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Research paper

Oxidative metabolism of the anti-cancer agent mitoxantrone by horseradish, lacto-and lignin peroxidase

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ABSTRACT

Mitoxantrone (MH_2X), an anthraquinone-type anti-cancer agent used clinically in the treatment of human malignancies, is oxidatively activated by the peroxidase/H_2O_2 enzyme system. In contrast to the enzymatic mechanisms of drug oxidation, the chemical transformations of MH_2X are not well described. In this study, MH_2X metabolites, produced by the horseradish, lacto- or lignin peroxidase (respectively HRP, LPO and LIP)/H_2O_2 system, were investigated by steady-state spectrokinetic and HPLC–MS methods. At an equimolar mitoxantrone/H_2O_2 ratio, the efficacy of the enzyme-catalyzed oxidation of mitoxantrone decreased in the following order: LPO > HRP > LIP, which accorded with the decreasing size of the substrate access channel in the enzyme panel examined. In all cases, the central drug oxidation product was the redox-active cyclic metabolite, hexahydronaphtho-[2,3-f]-quinoxaline-7,12-dione (MH_2), previously identified in the urine of mitoxantrone-treated patients. As the reaction progressed, data gathered in this study suggests that further oxidation of the MH_2 side-chains occurred, yielding the mono- and dicarboxylic acid derivatives respectively. Based on the available data a further MH_2 derivative is proposed, in which the amino-alkyl side-chain(s) are cyclised. With increasing H_2O_2 concentrations, these novel MH_2 derivatives were oxidised to additional metabolites, whose spectral properties and MS data indicated a stepwise destruction of the MH_2 chromophore due to an oxidative cleavage of the 9,10-anthracenedione moiety. The novel metabolites extend the known sequence of peroxidase-induced mitoxantrone metabolism, and may contribute to the cytotoxic effects of the drug *in vivo*. Based on the structural features of the proposed MH_2 oxidation products we elaborate on various biochemical mechanisms, which extend the understanding of mitoxantrone's pharmaceutical action and its clinical effectiveness with a particular focus on peroxidase-expressing solid tumors, such as breast carcinoma.

1. Introduction

Mitoxantrone (MH_2X, Novatrone, 1,4-dihydroxy-5,8-bis[2-(2-hydroxyethylamino)ethyl-amino]-anthracene-9,10-dione), a synthetic anti-cancer analog of anthracycline antibiotics, has demonstrated significant clinical effectiveness in the treatment of human malignancies [1–5]. The drug's oxidative activation and metabolism contrasts with the reductive processes that are believed to be responsible for the activation of other anthracyclin-type drugs, such as doxirubicin [6–16]. *In vivo* and *in vitro* experiments suggest that mitoxantrone metabolism and clearance proceed via numerous oxidised intermediates [1,6,8]. Interestingly, mitoxantrone shows particular effectiveness against tumors (myeloid leukaemia, breast and ovarian carcinoma) that express high titers of heme-peroxidases [1,3,13]. Therefore, peroxidase-catalyzed mitoxantrone oxidation may be important for *in vivo* drug activation, and the resulting metabolites could actually be the causative agents for its cytotoxic effects.

Consequently, the enzymatic mechanisms of peroxidase-catalyzed mitoxantrone oxidation have recently been subject to intense research efforts [17–20].

The model peroxidase, horseradish peroxidase (HRP), mammalian lactoperoxidase (LPO) and myeloperoxidase (MPO) catalyse the one-electron oxidation of mitoxantrone ($E^{0'}$ ($MH_2X/MHX(O)^{+*}$ = +0.38 V; pH = 7) via the catalytic intermediates Compounds

Abbreviations: HRP, horseradish peroxidase; LIP, lignin peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; HPLC, high performance liquid chromatography; RP, reversed phase; RT, retention time; AU, arbitrary absorbance unit; MH_2X, mitoxantrone (1,4-dihydroxy-5,8-bis{2-[(2-hydroxyethyl)-amino]-ethyl}-amino-9,10-anthracenedione); MH_2, 8,11-dihydroxy-4-(2-hydroxyethyl)-6-[[2-[(2-hydroxyethyl)-amino]-ethyl]-amino]-1,2,3,4,7,12-hexahydronaphtho-[2,3-f]-chinoxaline-7,12-dione.

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I ($O = Fe^{IV}P^{+\bullet}$) and II ($O = Fe^{IV}P$), using H_2O_2 as the primary oxidant [18,21–23]. In the presence of excess H_2O_2, the catalytically dead-end intermediate Compound III ($O_2^- - Fe^{II}P$) is formed. Whereas in the presence of a large excess of H_2O_2 peroxidase Compound III is irreversibly degraded, a moderate excess allows a slow conversion of Compound III back to the native (ferric) state ($Fe^{III}-P$) via an autocatalytic process, thereby releasing a superoxide ($O_2^{-\bullet}$) anion [21,24]. As the superoxide anion ($E^{0'}$ ($H_2O_2/O_2^{-\bullet}$) = +0.89 V; pH 7), [25] itself is a powerful oxidant, it can potentially react with substrate molecules, which would complicate the interpretation of enzymatic reactions and detection of associated metabolites.

While the enzymatic mechanisms of mitoxantrone oxidation are reasonably well understood, the detailed structural identification of mitoxantrone metabolites is complicated by the formation of mixtures containing monomeric, dimeric and higher molecular aggregates [14,26,27].

Various combinations of spectrophotometry, NMR spectroscopy and LC–MS have been used to assign structures of key mitoxantrone metabolites, but so far a uniform mechanism outlining drug transformation has not evolved [6,9,20]. In concert with the proposed enzymatic mechanisms, the initial one-electron oxidation of MH_2X results in the formation of mitoxantrone radical cation ($MHX(O)^{+\bullet}$) intermediates, which have been detected by EPR spectroscopy [19,23].

A recent LC–MS-based study suggested that both electrochemical and HRP-catalyzed oxidations of mitoxantrone lead to a stepwise dehydration of the hydroquinone moieties, forming quinone- and quinonedi-imine-based metabolites respectively [20].

By contrast, other investigators suggest that disproportionation of mitoxantrone radicals leads to internal cyclisation of an aminoalkyl side-chain and subsequent formation of the redox-active naphthoquinazoline derivative, MH_2 [22,23]. The presence of MH_2 (8,11-dihydroxy-4-(2-hydroxyethyl)-6-[[2-[(2-hydroxyethyl) ami no]-ethyl]amino]-1,2,3,4,7,12-hexahydronaphtho-[2,3-f]-chinoxaline-7,12-dione) has been detected in the urine samples of mitoxantrone-treated patients [6] and its structure has been established by a combination of NMR spectroscopy, LC–MS and spectrophotometry. Depending on the H_2O_2 concentration, peroxidases are able to oxidise MH_2 to the putative metabolite MH_2^{2+} via the radical intermediate $MH_2^{+\bullet}$ [22]. Although the spectrophotometric properties of MH_2^{2+} have been reported, its structure remains to be elusive, possibly due to rapid degradation or equilibrium effects with other metabolites.

At present, studies that attempted to identify metabolites resulting from the peroxidase-catalyzed oxidation of mitoxantrone, employed steady-state conditions with a significant H_2O_2 excess. Previous kinetic studies, however, indicated that the mitoxantrone to H_2O_2 ratio in the reaction mixture has to be considered because it is the key factor controlling both the enzymatic oxidation pathway and the extent of mitoxantrone oxidation [18,19]. Identification of mitoxantrone metabolites is often complicated by ongoing radical reactions of drug metabolites even after the enzymatic reaction has terminated [22]. It is therefore imperative to establish an equilibrium between the different chemical species prior to analysis.

In this study, we will show the effects of varying H_2O_2 concentration on peroxidase-catalyzed mitoxantrone oxidation and subsequent metabolite formation. We will also demonstrate that under equilibrium conditions the extent of mitoxantrone metabolite formation depends on the type of peroxidase used. The peroxidase panel employed in this study encompassed the model peroxidase HRP, fungal lignin peroxidase and mammalian LPO. As these enzymes differ in oxidation potential, pH optimum and the size of their substrate access channel, their reactions potentially lead to different metabolites [18,21,28,29]. The reactions of LPO are of particular pharmacological relevance, as this enzyme is constitutively expressed in breast carcinomas [30]. We will assign key metabolites and suggest a consistent chemical pathway with novel intermediates, using a combination of spectrophotometry and LC–MS.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma Chemical Corp., except for LIP, which was produced according to the methods of Zacchi et al. [31]. LPO and LIP were purified and stored as previously described [18,21,28]. Concentrations of native HRP, LIP and LPO were determined spectrophotometically as described previously [18,21,28]. Preparations of HRP C (pI = 8.5), bovine LPO (pI = 8.8), and LIP (pI = 4.59) from *Phanerochaete chrysosporium* showed purity numbers (RZ) of 3.3, 2.0, and 4.8 respectively [21,31–33].

2.2. Steady-state mitoxantrone oxidation catalyzed by peroxidases

Mitoxantrone and its metabolites (λ_{max} = 550–800 nm) absorb at a different wavelength range than peroxidase intermediates (λ_{max} = 350–550 nm), which enables simultaneous monitoring during the reaction [22,23]. Mitoxantrone concentrations were measured at the isosbestic point between the monomer/dimer transitions at 682 nm ($\varepsilon_{682} = 8.36 \times 10^{-3}\ M^{-1}\ cm^{-1}$) [26].

Steady-state experiments were carried out using an HP8553 diode array spectrophotometer (Agilent Ltd., Stockport, UK) fitted with a dual syringe stopped-flow attachment (Hi-Tech Scientific Ltd., Bradford-on-Avon, UK), where syringe A contained H_2O_2 (34 or 340 μM; $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$) [11] and syringe B a mixture of mitoxantrone (34 μM) and native (ferric) peroxidase (2 μM). Reactions were performed in 50 mM-phosphate buffer at either pH 7.2 (for HRP and LPO) or pH 4.6 (for LIP). Manual triggering of the stopped-flow mechanism resulted in even mixing of the reactants and initiated automatic monitoring (range: 190–800 nm) of the reaction. The spectrokinetic data sets (spectral resolution: 1 nm; temporal resolution: 200 ms) were subjected to singular value decomposition (SVD) in combination with evolving factor analysis (EFA) [34] using the Specfit/32 (Spectrum Associates, Milford, CT, USA) software package. The calculated spectral profiles were used to identify the various peroxidase intermediates (ferric peroxidase, Compounds I, II and III) and mitoxantrone metabolites in the reaction. The time-dependent concentration profiles of the mitoxantrone metabolites generated by EFA were used to determine the reaction sequence for mitoxantrone oxidation.

2.3. HPLC analysis

HPLC experiments were carried out on a Model LC-10 (Shimadzu Ltd., Munich, Germany) unit equipped with an automatic sample injection system (SIC-10A), system controller (SCL-10A VP) column oven (CTD-10A VP, at 25 °C), and diode array detector PDA (SPD-M10A; 190–800 nm; time resolution: 0.5 s; spectral resolution: 2 nm). The system was fitted with a 50 μl injection loop.

Samples were analysed by HPLC on a 10 cm × 0.5 cm RP C-18 column (Chromolith® RP-18e 100-4.6; Merck, Darmstadt, Germany). Samples were eluted at a rate of 2 ml × min^{-1} with a gradient of 10 mM-NaH_2PO_4, pH: 3.0 (Solvent A) and acetonitrile (Solvent B), commencing with 90% (v/v) A/10% (v/v) B for 1 min. The percentage of acetonitrile was ramped linearly to 80% B over a time span of 19 min. The column was then flushed for 5 min at 20% A/80% B and re-equilibrated for 10 min with Solvent A. Sample elution was monitored over 190–800 nm. Acquired data were processed