siveness of inhibited NTE to KF is clearly evident. The progressive loss of responsiveness to KF enables us to conclude that aging of DFP-inhibited NTE is a biochemical reaction common to human and hen NTEs.

In conclusion, NTE activity is widely present in human organs and some of its biochemical characteristics, like sensitivity to inhibitors and aging of the phosphorylated enzyme, are similar among different tissues and comparable to those of hen brain NTE.

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Modification of keratin by the chemotherapeutic drug mitoxantrone

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A major focus of research on the mechanism of action of anthracycline and anthracene chemotherapeutic drugs has been on the interactions of these drugs with cellular DNA [1, 2]. Mitoxantrone is a member of a series of anthraquinones which was originally synthesized as a DNA-intercalating drug. This drug has proven to be an active agent in the treatment of human breast carcinoma, acute leukemia, malignant lymphomas, and hepatoma [3]. Studies on the mechanism of cytotoxic action of mitoxantrone have indicated that in intact cells the drug binds DNA by a non-

intercalative, electrostatic interaction and induces non-protein associated DNA strand breaks. The degree of cytotoxicity could not simply be related to the observed DNA damage [4]. Other investigators have also found that with drugs such as nitrogen mustards and chloroethylnitrosourea no clear correlation exists between DNA damage and cytotoxicity [5]. Alternative biological targets are being investigated, and the change in the morphological appearance of the cytoskeleton by different compounds has been described by others [6–8]. The intracellular protein association of chemotherapeutic drugs is also of increasing interest since the discovery of two cellular proteins which interact with chemotherapeutic agents, a transmembrane glycoprotein which is implicated in acquired pleotropic drug resistance and DNA topoisomerase whose activity can be altered by chemotherapeutic agents [9, 10]. Since these two protein components are considered to be intracellular sites of drug interaction, we were interested to discover if other cellular elements would interact with a specific chemotherapeutic agent. Mitoxantrone was used to study the intracellular binding of a chemotherapeutic drug within the cytoskeletal fraction of a human colon carcinoma cell line designated WidR [11]. Transmembrane glycoproteins are known to be associated with cytoskeletal elements, and it was of interest whether a specific chemotherapeutic drug would be associated with any cytoskeletal element.

The major components of the cytoskeleton are the microtubules, intermediate filaments and the microfilaments [12]. The isolation of the skeletal structures from intact cells can be reproducibly accomplished and the proteins analyzed by two-dimensional gel electrophoresis. Within the intermediate filament class, there exist six classes of proteins which have been found in the different types of filaments [13–16]. In this study we used a human cell line treated with the chemotherapeutic agent mitoxantrone to determine whether the drug was intracellularly bound to any cytoskeletal element.

Methods and results

The WidR cell line was treated with 20 μM mitoxantrone for 1 hr at 37° and fractionated into various cellular components. The fractionation scheme was based upon the extraction conditions of Staufenbiel and Deppert [17]. The cells were extracted with Buffer A (60 mM PIPES*, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 10 mM NaCl, pH = 6.2) containing one of the following: 10% Triton X-100, 50 μg/ml DNase I and RNase A, or 2 M NaCl. Table 1 indicates the distribution of protein within the fractions as well as the distribution of radiolabeled drug. Cellular protein was labeled by an 8-hr incubation of cells with medium containing 5 μCi/ml of [^{35}S]-methionine (sp. act. = 1200 Ci/mmol).

These data indicated that the bulk of the protein within the cell was extractable and that the amount of protein within the cytoskeleton was approximately 9% of the total cellular protein. The concentration of mitoxantrone was 20 μM and the incubation time was 1 hr at 37°. Under these conditions, the cell survival is approximately 70%. Analysis of the drug distribution by the use of radiolabeled mitoxantrone revealed that approximately 40% of the drug was readily extractable and 46% could be solubilized by the use of 2 M NaCl. An interesting finding was that 4% of the drug remained associated with the cytoskeleton after the extractions were performed. Additional data suggest that the drug persisted in the cytoskeletal fraction for up to 72 hr after removal of the drug from the medium (data not shown). The drug did not remain in the cytoskeletal fraction due to RNA or DNA interactions since the fractions were digested extensively with DNase and RNase.

Table 1. Distribution of protein and mitoxantrone in cellular fractions*

Fraction	Protein (%)	Drug (%)
Triton X-100†	46 ± 1	21 ± 1
DNase‡	22 ± 2	21 ± 1
NaCl§	21 ± 1	46 ± 1
DNase/RNase‖	2 ± 1	8 ± 1
Cytoskeleton	9 ± 2	4 ± 1

* The percentage distribution of protein is based upon a total incorporation of 7.5×10^6 dpm per 2.0×10^6 cells. The total incorporation of drug was 3.6×10^4 dpm (10 μmol) per 2.0×10^6 cells. Each value is the mean ± SE of at least six determinations.

†–‖ Cellular fractions were obtained by sequential treatment and harvest of solubilized proteins under the following conditions: † The Triton X-100 fraction represents a treatment of 5 min with 10% Triton X-100 and 5 mM DTT in buffer A; ‡ Treatment was 50 μg/ml DNase I in buffer A for 15 min at 37°; § 2 M NaCl and 5 mM DTT in buffer A for 10 min; and ‖ 50 μg/ml of DNase/RNase in buffer A for 15 min at 37°.

The two-dimensional gel electrophoresis pattern of the proteins present in the cytoskeletal fraction is shown in Fig. 1A. The major proteins corresponded in position to the keratins 8 (M_r = 53,000; pI = 6.1), 18 (M_r = 45,000; pI = 5.7), and 19 (M_r = 40,000; pI = 5.2). An immunoblot of the proteins within the cytoskeletal fraction is shown in Fig. 1B. Using an antibody specific for cytokeratin (antibody designated 10,11 and supplied by Dr. Raymond B. Nagle, University of Arizona), we verified the identity of the major proteins as the cytokeratins 8, 18, and 19. Analysis of the cytoskeletal preparation for the residual drug mitoxantrone indicated that all three cytokeratins had drug associated with them and that the majority of the drug comigrated with the cytokeratin 8. The drug mitoxantrone is a compound ring-labeled with ^{14}C (sp. act. = 8.1 mCi/mmol) (SRI, Menlo Park, CA) instead of side chain labeled. It is possible that the bound radioactivity was due to a metabolite of mitoxantrone and not the parent compound. However, mitoxantrone has a reduced ability to generate free radical intermediates as compared to adriamycin [20], and preliminary experiments have shown that adriamycin is not as effective as mitoxantrone in binding the cytokeratins (data not shown). Other metabolites of mitoxantrone have been reported [21], but they were not detectable in our cell line by HPLC analysis (data not shown).

Discussion

After treatment of WidR cells with mitoxantrone, approximately 4% of the drug was bound specifically to the keratin proteins 8, 18 and 19 with the majority of the drug binding cytokeratin 8. The drug binding was resistant to protein-denaturing conditions since the association survived the process of two-dimensional gel electrophoresis. We investigated the fate of the 96% of the drug which was not bound to the keratin and found that, although some drug binding occurred which was recoverable after salt extractions were performed (Table 1), the identification of the alternative binding sites was not possible by the method used here since the majority of the binding did not withstand the denaturing conditions inherent in the electrophoresis procedure. We are presently optimizing non-denaturing methods to isolate the additional intracellular protein binding sites. It is also important to note that we are investigating here intracellular protein binding which is

* Abbreviations: PIPES, piperazine-*N*,*N*′<-bis(2-ethanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid; and DTT, dithiothreitol.

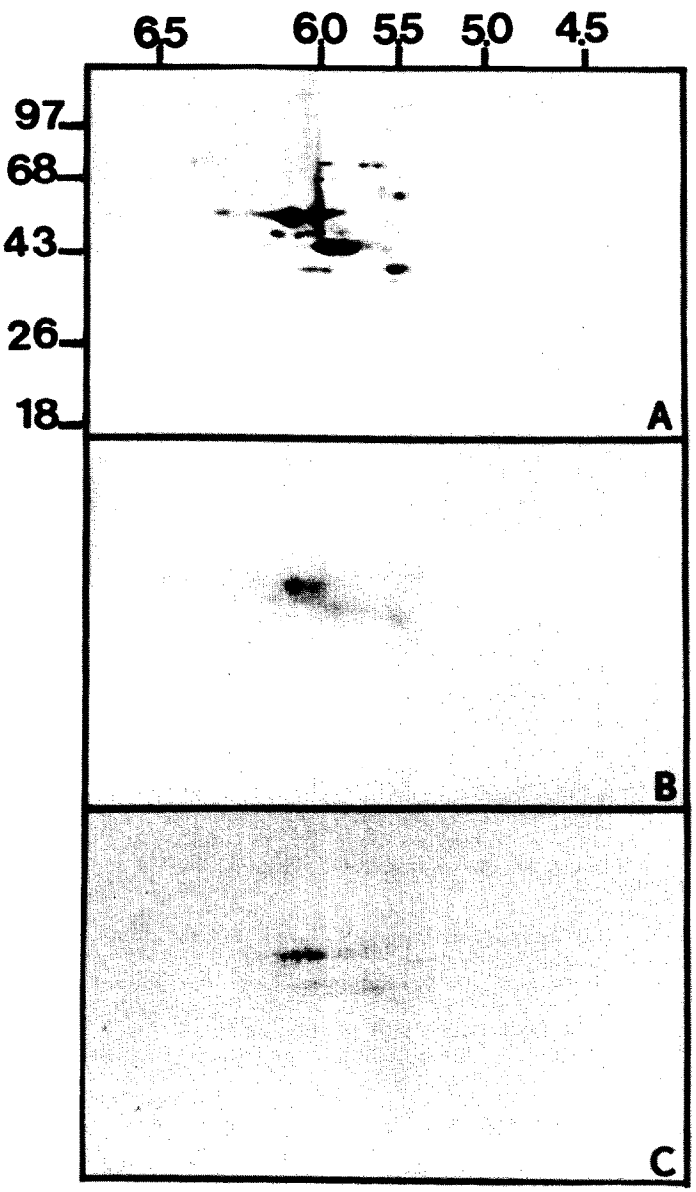

Fig. 1. Association of mitoxantrone with the keratin proteins 8, 18 and 19. The two-dimensional gel pattern in panel A illustrates the proteins that were present in a cytoskeletal preparation from WidR cells. The protein samples were applied to an IEF gel in the first dimension according to the procedure of O'Farrell [18] followed by a 12.5% polyacrylamide slab gel [19]. The approximate pH gradient within the gels and immunoblots is shown at the top of the figure; the approximate $M_r \times 1000$ is shown at the left of the figure. The distributions of pH and M_r are identical in all three panels. Panel B is an immunoblot of the protein gel identifying the primary position of the keratin proteins by the use of a monoclonal antibody and an ^{125}I-labeled goat antimouse antibody (Cappel Labs). Panel C illustrates the distribution of the [^{14}C]mitoxantrone which was present in the two-dimensional gels.

distinct from the known binding to serum albumin which is primarily ionic in nature [22].

Although only 4% of the drug is bound to keratin, the possible significance of this finding relates to the fact that the keratin molecule is part of a family of intracellular proteins which have large portions of their amino acid sequences in common [15]. An interesting example of other intermediate filament proteins are the lamins A, B, and C whose assembly and disassembly are critical for ordered cell division [23]. Therefore, although only a small portion of the drug was found associated with the filament system, the biological consequences may be profound. Taken one step further, if the drug bound to the keratin is a universal finding, it may well explain the well documented G_2 progression blocks which are known to occur with a variety of chemotherapeutic drugs [24, 25].

These data suggest that the intermediate filament system involving keratin is a previously unappreciated site for drug binding. The keratin proteins contain a constant central alpha helical domain of 300 amino acid residues flanked by non-helical termini of variable length and sequence. The end domains of the keratins are unusually abundant in glycine and serine rich sequences and are implicated in providing the functional uniqueness of the keratin protein filaments; the serine residues are available for modification by phosphorylation [26–30]. Mitoxantrone appeared not to

bind to the keratin in equal molar ratios as judged by comparing the amount of protein present (approximately 50 μg) to the amount of drug (approximately 1×10^4 cpm or 10 mmol) which was bound. It would be interesting to discover whether the drug binds primarily in the central core region or principally at the end domains of the keratin filaments. We also are interested in the details of the turnover rate of the drug within the cytoskeletal fraction since the drug binding could represent long term drug binding, i.e. that which is retained after the majority of the drug leaves the cell.

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Effect of chloramphenicol administration *in vivo* on cytochrome P-450-dependent monooxygenase activities in liver microsomes from uninduced male rats

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The cytochrome P-450-dependent monooxygenase system plays a key role in the detoxification and bioactivation of a wide variety of xenobiotics and endogenous compounds [1]. One important means of enhancing or attenuating the *in vivo* effects of compounds that are normally metabolized by cytochromes P-450 is through the use of chemical inhibitors. A common complication, however, is that many classical cytochrome P-450 inhibitors, including isosafrole, allylisopropylacetamide, metyrapone, and SKF-525A, that decrease monooxygenase activity within a few hours after *in vivo* administration, induce cytochromes P-450 after longer times, especially when given in multiple doses [2].

Our own efforts have focused on the inhibition of rat liver cytochromes P-450 by chloramphenicol and analogs. Chloramphenicol inactivates major forms of cytochrome P-450 induced by phenobarbital and pregnenolone-16α-carbonitrile, as well as several important cytochromes P-450 in uninduced rats [3, 4]. Chloramphenicol has also been shown to prevent the toxicity of such compounds as carbon tetrachloride, which require cytochrome P-450-dependent