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Formation of reactive intermediates by cytochrome P-450 mediated oxidation of the anti-cancer drug mitoxantrone

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Abstract: Oxidative activation of mitoxantrone (**1**) is involved in the biotransformation of the anti-cancer drug. Two distinct reactive intermediates (**5** and **6**) interconnected by a tautomeric equilibrium are formed by enzymatic oxidation. Intermediate **5** showing Michael acceptor properties at ring C reacts intramolecularly to afford metabolite **7**. In contrast, intermediate **6** presents electrophilic character at ring A and reacts intermolecularly with nucleophiles. Nucleophilic attack of glutathione gives rise to the formation of thioether derivatives (**8** - **11**). In the presence of the cytochrome P-450 inhibitor metyrapone, the oxidation of mitoxantrone is blocked as shown by the absence of formation of the metabolites **7** and **8**. Under these conditions, the damaging effect of mitoxantrone on rat hepatocytes and human derived hepatoma (HepG2) cells is lost. Thus, it can be concluded, that the oxidative activation of mitoxantrone is involved in the cytotoxic effect of the drug.

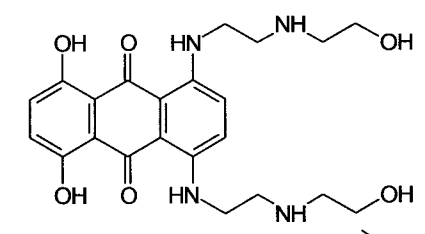
The anti-cancer agent mitoxantrone (**1**) is currently used for the treatment of breast cancer, acute nonlymphocytic leukaemia, and non-Hodgkin's lymphoma (1,2). Interaction with DNA by intercalation and electrostatic binding, trapping of the DNA topoisomerase II complex, inhibition of DNA synthesis, and other mechanisms have been suggested to explain the mode of action of the drug (3). In contrast to anthracyclines, mitoxantrone is evidently resistant to reduction (4) but is subject to facile enzymatic oxidation (5, 6).

The known biotransformation of **1** includes side chain oxidation to the mono (**2**) and dicarboxylic acid derivative (**3**) and glucuronidation to **4** (7). These metabolites can be regarded as detoxification and excretion products.

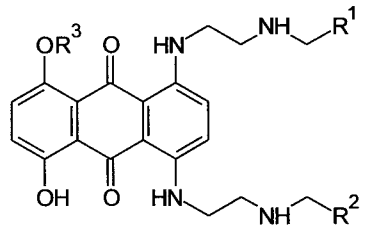
Recently, it has been shown that **1** yields the tetrahydronaphthoquinoxaline derivative **7** by horseradish peroxidase-catalysed oxidation (5). In the meantime this compound has been identified as an additional metabolite in the urine of different mitoxantrone treated species including human (6, 7). Its formation can be explained by the oxidation of **1** to the intermediate **5** followed by intramolecular Michael addition of the aliphatic side chain amino function.

In vitro oxidation of mitoxantrone with activated horseradish peroxidase in the presence of glutathione yields a glutathione conjugate of mitoxantrone. The same compound is obtained from mitoxantrone treated cultures of rat hepatocytes and human derived hepatoma (HepG2) cells (8). In addition, products formed by degradation of the glutathione moiety are detected in the cell culture experiments. The structure of the thioether derivatives of mitoxantrone have been determined by tandem mass spectrometry and nuclear magnetic resonance spectroscopy including two dimensional techniques. By means of these techniques it has been proved that the C,S-bond is formed at ring A (**8**- **11**) (9).

The formation of the tetracyclic metabolite **7** and the glutathione conjugate **8** reveals that two distinct electrophilic species (**5**, **6**) are present in the oxidatively activated mitoxantrone. This can be rationalised by the establishment of a tautomeric equilibrium. In the tautomer **5** the intramolecular Michael addition of



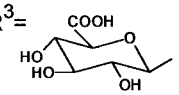
1

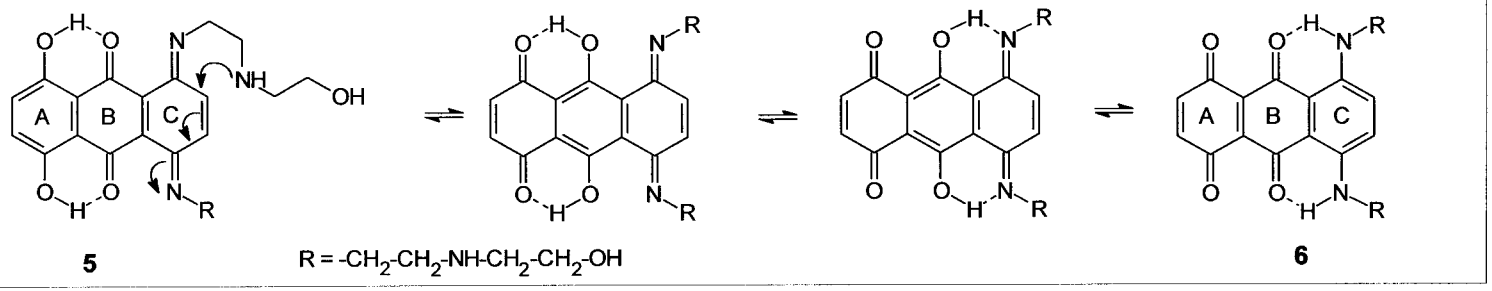


2 : $R^1 = CH_2OH$, $R^2 = COOH$, $R^3 = H$

3 : $R^1 = R^2 = COOH$, $R^3 = H$

4 : $R^1 = R^2 = CH_2OH$, $R^3 =$

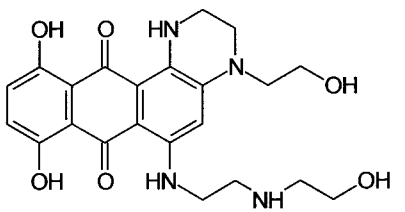




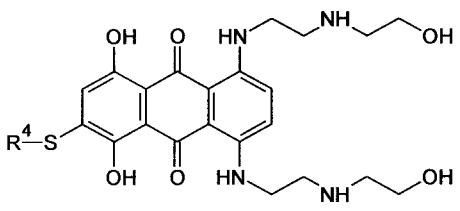
5

$R = -CH_2-CH_2-NH-CH_2-CH_2-OH$

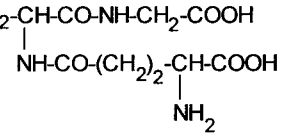
6



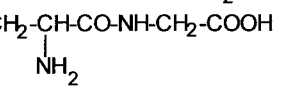
7



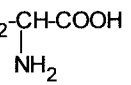
8 : $R^4 =$



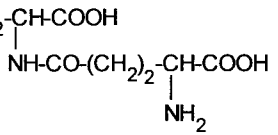
9 : $R^4 =$



10 : $R^4 =$



11 : $R^4 =$



the side chain nitrogen is favoured (neighbouring group effect) over the attack of external nucleophiles. In contrast, no such competitive situation exists for the tautomer **6** making this species a likely candidate for the intermolecular reaction with nucleophiles (e.g. glutathione).

The glutathione derivative **8** and its degradation products **9** - **11** have also been detected in the bile of mitoxantrone treated minipigs. This provides evidence for the corresponding biotransformation pathway in living organisms.

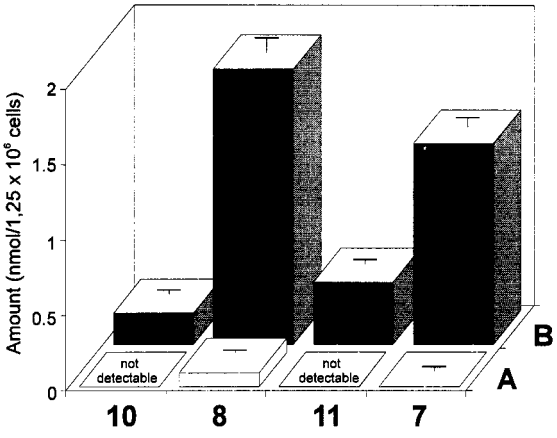


Figure 1

Effect of metyrapone (1 mM) on the formation of oxidation-related metabolites of mitoxantrone (50 µM) in incubations of rat hepatocytes (n=3).
[A, incubations in the presence of 1 mM metyrapone, B, controls without metyrapone. Quantitation was performed 19 hours after addition of mitoxantrone.
Results are expressed as means ± SD of three independent experiments.]

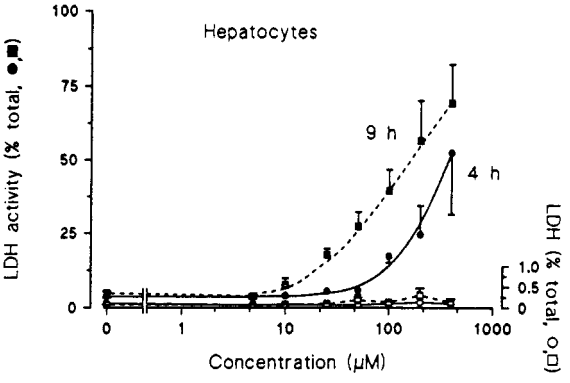


Figure 2.

Leakage of LDH from hepatocytes into the culture medium after exposure to various concentrations of mitoxantrone in the absence (*closed symbols*) or presence (*open symbols*) of metyrapone. Hepatocytes cultured for 2 h in medium containing 10 % newborn calf serum were washed extensively with saline and exposed to various concentrations in fresh serum-free culture medium. Metyrapone (1 mM) was added simultaneously to half of the cultures. After 4 h (circles, solid line) and 9 h (squares, dashed line) of incubation LDH activity was measured in the culture medium. Total LDH activity present in the hepatocytes was also determined. [Points represent means ± SD of 4 determinations of a representative culture.]

Some observations reported in the literature can be rationalised on the basis of the oxidative activation of mitoxantrone. This includes the depletion of intracellular glutathione in mouse liver cells 4 days after injection of mitoxantrone (10). Furthermore, the covalent binding of mitoxantrone to calf thymus DNA (11) could be explained by the reaction of the electrophilic intermediate **6** with nucleophilic centers in the DNA.

In a further set of experiments it could be shown that the glutathione conjugate **8** and the hexahydronaphthoquinoxaline derivative **7** are not formed in cultured rat hepatocytes and HepG2 cells when metyrapone is added (Figure 1). Since metyrapone is a well known inhibitor of cytochrome P-450 isoenzymes, these findings indicate that the oxidative activation depends on this enzyme system. This is exactly paralleled by the effect of mitoxantrone on cells in the presence and absence of metyrapone. The damaging effect of mitoxantrone expressed as leakage of lactate dehydrogenase (LDH) increases as expected with increasing amounts of mitoxantrone. However, in the presence of metyrapone almost no cytotoxic effect of mitoxantrone is observed (Figure 2). Thus, since inhibition of cytochrome P-450 suppresses both, the formation of metabolites via electrophilic intermediates **5** and **6** and the damaging effect on cells, it is justified to conclude, that the oxidative activation of mitoxantrone is involved in the cytotoxic effect of this agent.

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