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INTERACTIONS OF THE ANTI-TUMOR AMETANTRONE AND MITOXANTRONE WITH RAT HEPATIC MICROSOMES

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Abstract—The interaction of the anti-tumor anthraquinones, ametantrone and mitoxantrone, with rat hepatic microsomes has been studied with a fluorescence technique using 7,12-dimethylbenzanthracene as a new fluorescent probe. The two drugs were able to quench the intrinsic fluorescence of microsomal suspension.

Mitoxantrone was able to displace dimethylbenzanthracene bound microsomes with a linear representation of one ligand–one acceptor model, whereas bimodal shape was found in the case of ametantrone. The mechanism of quenching and/or binding is discussed.

Mitoxantrone and ametantrone are anti-tumor agents. They inhibit NADPH-cytochrome P-450 reductase and xanthine oxidase-catalyzed conjugated diene formation from linoleic acid in a concentration-dependent manner [1]. Other related compounds, like bisantrene [2] and ametantrone acetate [3, 4], were also tried as anti-cancer compounds in both humans and experimental animals. The binding of ametantrone to DNA was believed to take place by intercalation to double stranded nucleic acids, and although it is not certain whether this type of intercalation is responsible for their anti-tumor activities, there is evidence that nucleic acids are the targets of these drugs in living cells [5–7].

Recently, fluorescence quenching technique has been widely used to investigate the interaction of various drugs to proteins, enzymes and lipids [8–11]. This technique is simply defined as reduction of the quantum yield which can be calculated either as a physical phenomenon, in which the effect of the quencher acts according to static or dynamic mechanism against the intrinsic protein fluorescence, or by using the extrinsic fluorescent probes.

However, although binding of those anti-cancer compounds was studied with DNA, no reports have appeared to describe their binding with the microsomal protein, the site of xenobiotic metabolism. In the present work, dimethylbenzanthracene was introduced as a new fluorescent probe for microsomal protein and its probe properties were used to study the protein binding parameters of ametantrone and mitoxantrone.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA)† was obtained from Fluka AG (Switzerland); 7,12-dimethylbenzanthracene (DMBA) was obtained from Sigma Chemical Co. Ametantrone and mitoxantrone were the generous gift of Dr B. Woynarowska (Technical University of Gdansk, Poland). The organic solvents were of spectroscopic grade (Eastman).

Microsomes preparation. Microsomes were prepared from Wistar albino rats. The animals were sacrificed by cervical dislocation, and the livers were immediately removed and immersed in 0.15 M KCl solution followed by homogenization using an Elvejhem-glass homogenizer with a Teflon pestle. The suspension was centrifuged for 30 min at 12,000 rpm to remove nuclei, mitochondria and debris. The supernatant was centrifuged for 60 min at 40,000 rpm using an L8-80 Beckman ultracentrifuge. The temperature was kept at 0–4° during the process. The microsomes were immediately frozen, and for daily experiments the microsomes were diluted with 0.1 M phosphate buffer, pH 7.4, containing 1.0 mg/ml (unless stated otherwise). The protein content of the microsomal suspension was determined by the method of Lowry *et al.* [12] using BSA as standard.

Fluorescence measurements. Fluorescence measurements were made with Farrand spectrofluorometer mark-1 (Farrand Optical Co.) equipped with a 150-W Xenon source. Measurements were carried out at 25° with quartz cells. The microsomal excitation and emission wavelengths were 298 nm and 340 nm, respectively. The excitation and emission wavelengths used with the probe–protein complex were 367 nm and 405 nm. Quenching was obtained by successive additions of 5 μl of the drug using a 10-μl microsyringe (scientific glass engineering) in order to obtain maximum additions of 50 μl of the drug solution to 2 ml of microsomal protein. Quenching studies were presented by Stern–Volmer plots [8] according to the equation

$$\frac{F^0}{F} = 1 + KQ$$

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† Abbreviations used: DMBA, 7,12-dimethylbenzanthracene; BSA, bovine serum albumin.

where F^0 and F were the fluorescence intensities in the absence and presence of quencher, K was the Stern–Volmer constant, and Q the quencher concentration. In the case of upward deflection, the above equation must be modified as follows [10]:

$$\frac{F^0}{F} = (1 + KQ)\, e^{VQ}$$

where V is a parameter related to the probability of finding a quencher molecule close enough to the fluorophore to produce a static quenching.

Displacement titrations were performed by successive additions of microlitre quantities of the drug to the probe–protein complex. Results were presented according to the equation

$$\text{Percentage displacement} = \frac{F_1 - F_2}{F_1} \times 100$$

where F_1 was the fluorescence of probe bound to protein, and F_2 the fluorescence in the presence of the drug. The affinity constant was estimated by plotting $F_2/(F_1 - F_2)$ against the reciprocal of the quencher concentration.

RESULTS

The excitation spectrum of DMBA in aqueous solution revealed two peaks at 298 nm and 362 nm. Using either of those two wavelengths, two emission peaks were observed at 410 nm and 430 nm. When the compound was added to rat hepatic microsomes, the second excitation peak was shifted by 5 nm to become 367 nm, and the fluorescence peaks shifted to 405 nm and 425 nm with a greater than fourfold increase in fluorescence intensity as compared with that in the phosphate buffer. However, the 367 nm excitation wavelength was used mainly in order to avoid any overlap that may occur between the probe-microsomes excitation and the intrinsic microsomal excitation wavelength. The intrinsic fluorescence of the microsomal suspension was quenched by the addition of DMBA at the time when the probe was hydrophobically bound to microsomes. The highest quenching (78%) was obtained by the addition of 95 μM of DMBA.

The effect of ametantrone and mitoxantrone (Fig. 1) upon the intrinsic fluorescence of microsomes was investigated by fluorescence titration. The intrinsic fluorescence was quenched by the addition of any of the two compounds. This quenching was increased as the concentration of the drugs increased. The Stern–Volmer plot revealed a dynamic quenching for ametantrone (Fig. 2) and static for mitoxantrone (Fig. 3). The maximum percentage quenching obtained was 67% and 88%, respectively. The concentration needed to produce these percentages was 86 μM for ametantrone and 22 μM for mitoxantrone.

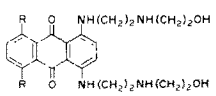

Fig. 1. Structures of ametantrone and mitoxantrone: R = H ametantrone; R = OH mitoxantrone.

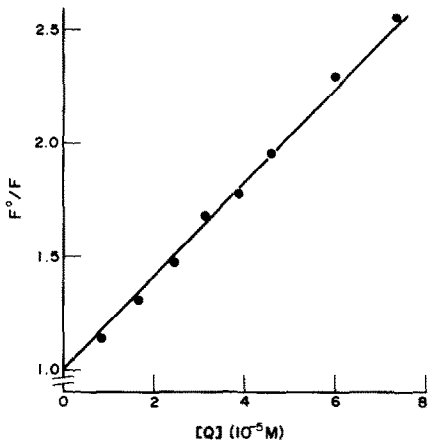

Fig. 2. Stern–Volmer plot of the quenching of the intrinsic fluorescence of rat hepatic microsomes by ametantrone.

The fluorescence enhancement of DMBA bound to rat hepatic microsomes decreases in the presence of either drug. The starting concentration was 8.0 μM and 13.5 μM for ametantrone and mitoxantrone. Figure 4 shows the Stern–Volmer plot of the quenching of DMBA-microsomal suspension by the two drugs. The quenching pattern was found to be dynamic in both cases. As the figure shows, mitoxantrone binds to microsomes more strongly than ametantrone, displacing DMBA at higher percentages than ametantrone. Association constants for the drug–microsomes interaction were calculated according to the method of Casanovas *et al.* [8] indicating a biphasic interaction for ametantrone with $K_{A1} = 875\ M^{-1}$ and $K_{A2} = 4000\ M^{-1}$ whereas the affinity constant of mitoxantrone was found to be $2500\ M^{-1}$. From the quenching results presented, percentage displacement might be calculated (Fig. 5). It reflects the ability of the anti-cancer drugs to displace the probe from its binding sites on the microsomes. Figure 6 shows the displacement caused by the two compounds and indicates that mitoxantrone displaces DMBA better than ametantrone.

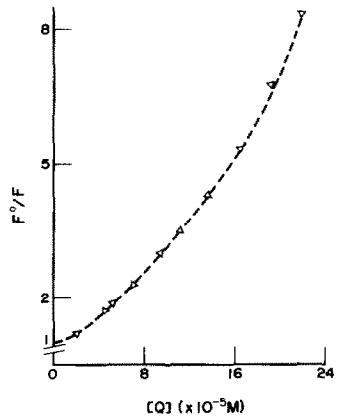

Fig. 3. Stern–Volmer plot of the quenching of the intrinsic fluorescence of rat hepatic microsomes by mitoxantrone.

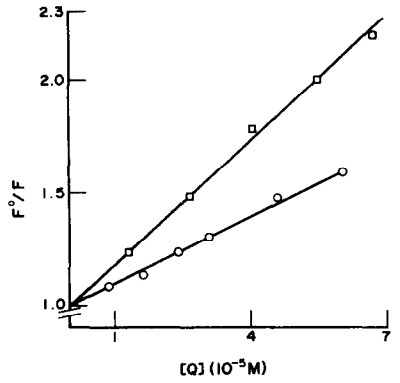

Fig. 4. Stern–Volmer plots of the quenching of rat hepatic microsomes bound DMBA by ametantrone (○) and mitoxantrone (□).

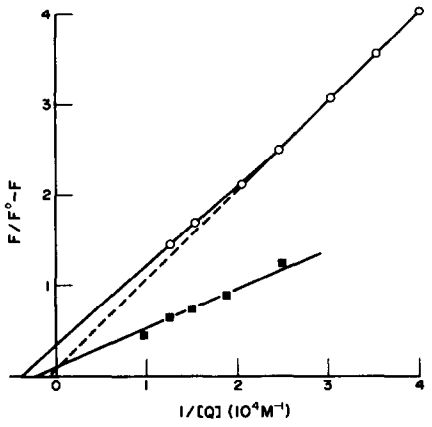

Fig. 5. Double reciprocal plot of Stern–Volmer equation for ametantrone (○) and mitoxantrone (■).

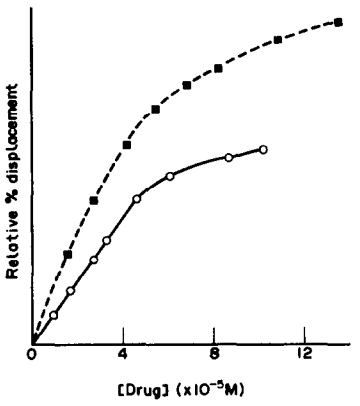

Fig. 6. The effect of ametantrone (○) and mitoxantrone (■) on the fluorescence of DMBA bound microsomes.

DISCUSSION

DMBA, a widely used chemical carcinogen, shows sufficiently marked fluorescence intensity and wavelength alterations with a change in solvent polarity [13], therefore its use as a fluorescent probe is justified. The addition of DMBA to the microsomes results in a quenching of the intrinsic protein fluorescence and an enhancement of the probe-protein fluorescence which was accompanied by a 5 nm shift in the emission maxima. This observation suggests that the microsomal binding site for DMBA is located in a hydrophobic region of the protein. This hydrophobic site also binds the anti-cancer drugs, ametantrone and mitoxantrone, since those compounds have been shown to displace DMBA from the microsomes, being able to quench the probe-protein fluorescence.

The fluorescence quenching of microsomes by DMBA is probably due to either energy transfer from the excited state of protein tryptophan residue to the bound drug, and if this is the case then the emission band of the donor must overlap the absorption band of the acceptor [14]; or DMBA was close enough to the tryptophan fluorescent residue (i.e. binds to, or near the active fluorescent site) to deactivate the excited molecules by random collision between the fluorophore and the quencher molecules. However, although the absorption of DMBA was in the same region of the fluorophore, it seems that the second possibility is more probable, since the fluorescence of DMBA-microsomes was easily quenched by the addition of ametantrone and mitoxantrone displacing DMBA from its binding site on the microsomal protein. The evidence for this explanation is that the excitation wavelength used (367 nm) was far away from the fluorescence of the protein (340).

The interaction of ametantrone and mitoxantrone was initially acknowledged by its ability to quench the intrinsic protein fluorescence (Figs 2 and 3), and then by displacing DMBA as a probe from its binding site on the microsomal protein. The binding affinity of ametantrone at low concentrations was found to be higher than mitoxantrone (Fig. 5), which is in accordance with the hydrophobicity requirements of the interaction, since DMBA was bound at the hydrophobic regions of the microsomes, but at high ametantrone concentration, it becomes weakly bound compared to mitoxantrone. This bimodal shape (Fig. 5) was reported previously for the interaction of pyridylalkanamides [15] and biphenyl [16] with hepatic microsomal cytochrome P-450. The bimodal kinetics were presented as evidence for the presence of high and low affinity binding sites of cytochrome P-450 [17]. However, it appears that mitoxantrone occupies one site throughout the concentrations used (13.5 μM up to 135 μM) with a maximum percentage displacement of 71%. The highest displacement produced by ametantrone against DMBA (43%) was found at 100 μM. The probability exists that ametantrone occupies the same hydrophobic site, being able to displace DMBA at low concentrations (8–45 μM), but at higher concentrations it prefers other site(s) not necessarily hydrophobic, still affecting, but only slightly, the DMBA-microsomes fluorescence. The evidence for this explanation is the low percentage displacement produced at high concentration, and the second value obtained for the binding affinity. It should be interesting to note that mitoxantrone was two orders of

magnitude more potent as an anti-tumor drug than ametantrone [6]. Furthermore, mitoxantrone has a higher affinity to calf thymus DNA than ametantrone [18]. However, these observations suggest further studies on the metabolism of both compounds by cytochrome P-450.

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