cellular organelles (131), and physically linked to nuclear matrix proteins or nuclear lamins (152). Organelles may in fact be interconnected by cytoskeletal proteins (131). Therefore, the ectopic expression of human cytoskeletal proteins in a mouse cell line could cause a reorganization of both cytoplasmic and nuclear regions, and possibly explain the unique nuclear distribution of doxorubicin in LK8+18 cells. The protection of specific nuclear sites from drugs could then be related to the drug resistance phenotype.

At this time, it is unknown if drug resistance in LK8+18 cells is conferred by cytokeratins or by the dual

expression of cytokeratins and vimentin. However, some human tumor types express both cytokeratin and vimentin (158). The dual expression of intermediate filament proteins could also have an important influence on the responses of these tumors to drugs. The LK8+18 fibroblasts should provide a useful model for investigating the protective mechanisms and factors utilized by cells to evade drug cytotoxicity.

CHAPTER 8. PERSPECTIVES

Organisms from simple procaryotes to complex human beings have developed protective mechanisms upon which their survival depends. Drug-resistant tumor cells are remarkable in that they are capable of both escaping the body's defense mechanisms, and surviving a barrage of chemotherapeutic agents.

The survival mechanisms used by drug-resistant tumor cells are often not different from the mechanisms used by normal cells for defense against exogenous toxicants. For example, the first line of defense in both normal and tumor cells is the semi-permeable plasma membrane, which creates a barrier between the cell and the external environment. Specific proteins embedded in the membrane remove toxicants that have penetrated the intracellular space. Both tumor and normal cells express the multidrug transporter P-glycoprotein, which may function in the secretion of such xenobiotics.

Alternatively, some cells retain xenobiotics but metabolically transform or deactivate them. Normal and tumor cells with high levels of metabolic enzymes are therefore protected from xenobiotics, including many chemotherapeutic agents.

The results presented herein suggest that cytokeratin filaments may partake in another defense mechanism in both

normal and tumor cells.

Several characteristics of cytokeratin make it an excellent candidate for a role in cellular defense. First, cytokeratin is produced abundantly throughout the epithelial cell cytoplasm, and is constructed as a highly branched network which surrounds the nucleus. As such, any xenobiotic entering epithelial cells is highly likely to encounter the cytokeratin network, especially prior to gaining access to the nucleus. In this sense, cytokeratin could serve as an obstacle to drugs en route to their target sites.

Second, the cytokeratin filaments could potentially interact with a large number of chemical compounds. The repeating hydrophobic residues of the central rods create hydrophobic domains along the filaments, whereas the glycine- or serine-rich termini form hydrophilic domains. Because anti-neoplastic agents generally contain both hydrophobic and hydrophilic functional groups, they are likely to associate with and potentially bind to cytokeratin filaments.

Third, because cytokeratins contact membrane-bound organelles including the nucleus, they are ideally situated for signal transduction and could transmit information regarding cellular drug damage throughout the cell in order to coordinate repair responses. One response might be the

induction of cytokeratin may be protein itself, as suggested by a recent study by Brambilla and colleagues (159). They found that untreated small-cell lung carcinomas are composed of fewer cytokeratin-expressing cells than carcinomas from patients treated with the combination of etoposide, doxorubicin and cyclophosphamide. Moreover, individual tumor cells from treated patients expressed higher levels of cytokeratin than the comparable cells from untreated patients (159).

If cytokeratin does act as a protective structure within the cell, then it could potentially limit the effective use of chemotherapeutic agents. As mentioned, cytokeratin proteins are abundant, so circumventing drug-cytokeratin interactions might be impractical. As a group, cytokeratins are also quite diverse, with at least 20 different monomers making at least 10 different kinds of filaments.

Moreover, because cytokeratin can confer resistance to multiple unrelated drugs, no rationale yet exists for designing drugs that will avoid interactions with cytokeratin. This situation is analogous to trying to design anti-cancer drugs that can not be exported by P-glycoprotein; the chemical groups that target drugs to P-glycoprotein for removal are unknown.

However, there may be strategies for circumventing *de*

novo multidrug resistance, just as there are strategies to circumvent acquired resistance mediated by P-glycoprotein. For example, chemomodifiers like verapamil modify the P-glycoprotein, rather than trying to modify individual drugs (160). A chemotherapeutic agent could be designed to modify cytokeratin prior to or in combination with DNA-damaging drugs. A possible candidate for such a modifier is cis-platin, an anti-tumor agent which both collapses cytoskeletal networks and cross-links DNA (161).

A better prospect for the treatment of drug-resistant epithelial cell tumors might be the use of radiation therapy. Irradiation bypasses cytokeratin filaments and directly damages DNA. The data herein using LK8+18 cells further suggest that expression of cytokeratin does not confer radioresistance.

Regardless of new and advanced chemotherapeutic agents, adaptive cellular responses remain. Problems in clinical drug resistance require continued research in both cell biology, to enable the prediction of cellular responses to drug; and in pharmacology, to rationally design drugs to circumvent these responses. Most importantly, however, drug resistance highlights the importance of cancer prevention, which is surely preferable to any cure.

APPENDIX 1. LABORATORY PROTOCOLS

1. GENERAL LABORATORY SOLUTIONS

Trypsin

Per 2 liters:	16	gm	NaCl
	0.8	gm	KCl
	2.5	gm	glucose
	0.7	gm	$NaHCO_3$
	0.6375	gm	EDTA
	1.25	gm	trypsin (Calbiochem)
			phenol red

The final pH was adjusted to 7 prior to filter sterilization.

Phosphate-buffered saline (PBS) - 20x stock

Per 1 liter:	175.5	gm	NaCl
	1.942	gm	KH_2PO_4
	14.93	gm	K_2HPO_4 (anh.)

Cytokeratin low salt extraction buffer (LSEB)

10 mM Tris-HCl
140 mM NaCl
0.5 % Triton X-100
5 mM EDTA

Cytokeratin high salt extraction buffer (HSEB)

LSEB
1.5 M KCl

Cytokeratin extraction buffer (L cell transfectants)

20 mM Tris, pH 7.4
0.6 M KCl
1% Triton X-100

2. SDS-PAGE SOLUTIONS

Soln. A: 36.6 gm Tris (Ultrol, Calbiochem)
5 ml conc. HCl
230 μl TEMED (N,N,N',N'tetramethylethylenediamine)
Add ~50 ml ddH_2O, pH to 8.9, then make up to final vol. of 100 ml with ddH_2O. Filter and store dark.

Soln. C: 28.8 gm acrylamide
0.735 gm DATD (N,N'-diallyltartardiamide)
Make up to 100 ml with ddH_2O, filter and store dark.

Soln. D: 11.64 gm Tris base
4 ml 20% SDS
250 μl TEMED
Make up to 100 ml with ddH_2O, pH to 7, filter and store dark.

Ammonium persulfate (AP): 0.014%

Running buffer: 57.6 gm glycine
12.12 gm Tris (Ultrol)
20 ml 20% SDS
Make up to 4 liters. For blotting buffer, omit SDS and add 800 ml methanol.

Cracking buffer - 4x stock: 4 ml 20% SDS
2 ml β-mercaptoethanol
2 ml of 1 M Tris, Ph 7
1.2 gm sucrose
Make up to 10 ml; add a pinch of bromophenol blue.

PBS-Tween: PBS + 0.06% Tween 20. Other solutions for blotting are made up in PBS-Tween: 0.1% Triton X-100; 0.5% Triton X-100; 0.5 M NaCl.

Ponceau stain: 2% Ponceau
3.5% sulfosalicylic acid
5% trichloroacetic acid

Coomassie blue: 0.04% Coomassie Brilliant Blue R-250
5% Glacial Acetic Acid
10% Isopropanol

Destain: 5% Glacial Acetic Acid
10% Isopropanol

SDS-PAGE GEL TABLE

% GEL	ML A	ML C	μL 20% SDS	ML H_2O	ML AP
5	2	2.82	80	6.9	4
7	2	3.96	80	5.76	4
7.5	2	4.24	80	5.48	4
8.5	2	4.80	80	4.88	4
10	2	5.72	80	4.00	4
12.5	2	7.08	80	2.60	4
15	2	8.48	80	1.24	4
gradient of 5 to 12%					
5	1	1.41	40	3.45	2
12	1	3.54	40	1.30	2

3. PLASMID PREPARATIONS

A. Solutions

Luria broth
10 gm bacto-tryptone
5 gm bacto yeast extract
10 gm NaCl
Make up to 1 liter and adjust Ph to 7.5 with NaOH.

TERB 450 ml of LB plus 50 ml of phosphate buffer

Phosphate buffer
0.17 M KH_2PO_4 (2.3 gm/100 ml)
0.72 M K_2HPO_4 (12.5 gm/100ml)
Autoclave.

Solution I
10 mM EDTA, pH 8.0
25 mM Tris, pH 8.0
50 mM glucose

Solution II
0.2 M NaOH
1% SDS

Solution III
6 ml of 5M KAc
1.15 ml of conc. HAc
2.85 ml water

TE 10 mM Tris-HCl, pH 7.4 (also can be 7.6, 8.0)
1 mM EDTA, pH 8.0

DNase-free Rnase stock 10 mg/ml of RNase A in 10 mM Tris, pH 7.5/15 mM NaCl
Heat to 100° for 15 min. and allow to cool slowly at room temperature. Aliquot and store at -20°.

Proteinase K stock 20 mg/ml in water. Freeze aliquots at -20°.

B. Method

Day 1

* Inoculate 2 ml of Luria Broth, supplemented with 5 ml of 20% glucose per liter and 25 μg/ml ampicillin in a tube, with plasmid-containing E. coli

* Incubate at 37° with shaking until culture is dense. Meanwhile, prewarm 500 ml TERB supplemented with 25 μg/ml

ampicillin.

* Add the pre-culture to the Erlenmeyer flask and incubate o/n, as above.

Day 2

* Centrifuge cells at 2000 rpm, 10 min. Make solution I.

* Add lysozyme to solution I, to a final concentration of 2 mg/ml. Pour off supernatant and resuspend cells in 2.6 ml solution I. Transfer to 13 ml Sarstedt tube. THE VOLUMES ARE IMPORTANT IF YOU DON'T WANT TO EXCEED TUBE CAPACITY

* Incubate on ice 30 min. Make solution II.

* Add 5.1 ml of solution II. Mix by GENTLY INVERTING the tube. Incubate on ice 5 min. Make solution III.

* Add 3.8 ml of solution III. Mix gently by inversion. Incubate on ice 60 min.

* Spin at 9000 rpm, 5 min. cold.

* Filter the supernatant through two Kleenex in a cone, by using a disposable sterile Pasteur pipet. (Large plastic ones with bulbs as part of the pipet are best.) Collect < 8 ml in each Sarstedt tube.

* Add 0.6 volumes of ice-cold isopropanol. Precipitate o/n at -20°.

Day 3

* Centrifuge DNA at 12,000 rpm at 4°, 10 min.

* Remove supernatant. Let tube stand upside down for ~ 1 min. If necessary, dry the walls of the tube with a Kleenex.

* Dissolve the pellet in 100 µl TE buffer.

* Transfer to sterile Eppendorf tube. Add 100 µl phenol (water-saturated, OBS the lower phase is the phenol.) Mix by inversion.

* Add 25 µl chloroform; mix by inversion. Spin in microfuge at 4°, 10 min.

* Transfer the water soluble phase (UPPER) to a new tube. Add 100 µl chloroform and mix by inversion. Spin as above.

* Transfer supernatant to a new tube. Add 50 µl of 7.5M ammonium acetate. Mix by inversion. Incubate on ice 20 min.

* Spin in a microfuge at 4°, 10 min.

* Transfer to a new tube. Add 375 µl ice-cold EtOH. Incubate o/n at -20°.

Day 4 - H_2O baths at 37° and 50°

* Spin in a microfuge at 4° for 10 min. Dispose of supe. Add about 600 µl of 70% EtOH and spin again.

* Dispose of supe. Let air dry upside down 5-10 min.

* Dissolve pellet in 1 ml TE buffer. Add DNase-free RNase (10 mg/ml stock) to final conc. of 100 µg/ml and incubate at 37° for 1 hour.

* Add SDS to a final conc. of 0.5% and proteinase K to 100 µg/ml and incubate at 50° for 1 hour.

* Extract with 100 µl phenol (aqueous is top), then 25 µl chloroform/isoamyl alcohol, mixing by inversion after each. Spin 4°, 10 min.

* Transfer aqueous phase to new tube and repeat with 100 µl chloroform/isoamyl alcohol. Transfer aqueous (top) to new tube.

* Add 0.1 volumes of 3 M Na acetate, Ph 5.2 and 2 volumes EtOH. Precipitate overnight at -20° or at -70° for more than 2 hours.

DAY 5

* Spin in microfuge for 20 min. at 4°. Discard supe and wash with 70% EtOH. Spin again. Dispose of supe and air dry. Resuspend in sterile water (50-200 µl, depending on size of pellet). Store at -20°.

4. CALCIUM PHOSPHATE TRANSFECTION OF ADHERENT CELLS WITH PURIFIED PLASMID

A. Solutions

2X HBSP buffer

1.5 Mm Na_2PO_4
10 Mm Kcl
280 mM NaCl
12 mM glucose
50 mM HEPES, pH 7

Test to see if buffer forms precipitate by performing steps 3 and 4 without DNA. Precipitate should appear after 15 min. Filter sterilize and store in 5 ml aliquots at -20°.

15 % glycerol in 1X HBSP - Make fresh.
2M $CaCl_2$ - Store at -20°.
PBS

B. Method

1. First test cells for glycerol sensitivity by adding the 15% glycerol to growing cells and incubating at 37° for different times up to 3 min. Examine microscopically. Do not use on intolerant cells.

2. Split cells and plate 3 x 10^5 cells per dish. Replace medium with fresh medium 2 to 4 hours prior to transfection.

3. Prepare $CaPO_4$/DNA mixture by combining in order:
   H_2O (bring final volume to 500 μl)
   plasmid DNA (5-12.5 μg)
   31 μl of 2M $CaCl_2$ (final concentration 125 mM)
   250 μl of 2X HBSP

Place H_2O in bottom of sterile 15 ml conical tube. Gently add DNA and **DO NOT MIX.** Add $CaCl_2$ and **DO NOT MIX.** Add 2X HBSP to **bottom** of the tube slowly and blow about 5 bubbles to gently mix ingredients.

4. Allow precipitate to form for 30 min. Then, pipette precipitate up and down once. Add very slowly to plated cells. Gently rock plate. Medium should turn yellow and turbid.

5. Incubate 4 hours at 37°. Look for precipitate after 10 min of incubation.

6. Glycerol shock, if cells will tolerate. See step 1.

7. Remove glycerol, wash with PBS, replace medium and incubate 36-48 hours.

8. After trypsinization, plate cells in selective medium.

5. LOW MELT GEL PURIFICATION OF DNA

A. Solutions

2X extraction buffer
- 1 M NaCl
- 40 mM Tris, pH 7.5
- 10 mM EDTA

buffer-saturated phenol
phenol/chloroform/isoamyl alcohol (25:24:1; P/C)
chloroform/isoamyl alcohol (24:1; C)
3 M Na acetate
100 mM $MgCl_2$
100% cold ethanol
70% cold ethanol

B. Method

* Cut out band of interest and place in tared tube. Add an equal volume of 2X extraction buffer and melt gel at 68° for about 15 min.

* Extract 3 times with equal volume of buffer saturated phenol. Remove top aqueous layer to new tube each time.

* Extract once with P/C, and twice with C. Use equal volumes, and take top aqueous layer to new tube each time.

* Concentrate to 100 µl with n-butanol. Take **BOTTOM** layer to new tube each time.

* Extract twice with C to remove butanol, and take top layer to new tube each time. Add 10 µl 3M NaAc, 5 µl 100mM $MgCl_2$, and 250 µl EtOH. Precipitate overnight at -20°.

* Next day, wash with 70% EtOH, dry and bring up in water as desired.

6. EXTRACTION OF GENOMIC DNA

A. Solutions

STE 0.1 M NaCl
1 mM EDTA
0.05 M Tris, pH 7.5

proteinase K - stock is 20 mg/ml in H_2O
10% SDS
P/C, as above
0.2 M Na acetate
ethanol, as above
TE - 10 mM Tris, pH 7.5
1 mM EDTA
DNase-free RNase - stock is 10 mg/ml

B. Method

* Pellet cells and resuspend in about 400 μl (for 1 or 2 T75's) in microfuge tube. Add proteinase K to a final concentration of 0.1 mg/ml and SDS to a final concentration of 0.5%. Incubate 3 hr to o/n at 50-55°.

* Add P/C and shake 10 min. Can set on ice to precipitate SDS. Spin in microfuge at 4 ° and take upper phase to new tube.

* Adjust supernatant to 0.2 M Na acetate and slowly add 2 volumes cold 100% EtOH. Mix and precipitate o/n at -20°.

* Pellet DNA, wash with cold 70% EtOH. Dry inverted.

* Dissolve DNA in 1 ml TE. Add RNase to a final concentration of 0.1 mg/ml and incubate 1 hr at 37°.

* Add SDS to 0.5% and proteinase K to 0.1 mg/ml and incubate 1 hr at 50-55°. Repeat from addition of P/C through the pelleting, washing and drying of DNA. Bring up in small volume TE

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