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Interaction of HPMA copolymer-doxorubicin conjugates with human liver microsomal cytochromes P450. Comparison with free doxorubicin.

Names of authors:

Vlastimil Mašek, Eva Anzenbacherová, Tomáš Etrych, Jiří Strohalm, Karel Ulbrich, Pavel Anzenbacher

Laboratory of origin:

Department of Pharmacology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic

Affiliations:

Department of Pharmacology (V.M., P.A.), Department of Medical Chemistry and Biochemistry (E.A.), and Institute of Molecular and Translational Medicine (V.M., P.A.), Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic; Institute of Macromolecular Chemistry v.v.i., AS CR, Prague, Czech Republic (T.E., J.S., K.U.).

1

RUNNING TITLE PAGE

Running title:

Doxorubicin conjugates interact with human cytochromes P450.

Corresponding author:

Vlastimil Mašek, Ph.D.

Department of Pharmacology, Faculty of Medicine and Dentistry, Palacky University

Olomouc,

Hnevotinska 3

CZ-775 15 Olomouc, Czech Republic

Tel. +420-58-563-2558

Fax +420-58-563-2966

E-mail: v.masek@gmail.com

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CYP, cytochrome P450; HPMA, *N*-(2-hydroxypropyl) methacrylamide; DOX, doxorubicin;

HPLC, high performance liquid chromatography.

ABSTRACT

Interaction of nine forms of human hepatic cytochromes P450 (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) with two HPMA copolymer-based doxorubicin (DOX) conjugates designed for passive tumor targeting was studied using pooled human microsomes. The compounds used in this study were two high-molecular-weight *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers bearing doxorubicin attached to the polymeric carrier by A) hydrazone bond enabling intracellular pH-controlled drug release; or B) amide bond through enzymatically cleavable tetrapeptide GlyPheLeuGly spacer. Both polymeric conjugates differing in mechanism of their anti-tumour activity and the free doxorubicin as control were tested for potential inhibition activity.Among nine cytochrome P450 forms studied, no HPMA copolymer with bound DOX caused an inhibition of potential clinical significance. The extent of inhibition of enzymatic activities of the cytochrome P450 (CYP) forms studied was negligible with exception of CYP2B6 and was apparently caused by DOX as no inhibition was observed with polymers alone and the extent of inhibition by the complex corresponded to this of the free DOX at the same concentration. In conclusion, the polymers as well as their conjugates with DOX appear to be relatively safe at least in this respect, i.e. of inhibition of the liver microsomal drug metabolizing enzymes.

INTRODUCTION

Foreign substances entering the body are in the most cases biotransformed to more

polar compounds to facilitate their excretion from the organism (Parkinson and Ogilvie,

2008). Among enzymes taking part in these reactions, the liver microsomal cytochromes P450

(CYPs) are the most important ones as they are responsible for more than one half of known

pathways of drug metabolism in the liver and other organs (Anzenbacher and

Anzenbacherova, 2001). CYPs form a superfamily of enzymes localized in the liver, lung,

gastrointestinal tract, brain, heart, and in other tissues. Among liver microsomal CYPs, nine

forms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and

CYP3A4) are active in metabolism of more than 90% reactions catalyzed by these enzymes.

The importance of studies aimed at investigating their interactions with drugs and newly

designed active compounds is given by the fact that many compounds may be converted by

these enzymes either to less active or on the contrary to more active molecules and that there

is a possibility of a competition of a drug for these proteins causing drug interactions leading

to increasing even toxic levels of the respective drugs (Bachmann et al., 2004).

Two types of HPMA copolymer-doxorubicin (DOX) have been developed exhibiting

significant anticancer activity (as demonstrated in mice bearing various tumor models)

(Duncan et al., 1989; Kovar et al., 2002; Ulbrich et al., 2003). In the conjugates DOX was

attached to the HPMA copolymer carrier either via an oligopeptide GlyPheLeuGly spacer

susceptible to enzymatic hydrolysis with lysosomal enzymes in lysosomes of target cells

(amide conjugate, Fig. 1) (Kopecek et al., 2000) or via a hydrazone bond and aminohexanoic

acid residue in a spacer susceptible to pH-dependent hydrolysis (hydrazone conjugate, Fig. 2)

(Etrych et al., 2001; Etrych et al., 2002). Both conjugates were found to be fairly stable in

blood circulation and releasing DOX in lysosomes (amide conjugate) or in mild acidic

4

environment of tumor tissue or in endosomes of cancer cells (hydrazone conjugate). Both

polymer conjugates exhibited significantly prolonged blood clearance and enhanced

accumulation in solid tumors in mice (passive targeting) due to EPR effect (Etrych et al.,

2001; Lammers et al., 2007; Etrych et al., 2008a). Moreover, two amide conjugates,

nontargeted and galactose-targeted HPMA copolymer-DOX conjugates (Vasey et al., 1999;

Seymour et al., 2009) showed in Phase I and II of clinical studies limited side-effects and

improved anticancer activity suggesting the polymer-doxorubicin conjugates for further

development as promising drugs for treatment of cancer in humans (breast, lung, colorectal).

It was shown that detailed structure of the spacer and form of drug attachment in the

HPMA copolymer-DOX conjugates significantly influence mechanism of drug release,

interaction of the conjugates with cells, the intracellular distribution of the conjugates, their

antiproliferative effect and cell death signals they trigger (Hovorka et al., 2006; Kovar et al.,

2007).

In this paper additional results of detailed study of properties of both types of polymer

drugs are shown, namely, results of evaluation of interaction of the conjugates with the liver

microsomal cytochromes P450 bringing information on susceptibility of the conjugates to

metabolic elimination processes in liver. Such information is important for understanding of

mechanism of action of the polymer-DOX drugs and proper design and further development

of the conjugates. In the study behavior of two polymer conjugates with DOX (amide and

hydrazone) and respective polymer carriers used as controls (for conjugates structures see

Figure 1 and Figure 2) was compared with properties of the free drug (DOX.HCl).

MATERIALS AND METHODS

Chemicals

1-Aminopropan-2-ol, methacryloyl chloride, 2,2′-azobis(isobutyronitrile) (AIBN), methyl 6-aminohexanoate hydrochloride (ah-MeO), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-ethyldiisopropylamine, dimethyl sulfoxide (DMSO), hydrazine hydrate, amino acids glycine (G), phenylalanine (F) and leucine (L) and solvents (p.a. quality) were purchased from Fluka (Switzerland). Solvents were dried and purified by conventional procedures and distilled before use. Doxorubicin hydrochloride (DOX.HCl) was purchased from Meiji Seika, Japan.

For determination of CYP activities, chlorzoxazone, 6-hydroxychlorzoxazone, diclofenac, 4-hydroxydiclofenac, bufuralol, 6-hydroxybufuralol and 6β-hydroxytestosterone were supplied by Cerilliant Corporation (Round Rock, Texas, USA). P450-Glo® substrates luciferin ME (luciferin-6′ methyl ether), luciferin H (6′-deoxyluciferin) and luciferin BE (luciferin-6′ benzyl ether) for evaluation of CYP2C8, CYP2C9 and CYP3A4 activities by luminescence spectrometry were products of Promega (Madison, Wisconsin, USA) obtained through East Port (Prague, Czech Republic). 7-ethoxy-4-(trifluoromethyl)coumarin was supplied by Fluka (Buchs, Switzerland). Cryopreserved human liver microsomes (pooled) were purchased from Advancell (Barcelona, Spain). Microsomes were obtained under approval of the local Ethic committee and in accordance with the ethic regulations of the country of origin (Spain). They were from 5 males and 5 females with protein content 38.4 mg/mL; the CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1 and CYP3A4 enzyme activities are accessible at the Advancell web site (www.advancell.net, batch reference 102091201). All other chemicals were supplied by Sigma Aldrich (Prague, Czech Republic).

Synthesis of monomers, polymer precursors and polymer conjugates

N-(2-Hydroxypropyl)methacrylamide, methacryloylglycylphenylalanylleucylglycin and its 4-nitrophenyl ester (MA-GFLG-ONp) were synthesized as described in (Ulbrich et al., 2000). 6-Methacrylamidohexanohydrazide (Ma-ah-NHNH$_2$) was prepared by the reaction of methyl 6-aminohexanoate hydrochloride with methacryloyl chloride followed by hydrazinolysis of the methylester as described in (Etrych et al., 2008b). Purity and composition of all monomers was tested by NMR, HPLC using RP C18 column, m.p. and elemental analysis. Monomers were chromatographically pure (> 98%) and their characteristics corresponded with those given in respective papers (Ulbrich et al., 2000; Etrych et al., 2008b).

Polymer precursor, a copolymer of HPMA with MA-GFLG-ONp was prepared by radical precipitation polymerization in acetone using AIBN as initiator. Polymer precursor contained 4.5 mol% of 4-nitrophenoxy (ONp) groups (determined by UV spectrophotometry ($\varepsilon = 9\,500$ L mol^{-1} cm^{-1}, $\lambda = 274$ nm, DMSO). Polymer amide conjugate with DOX (poly(HPMA-co-MA-GFLG-DOX)) was prepared by aminolysis of the polymer precursor with DOX.HCl in DMSO using N-ethyldiisopropylamine as a base. For detailed synthesis and characteriation see (Ulbrich et al., 2000). Molecular weight (M$_w$) of the polymer conjugate was 66 000 g.mol^{-1}, polydispesity was 1.89 and content of DOX was 7.9 wt% (determined spectrophotometrically in distilled water, $\varepsilon = 11\,500$ L mol^{-1} cm^{-1} at $\lambda = 488$ nm). Content of free DOX in the conjugate was < 0.02 wt% (related to total DOX content). Control sample, polymer carrier poly(HPMA-co-MA-GFLG-AP) was prepared by aminolysis of the polymer precursor with 10-fold excess of 1-aminopropan-2-ol in DMSO, the product was isolated and purified by precipitation and re-precipitation into mixture aceton:diethyl ether (3:1). Content of oligopeptide groups terminating in AP was 4.5 mol% (estimated by amino acid analysis).

Copolymer of HPMA with Ma-ah-NHNH$_2$ (poly(HPMA-co-MA-ah-NH-NH$_2$) containing free hydrazide groups was prepared by radical solution copolymerization of respective monomers in methanol using AIBN as described earlier (Etrych et al., 2008b). Molecular weight (M$_w$) of the copolymer was 27 000 g.mol^{-1}, polydispersity 1.8 and content of hydrazide groups determined by TNBS method was 5.8 mol%. The hydrazone conjugate poly(HPMA-co-MA-ah-NHN=DOX was prepared by the reaction of polymer precursor poly(HPMA-co-MA-ah-NH-NH$_2$ with DOX.HCl in methanol containing catalytic amount of acetic acid. The reaction was carried out in the dark as described earlier (Etrych et al., 2001). Molecular weight (M$_w$) of the copolymer was 32 000 g.mol^{-1}, polydispersity 1.8 and content of DOX was 9.8 wt% (UV/VIS spectrophotometry). Content of free DOX was < 0.1 wt% (related to total DOX content).

Molecular weights of all copolymers were determined with FPLC Pharmacia system equipped with RI, UV and multiangle light scattering DAWN DSP-F (Wyatt Co., USA) detectors using 0.3 M acetate buffer pH 6.5 and Superose™ 6 column.

Determination of CYP activities

Activities of individual CYP forms were measured according to published protocols. All the tested microsomal CYP activities and corresponding methods of specific product detection are listed in the Table 1.

A TECAN Infinite M200 microplate reader (Tecan Austria, Vienna, Austria) was used for detection of the fluorescence- and luminescence-based assays. HPLC analyses were performed using the Shimadzu Prominence system (Kyoto, Japan).

Fresh stock solutions of hydrazone and amide conjugate, polymer precursors and DOX.HCl in ultrapure water were made before each CYP activity assay. In hydrazone and amide conjugate stock solution, the concentration of DOX was 40 µg/ml (74 µM). Polymer

precursors were dissolved to concentration that is equivalent to the respective polymer

conjugate solutions, free DOX.HCl stock solution concentration was 400 µg/ml (0,74 mM).

The stock solutions described here were added to reaction mixtures to obtain desired final

concentration of the tested drug, to the control sample no drug was added, only ultrapure

water.

Final incubation mixture volumes were: CYP1A2, CYP2A6 and CYP2B6, 100 µl;

CYP2C8, 100 µl; CYP2C9, 100 and 50 µl for diclofenac or 6´-deoxyluciferin as substrates;

CYP2C19, and CYP2D6, 200 µl; CYP2E1, 500 µl; CYP3A4 activities, 500 µl for testosterone

and 50 µl for luciferin derivative as substrate. The reaction mixtures of all CYP activities

tested were buffered by 75 mM K-phosphate buffer, pH 7.4.

For each enzyme assay, a preliminary experiment was done to determine the K_M and

V_{max} for a given enzyme reaction and to obtain the values of substrate concentrations suitable

for the inhibition experiments (as a rule, substrate concentration was chosen in the range

corresponding to the value of the K_M). Inhibition experiments were routinely performed with

up to seven concentrations of the tested drug. A control experiment with known reference

inhibitors was implemented in cases where a significant degree of inhibition was presumed,

namely, with sulfaphenazole (CYP2C9) (Brown et al., 2006), methoxsalen (CYP2A6)

(Kharasch et al., 2000), diethyldithiocarbamate (CYP2E1) (Bourrie et al., 1996; Guengerich,

2006) and 3-isopropenyl-3-methyldiamantane (CYP2B6) (Stiborova et al., 2002).

Inhibition of individual CYP activities by HPMA copolymer-DOX conjugates or free

DOX was in all cases evaluated by plotting the respective remaining activity against the

inhibitor concentration; as a rule, the results were expressed as means of two to five

independent determinations with the difference between duplicates being lower than 15%.

When an inhibition was pronounced, the K_i values were determined from Dixon plots with

three substrate concentrations used (corresponding to $0.5K_M$, K_M and $2K_M$). To get an

9

information on the mechanism of inhibition, both Dixon and Lineweaver-Burk plots were

used (Segel, 1993). To analyze the course of the enzyme kinetics, parameters of enzyme

kinetics (K_M, V_{max}) as well as the intercepts of the respective plots were obtained using the

Sigma Plot 8.0.2 (SPSS, Chicago, Illinois, USA) and GraphPad Prism 5 (GraphPad Software,

San Diego, California, USA) scientific graphing software.

RESULTS

Possible interactions of the conjugates, poly(HPMA-co-MA-GFLG-DOX) and poly(HPMA-co-MA-ah-NHN=DOX), as well as their polymer precursors and free DOX.HCl with activities of nine forms of CYP enzymes present in human liver microsomes (CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) were studied. The results show that within DOX concentration range up to 18 µM (corresponding approximately to levels attainable in human plasma (Vaclavikova et al., 2008)), none of the studied CYP activity was affected except for CYP2D6 (activity decrease between 10%-25%).

For comparison, the free DOX.HCl and the polymeric precursors without bound DOX were also tested with respect to their ability to influence CYP activities. Polymeric precursors did not affect any of the CYP forms studied (at the same concentration range as HPMA copolymer-DOX conjugates). The results of inhibition studies with the free DOX.HCl were nearly identical as with DOX bound through both types of conjugates (hydrazone and PK1), which means that only CYP2B6 activity was remarkably affected (decrease to 60% of control), the CYP1A2, 2A6, 2C8 and 2E1 forms were affected only slightly (activity decrease between 10%-25%).

Inhibition of CYP2B6 activity by free DOX as well as by DOX bound in hydrazone or amide conjugate (at concentrations 0 to 18 µM) is shown in Fig. 3). The results clearly show that independently on the mode of binding, the DOX acts as a moderate inhibitor of CYP2B6 activity. In the Fig. 3 inhibition data for free DOX at higher levels are also presented.

To obtain an information whether free DOX.HCl at higher concentrations is able to inhibit CYP enzymes in a more pronounced extent, the free DOX.HCl was tested also for ten times higher concentration equal to 184 µM. Here, the concentrated DOX.HCl affected markedly almost all studied CYP activities. The most prominent effect was observed at CYP2B6 form which was nearly completely inhibited with DOX at 184 µM (Fig. 4).

11

Activities of CYP2C8, CYP2C19, CYP3A4 forms (all of them were inhibited down to 30% of control) and CYP1A2 form (decrease to 40% of control) were affected significantly as well (Fig. 4). Apparent inhibition was observed also at CYP2A6, CYP2E1 and CYP2D6 activities, however, the remaining activity was still higher than 60% of control even at DOX concentration of 184 μM. The last activity tested, the CYP2C9 one, was not affected at all even at the DOX.HCl concentration reaching 184 μM.

The enzyme activities of CYP forms which exhibited the decrease by DOX exceeding 50% of control (i.e. CYP1A2, 2B6, 2C8, 2C19 and 3A4) (Fig. 4) were analyzed using Dixon and Lineweaver-Burk plots (Segel, 1993) to evaluate the possible mechanisms of enzyme inhibition (Burlingham and Widlanski, 2003). In the case of CYP1A2, CYP2C8, CYP2C19 and CYP3A4, the courses of the Dixon and Lineweaver-Burk plots indicate a non-competitive mechanism of inhibition which means an inhibition of product formation by the presence of an inhibitor (the inhibitor is bound in a site close to the substrate binding site; however, the inhibition is not conditioned by a conformational change of the active site due to the previous binding of a substrate). Data for CYP2C8 and CYP2B6 are shown (Fig. 5A, 5B, 6A and 6B), the courses of the Dixon and Lineweaver-Burk plots are similar for CYP3A4. The K_i value is at about 75 μM for CYP1A2; at about 30 μM for CYP2C19 activity and about 55 μM for the CYP3A4 activity. Similar case of non-competitive inhibition was also observed for inhibition of the CYP2C8 activity. The course of Dixon plot corresponds well to non-competitive inhibition for lower concentrations of DOX (up to approximately 110 μM) (Fig.5A); with the K_i matching 90 μM.

In the case of inhibition of CYP2B6 activity (which was the most affected one, see Fig. 3 and Fig. 4), the Dixon as well as the Lineweaver-Burk plot indicated a strongly bound inhibitor. Dixon plot shows an upright curvature typical for a strongly bound inhibitor (Fig. 6A), and a Lineweaver-Burk plot (Fig. 6B) is known to exhibit in this case (of strongly bound

12

inhibitior) the same course as for non-competitive with an intercept at the x-axis indicating

the K_i value of about 90 µM.

DISCUSSION

The results obtained in this study with two HPMA copolymer-DOX conjugates differing in mechanism of DOX release and mechanism of cytotoxic activity point to identical character of their interactions with the main liver microsomal systems of drug biotransformation, namely, with the CYP enzymes. Surprisingly, both polymer conjugates, the hydrazone conjugate and the amide conjugate exhibit only minor effect on activities of selected microsomal CYPs being thus safe drugs in the light of drug interactions based on this effect. In fact, within common therapeutic concentration levels of DOX, i.e. below 18 µM, hydrazone and amide conjugates inhibited markedly only one activity - CYP2B6. CYP1A2, 2A6, 2C8, 2E1 activity as well as the activity of the most important CYP form, CYP3A4 (metabolizing majority of drugs converted by CYP enzymes) were inhibited at the therapeutic concentration only slightly and the decrease was not greater than 20 % at the highest concentration of the drug conjugate. Polymer precursors of both conjugates did not inhibit CYP activities at all.

In other words, the inhibition experiments with both types of HPMA polymers either with DOX conjugated or without bound drug yielded rather weak and most probably clinically not relevant inhibition of activities of individual CYP enzymes. As it has been described earlier, the CYP2B6 was the only exception. The extent of inhibition at concentrations of DOX corresponding to levels in plasma was more than 40% and corresponded to extent of inhibition of CYP activities with free DOX.HCl at the same concentration.

The comparative experiments performed with free DOX.HCl revealed that the free drug affects the same CYP activities and in the same extent as conjugates with bound drug. Therefore DOX is responsible for inhibition properties of the HPMA copolymer conjugates

14

and attachment of the drug to a macromolecular carrier do not significantly influence its

inhibitory properties although conjugation of DOX with the same polymer carriers results in

dramatic decrease in systemic toxicity and significant improvement in anti-cancer activity

depending on the detailed structure of the polymer carrier system.

At higher DOX concentrations, the in vitro inhibition experiments were performed.

The mechanism of CYP inhibition was according to the kinetic analysis noncompetitive and

for the CYP2B6 form the results were interpreted in favor of a strongly bound inhibitor

(DOX). In other words, the results indicated possible influence of DOX.HCl on processes not

directly related to substrate binding, however, hampering the enzyme reaction. Here, the

possibility of influencing the electron transfer by interaction with residues at the surface of the

enzyme or, blockade of the substrate/product access/egress channels may be speculated

(Otyepka et al., 2007). The inhibition of CYP2B6 activity was markedly less specific (with Ki

value of 90 µM) in comparison with the specific inhibitor used, 3-isopropenyl-3-

methyldiamantane, which exhibits Ki of 2 µM (Stiborova et al., 2002).

This mode interaction (not affecting directly the substrate binding site of CYP

enzymes) may explain both (i) relatively low specificity of DOX.HCl or DOX conjugate –

CYP interactions as well as (ii) the fact, that both types of HPMA conjugates (although of

different properties and structurally diverse) behave in this respect a similar way. The fact,

that only one CYP enzyme (CYP2B6) exhibited a relatively specific inhibition by DOX.HCl,

is in line with an earlier finding that also the rat CYP2B1 form was found to be inhibited by

this drug. The explanation of the inhibition was based on an impairment of the electron

transfer, which may support the reasoning on possible mechanism of CYP inhibition by

influencing the residues at the surface of CYP macromolecule in both the enzymes of CYP2B

family.

Taken together, the DOX conjugates may be expected to be relatively safe anticancer therapeutic agents, at least most probably not influencing the enzyme activities of drug liver CYP enzymes.

AUTHORSHIP CONTRIBUTION

Participated in research design: Mašek, Anzenbacherová, Anzenbacher

Conducted experiments: Mašek, Anzenbacherová

Contributed new reagents or analytic tools: Etrych, Strohalm, Ulbrich

Performed data analysis: Mašek, Anzenbacher

Wrote or contributed to the writing of the manuscript: Mašek, Anzenbacher, Ulbrich

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FOOTNOTES

Reprint requests:

Vlastimil Mašek, Ph.D.

Department of Pharmacology, Faculty of Medicine and Dentistry, Palacky University

Olomouc,

Hnevotinska 3

CZ-775 15 Olomouc, Czech Republic

Tel. +420-58-563-2558

Fax +420-58-563-2966

E-mail: v.masek@gmail.com

LEGENDS TO FIGURES

Fig. 1. Structure of the amide HPMA copolymer conjugate with DOX (poly(HPMA-co-MA-GFLG-DOX)) (A) and its aminolyzed polymer precursor, polymer carrier poly(HPMA-co-MA-GFLG-AP) (B).

Fig. 2. Structure of the hydrazone HPMA copolymer conjugate with DOX (poly(HPMA-co-MA-ah-NHN=DOX)), (A) and its polymer precursor (carrier) poly(HPMA-co-MA-ah-NH-NH$_2$) (B).

Fig. 3. Inhibition effects of the hydrazone conjugate, amide conjugate and the free doxorubicin on CYP2B6 activity. Comparison demonstrates evident correlation between inhibition effects of both conjugates and inhibition effect of free doxorubicin. Mean and standard deviation (SD) values are shown.

Fig. 4. Effect of DOX.HCl on five specific activities of cytochrome P450. Activities tested: CYP1A2,7-ethoxyresorufin O-deethylation; CYP2B6, testosterone 6β-hydroxylation; CYP2C8, luciferin-6´ methyl ether demethylation; CYP2C19, (S)-mephenytoin 4´-hydroxylation; CYP3A4, 7-ethoxy-4-(trifluoromethyl) coumarin O-deethylation. Concentrations of DOX.HCl in reaction mixture were 0, 15, 29, 44, 74, 118, 147 and 184 μM. Mean and SD values are shown.

Fig. 5A. Dixon plot for inhibition of CYP2C8 enzymatic activity by DOX.HCl at three substrate concentrations (75 μM, squares; 150 μM, triangles; 300 μM, circles). 1/v, reciprocal velocity; RLU, relative luminescence unit. Mean and SD values are shown.

Fig. 5B. Lineweaver-Burk plot for inhibition of CYP2C8 enzymatic activity by DOX.HCl at three substrate concentrations (75 μM; 150 μM; 300 μM). Concentrations of DOX.HCl in reaction mixture were (from up to down): 184; 147; 118; 74; 44; 29; 15 μM and control with no inhibitor). 1/v, reciprocal velocity; 1/[S], reciprocal substrate concentration; RFU, relative fluorescence unit. Mean and SD values are shown.

Fig. 6A. Dixon plot for inhibition of CYP2B6 enzymatic activity by DOX at three substrate concentrations (7,5 μM, squares; 15 μM, triangles; 30 μM circles). Mean and SD values are shown.

Fig. 6B. Lineweaver-Burk plot for inhibition of CYP2B6 enzymatic activity by DOX at three substrate concentrations (7,5 μM, ; 15 μM, ; 30 μM) and 8 inhibitor concentrations (from up to down: 184; 147; 118; 74; 44; 29; 15 μM and control with no inhibitor). Four lowest concentrations are very close and data are overlapping. 1/v, reciprocal velocity; 1/[S], reciprocal substrate concentration. Mean and SD values are shown.

TABLES

Table 1. The list of CYP activities tested and references to corresponding methods of

individual CYP activity product detection.

CYP450	CYP activity	Detection	Reference
1A2	7-ethoxyresorufin O-deethylation	fluorescence	(Chang and Waxman, 1998)
	7-ethoxyresorufin O-deethylation	HPLC	(Chang and Waxman, 1998)
2A6	coumarin 7-hydroxylation	fluorescence	(Waxman and Chang, 1998)
2B6	7-ethoxy-4-(trifluoromethyl) coumarin O-deethylation	fluorescence	(Donato et al., 2004)
	7-ethoxy-4-(trifluoromethyl) coumarin O-deethylation	HPLC	(Morse and Lu, 1998)
2C8	luciferin-6´ methyl ether demethylation (P450-Glo substrate)	luminescence	Tech. Bulletin No 325 www.promega.com
2C9	diclofenac 4´-hydroxylation	HPLC	(Crespi et al., 1998b)
	6´-deoxyluciferin hydroxylation (P450-Glo substrate)	luminescence	Tech. Bulletin No 325 www.promega.com
2C19	(S)-mephenytoin 4´-hydroxylation	HPLC	www.cypex.co.uk Cypex 2C19 QC assays
2D6	bufuralol 1´-hydroxylation	HPLC	(Crespi et al., 1998a)

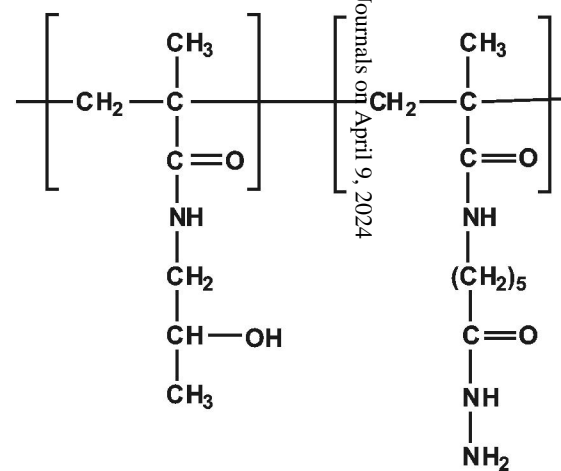| 2E1	chlorzoxazone 6-hydroxylation	HPLC	(Lucas et al., 1996)
3A4	testosterone 6β-hydroxylation	HPLC	(Guengerich et al., 1986)
	luciferin-6′ benzyl ether	luminescence	Tech. Bulletin No 325
	debenzylation		www.promega.com
	(P450-Glo substrate)		

27

Figure 1

A

B

Figure 2

A

B
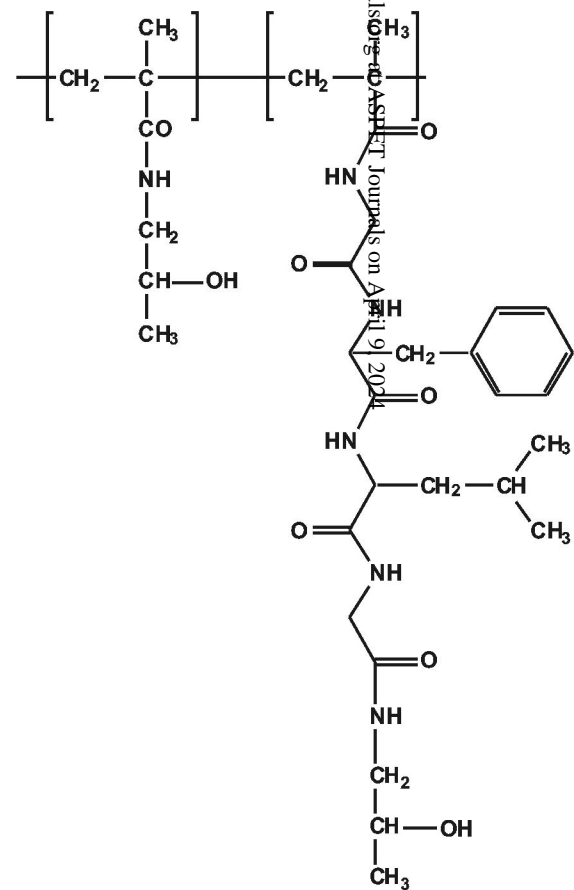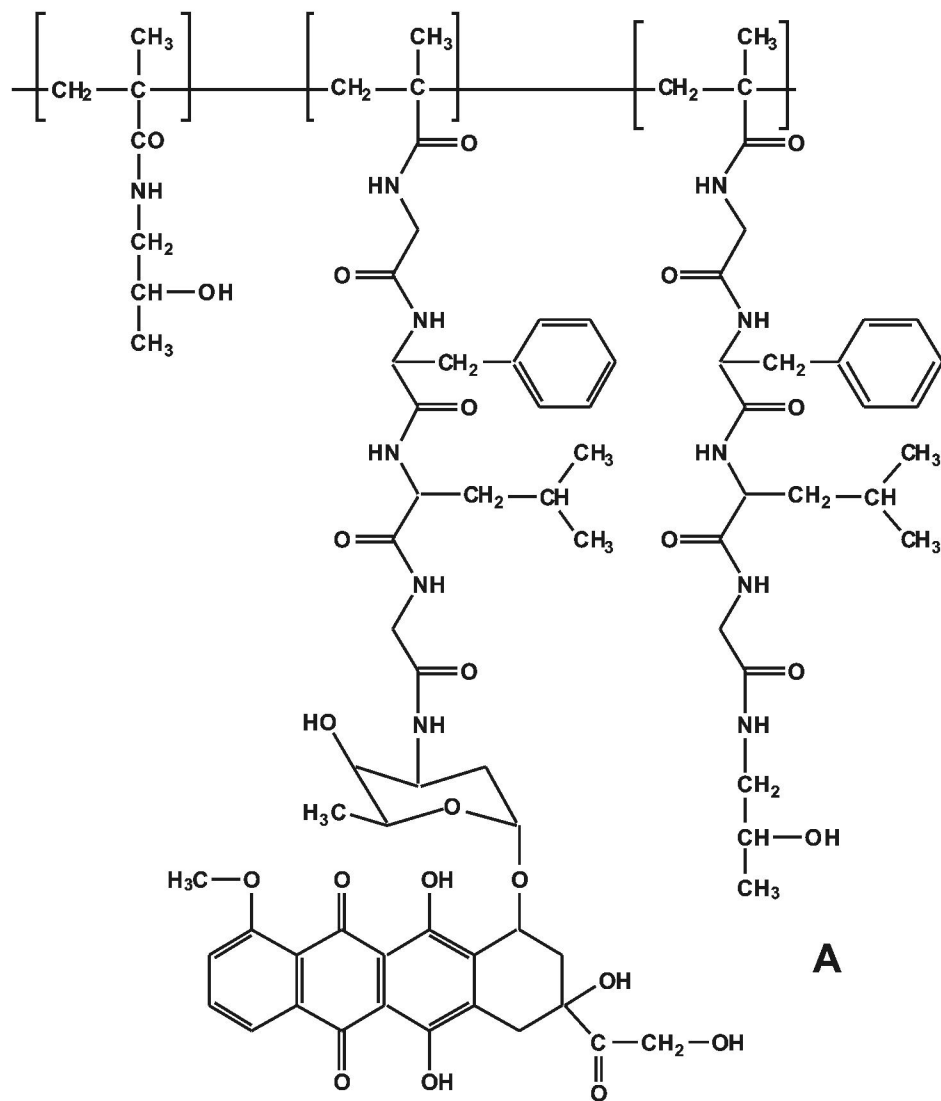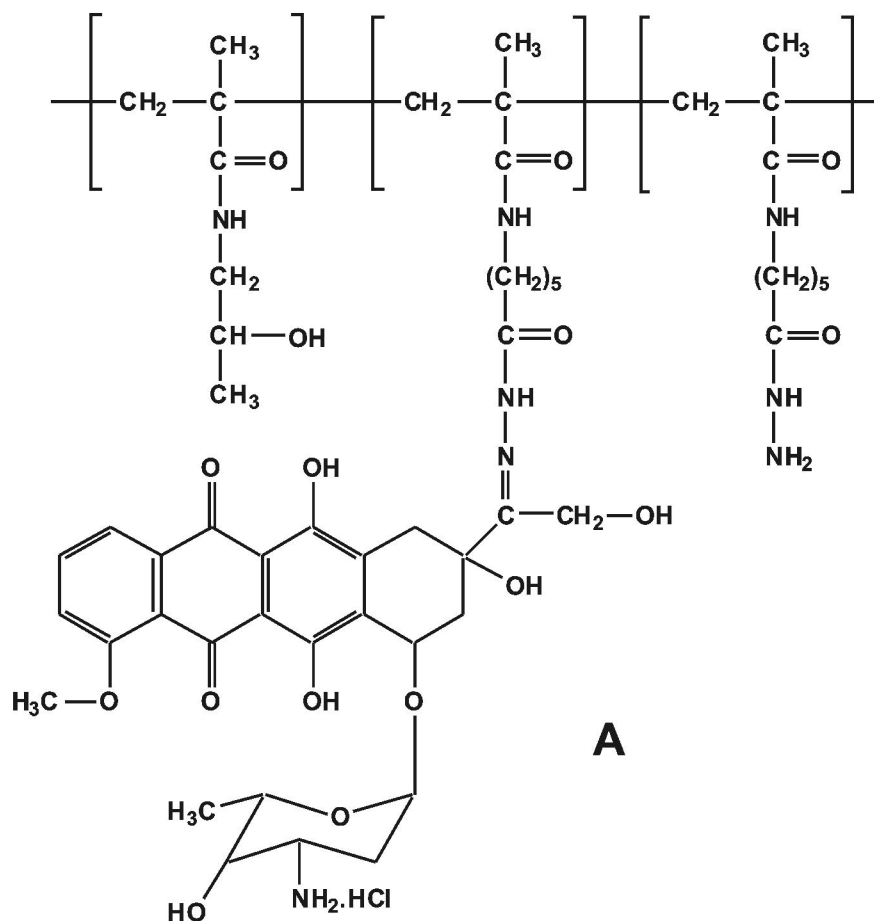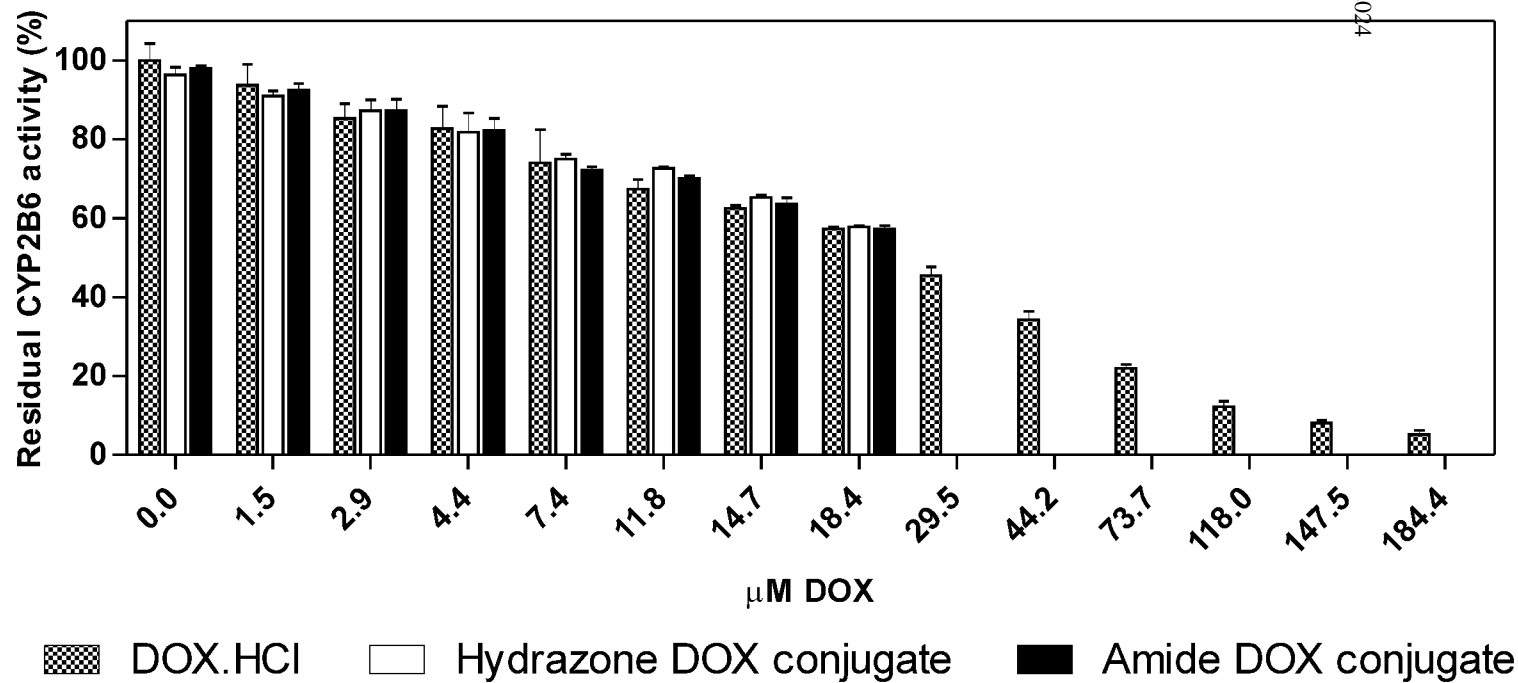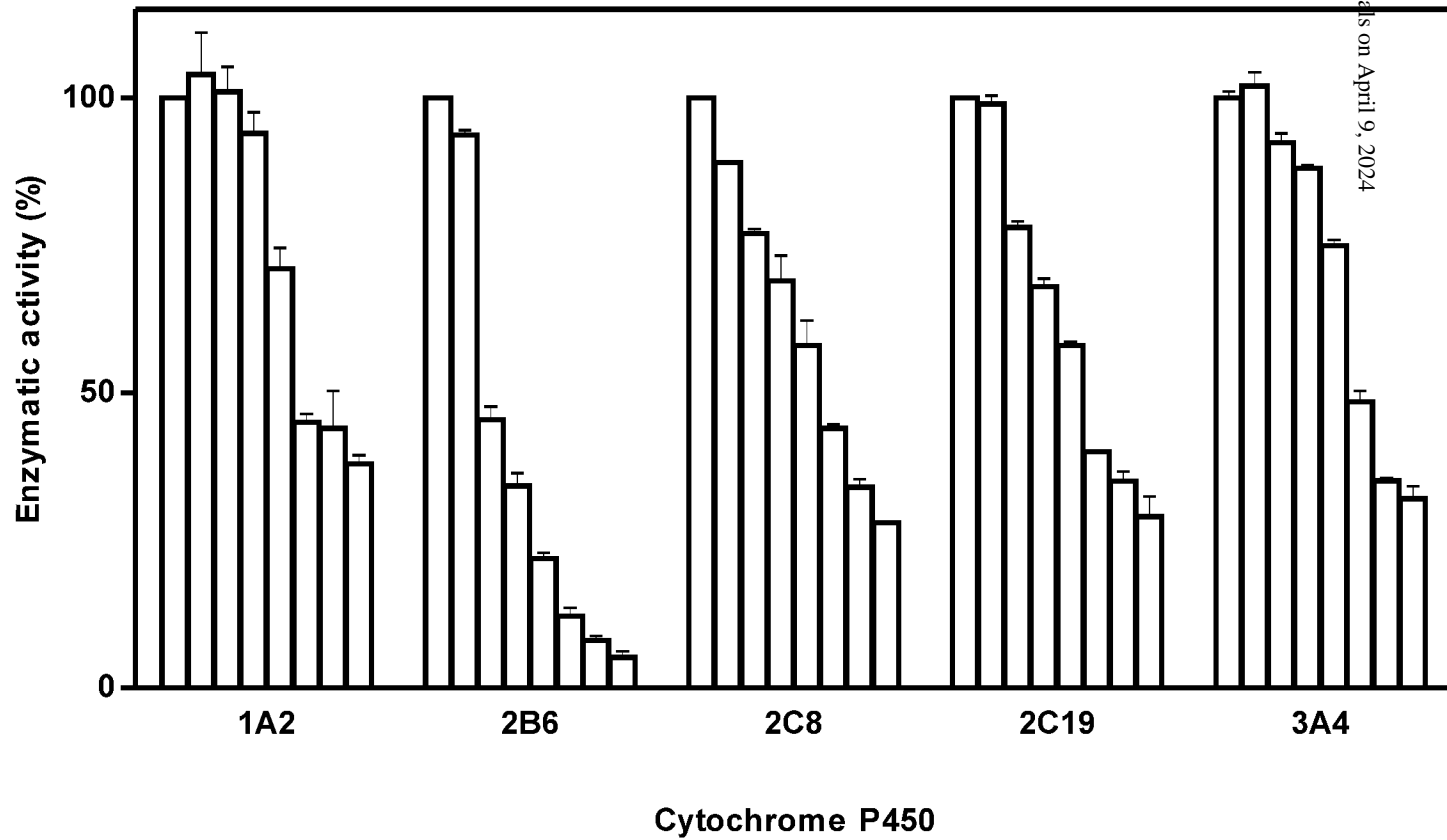
Figure 3

Figure 4

Figure 5A
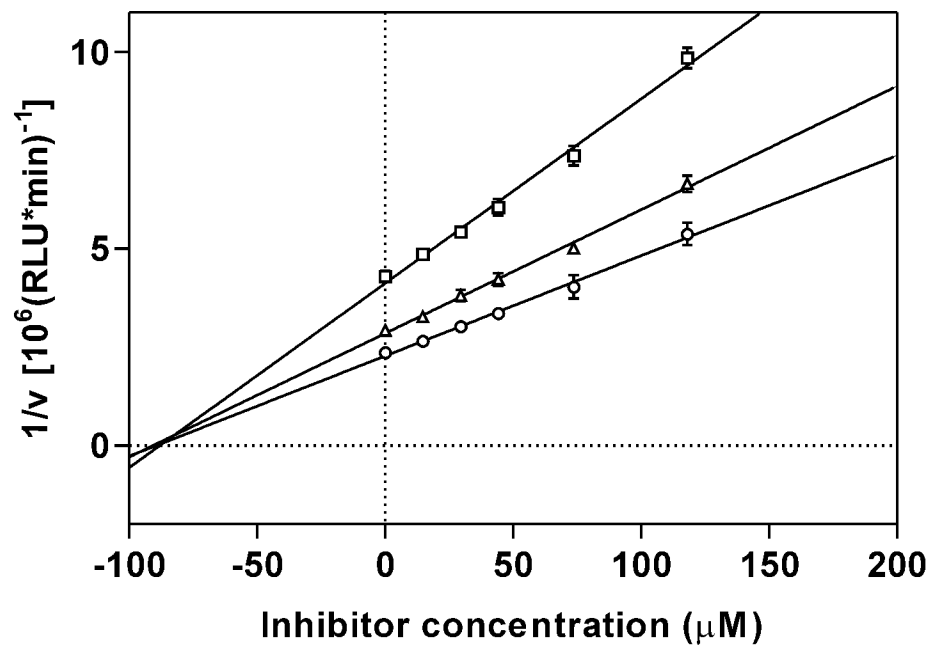
Figure 5B

Figure 6A

Figure 6B