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Reversible inhibition of four important human liver cytochrome P450 enzymes by diethylstilbestrol

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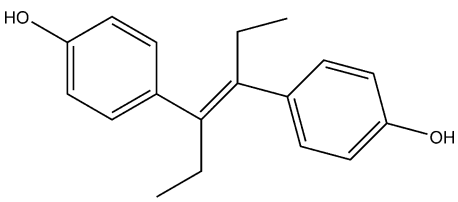
Diethylstilbestrol (DES), a synthetic estrogen clinically used to treat threatened abortion between the 1940 s and the 1970 s, has been restricted to treat certain cases of prostatic and breast cancer due to its adverse drug responses such as teratogenicity and carcinogenicity. Some reports have demonstrated that the addition of DES to docetaxel could modify tubulin composition and improve response of prostate cancer to chemotherapy. Given that DES might be co-administered with other drugs such as docetaxel, the present study focused on CYP-based drug-drug interaction (DDI). *In vitro* inhibitory effects of DES on CYP isoforms were investigated, and the results showed that DES could competitively inhibit CYP3A4, CYP2C8, CYP2C9 and CYP2E1. The inhibition constants (K_i) were calculated to be 4.4 μM, 3.0 μM, 5.0 μM and 8.0 μM for CYP3A4, CYP2C9, CYP2E1 and CYP2C8, respectively. Based on peak serum DES level after drip influsion of 500 mg of fosfestrol (DES diphosphate) in patients, [I]/K_i was calculated to be 4.3, 6.2, 3.7 and 2.3 for CYP3A4, CYP2C9, CYP2E1 and CYP2C8, which suggested that DES was likely to induce *in vivo* DDI through inhibition of these four major CYP isoforms. These results collectively demonstrate that adverse drug responses might exist when DES is co-administered with other drugs.

1. Introduction

Diethylstilbestrol (DES) is a synthetic estrogen clinically used to treat threatened abortion during the time between the 1940 s and 1970 s. In the early 1950 s, DES was also approved as a growth promoter for livestock, given either as a feed additive or as an implant (McMartin et al. 1978). In contrast to its wide utilization, toxicities associated with DES have been demonstrated, including teratogenicity (Nomura et al. 1977) and carcinogenicity (Gardner et al. 1944). Therefore, controversies existed when referring to the utilization of DES. Nowadays, the use of DES has been restricted to certain cases of prostatic and breast cancer (Smith et al. 1998; Peethambaram et al. 1999), and it is locally applied for postmenopausal estrogen deficiency. A previous phase II clinical trial has demonstrated that the addition of DES to docetaxel could modify tubulin composition and improve the response of prostate cancer to chemotherapy (Montgomery et al. 2007).



Diethylstilbestrol

Cytochrome P450 s (CYPs), a superfamily of heam-containing isoenzymes located primarily in hepatocytes, are major enzymes involved in phase I metabolic reactions of drugs (Guengerich 2006). Inhibition of CYP isoforms might influence the elimination of drugs and induce serious adverse drug response (Purnapatre et al. 2008). Previously reported cases have demonstrated that inhibition of CYPs by drugs have induced many clinical drug-drug interactions (DDI), including noscapine (Fang et al. 2010) and MPA (Zhang et al. 2006). In order to reduce attrition rate in drug development, many *in vitro* systems have been established to evaluate the CYPs' inhibitory potential of drug, including human liver microsomes and recombinant CYP isoforms (Ito et al. 1998).

Given that DES might be used in localization with other drugs such as docetaxel, the objectives of the study described herein are 1) to evaluate the inhibitory potential of DES on five major human CYP isoforms *in vitro* using a microsomal incubation system, including CYP3A4, CYP2C8, CYP2C9, CYP2E1 and CYP1A2, 2) to investigate the inhibitory type and kinetic parameters of DES towards these five CYP isoforms, and 3) to predict *in vivo* the DDI magnitude using *in vitro* data.

2. Investigations and results

As shown in Fig. 1, all positive inhibitors significantly inhibited corresponding probe substrate reactions with more than 80% of the control activity inhibited. 100 μM DES strongly inhib-

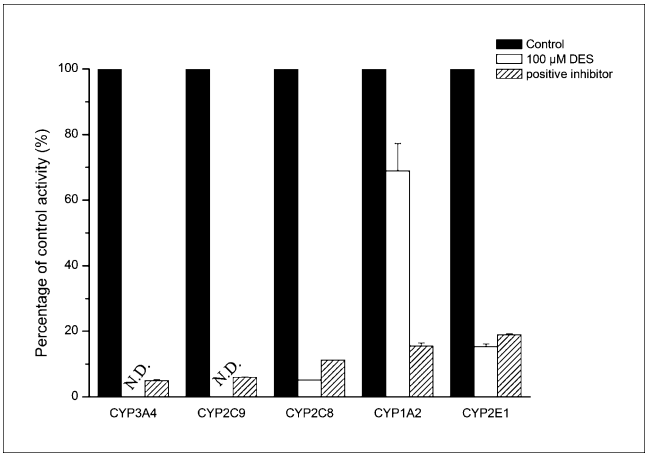


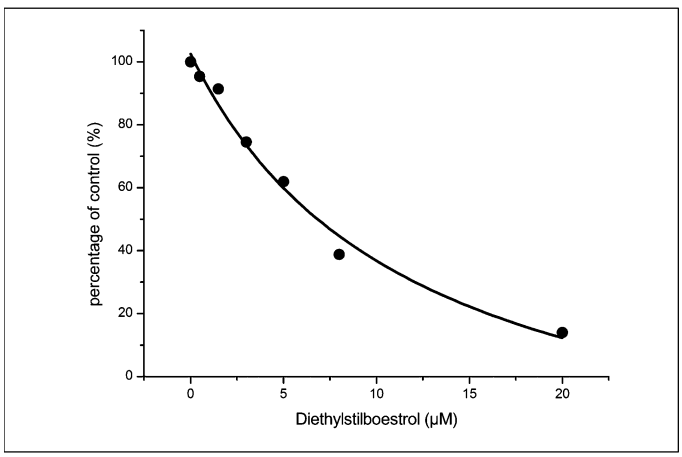
Fig. 1: Inhibition of five major CYP isoforms by DES (100 μM). Incubation conditions were described in Materials and Methods

ited the activity of CYP3A4, CYP2C9, CYP2E1, and CYP2C8, with the activity of CYP1A2 negligibly influenced. Furthermore, kinetic analysis was carried out for CYP3A4, CYP2C9, CYP2E1 and CYP2C8, whose activities were inhibited by more than 50%. Lineweaver-Burk and Dixon plots showed that the inhibition of CYP3A4, CYP2C9, CYP2E1 and CYP2C8 by DES was all best fit to a competitive way (Figs. 2–5). K_i value was calculated from second plot of the slopes from Lineweaver-Burk plots vs. the concentrations of DES. The results showed that K_i values were 4.4 μM, 3.0 μM, 5.0 μM and 8.0 μM for CYP3A4, CYP2C9, CYP2E1 and CYP2C8, respectively. Time- and NADPH-dependent inhibitions were also evaluated and the results demonstrated that the inhibition of all tested CYP isoforms by DES was increased by less than 15% (data not shown) in a single-point inactivation experiment, which suggested that there was no mechanism-based inhibition of CYP isoforms by DES.

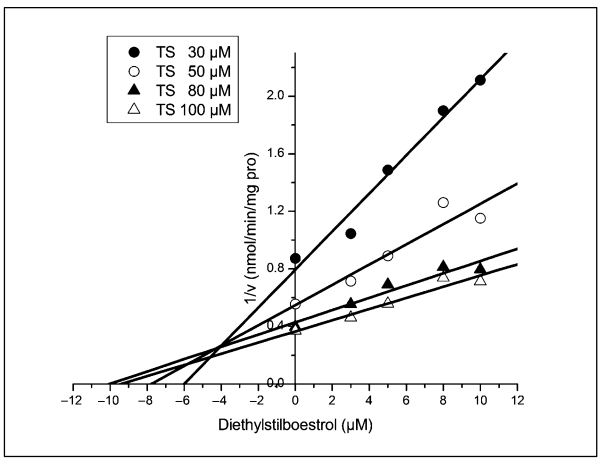
Peak serum DES level could reach approximately 5 μg/ml (18.7 μM) after a drip influsion of 500 mg of fosfestrol (DES diphosphate) in patients (Nakamura et al. 1986). Based on this *in vivo* concentration of DES, the value of $[I]/K_i$ was calculated to be 4.3, 6.2, 3.7 and 2.3 for CYP3A4, CYP2C9, CYP2E1 and CYP2C8, respectively.

3. Discussion

CYPs are actively involved in the elimination of many drugs (Soars et al. 2007) and CYP inhibition has been implicated in a majority of reported clinically relevant drug-drug interactions (Fang et al. 2010; Zhang et al. 2006). A good example is the DDI between ketoconazole or itraconazole (strong CYP3A4 inhibitors) and triazolam (a CYP3A4 substrate), in which the exposure to triazolam increased 22- or 27-fold following co-administration of ketoconazole or itraconazole, respectively (Varhe et al. 1994). Elevation of plasma drug concentrations could result in serious adverse drug responses (ADRs) for drugs with narrow therapeutic index.



(A)



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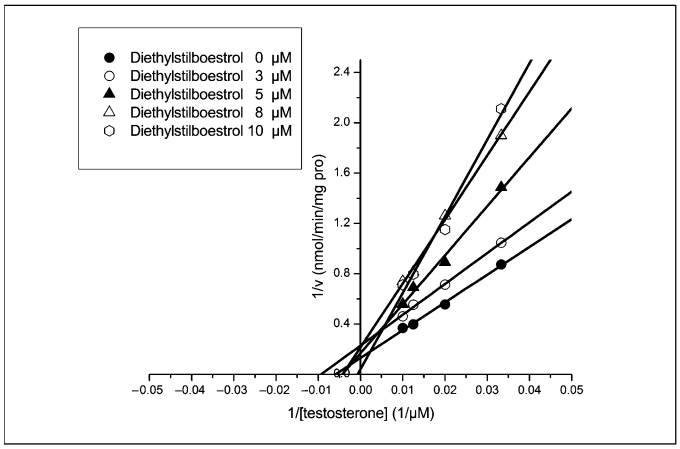
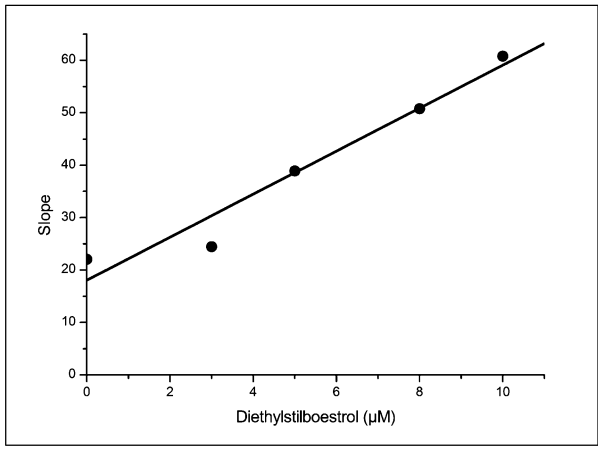


(C)



(D)

Fig. 2: A: Inhibitory effect of DES on testosterone (TS) 6β-hydroxylation activity (CYP3A4). B:Dixon plot of inhibitory effect of DES on testosterone 6β-hydroxylation activity (CYP3A4). C: Lineweaver-Burk plot of inhibitory effect of DES on testosterone 6β-hydroxylation activity (CYP3A4). D: Second plot of slopes from Lineweaver-Burk plot versus DES concentrations

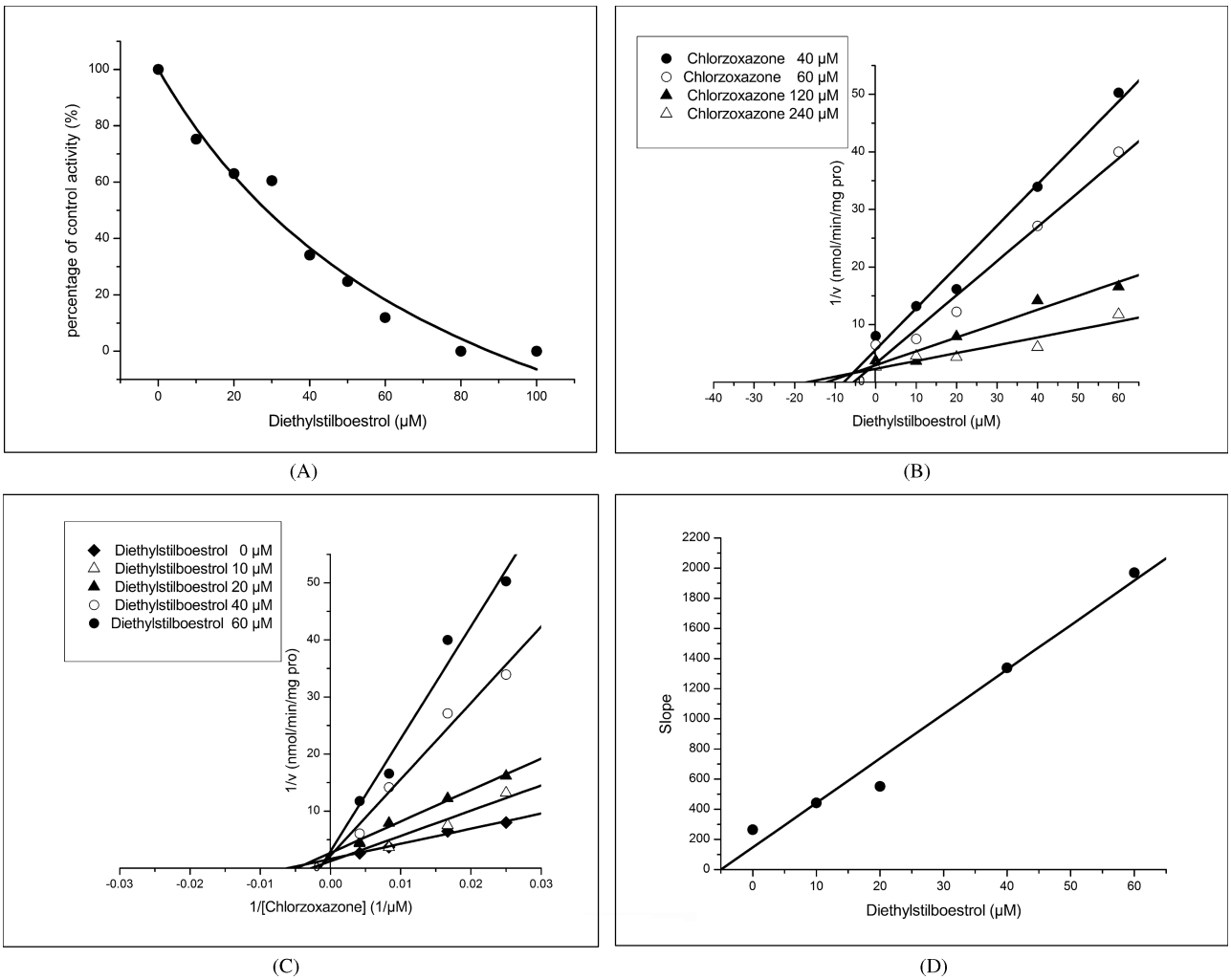


Fig. 3: A: Inhibitory effect of DES on chlorzoxazone 6-hydroxylation activity (CYP2E1). B:Dixon plot of inhibitory effect of DES on chlorzoxazone 6-hydroxylation activity (CYP2E1). C: Lineweaver-Burk plot of inhibitory effect of DES on chlorzoxazone 6-hydroxylation activity (CYP2E1). D: Second plot of slopes from Lineweaver-Burk plot versus DES concentrations

Adverse drug responses (ADR) associated with DES have restricted its utilization (Nomura et al. 1977; Gardner et al. 1944). However, this drug remains to be clinically employed to treat prostate and breast cancer alone or in combination with other anticancer agents such as docetaxel. Considering possible adverse drug responses caused by CYP inhibition by DES, inhibition of five major CYP isoforms was investigated *in vitro* using a human liver microsomal incubation system. The results demonstrated that DES could strongly inhibit the activities of CYP3A4, CYP2C8, CYP2C9 and CYP2E1 and the inhibition type all belongs to competitive inhibition. The reversible inhibition constant was calculated to be 4.4 μM, 3.0 μM, 5.0 μM and 8.0 μM for CYP3A4, CYP2C9, CYP2E1 and CYP2C8, respectively.

To predict the *in vivo* DDI magnitude from *in vitro* data, the peak serum DES level after a drip influsion of 500 mg of fosfestrol (DES diphosphate) in patients was adopted. According to the ratio of $[I]/K_i$ ($[I]/K_i > 1$), DES was likely to induce DDI via inhibition of CYP3A4, CYP2C8, CYP2C9 and CYP2E1. Inhibition of these CYP isoforms should be given enough attention because they were known to metabolize many drugs with narrow therapeutic index, such as docetaxel, paclitaxel, warfarin and phenytoin (Zhang et al. 2009; Kaminsky et al. 1997).

Taken together, our present study demonstrated that DES showed strong inhibitory effests towards CYP3A4, CYP2C9, CYP2C8 and CYP2E1, with CYP1A2 negligibly influenced. DES is likely to induce *in vivo* DDI via the inhibition of these four major CYP isoforms. Several other CYP isoforms such as CYP2D6, CYP2A6, CYP2C19 and CYP2B6 which have not been included in the present work will be investigated soon.

4. Experimental

4.1. Chemicals

DES (purity ≥ 99%), glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate, corticosterone, $NADP^+$, phenacetin, acetaminophen, 4′-hydroxydiclofenac, sulfaphenazole, 8-methoxypsoralen, chlorzoxazone, 6-hydroxychlorzoxazone, paclitaxel, 6β-hydroxytestosterone, clomethiazole and furafylline were obtained from Sigma-Aldrich (St. Louis, Miss., USA). Testosterone was purchased from Acros Organics (Morris Plains, N. J., USA). Montelukast was obtained from Beijing Aleznova Pharmaceutical (Beijing, China). Diclofenac and ketoconazole were from ICN Biomedicals (Aurora, Ohio, USA). All other reagents were of the highest purity commercially available or HPLC grade.

4.2. Human liver microsomes (HLMs)

Human liver samples were obtained from Dalian Medical University (Dalian, Liaoning province, China) with the approval of the local ethics committee at the university. Any information on the medication history of the autopsy samples is not gained. A panel of human liver microsomes (HLMs) was prepared from twelve liver samples obtained from male and female patients by differential ultracentrifugation as described previously

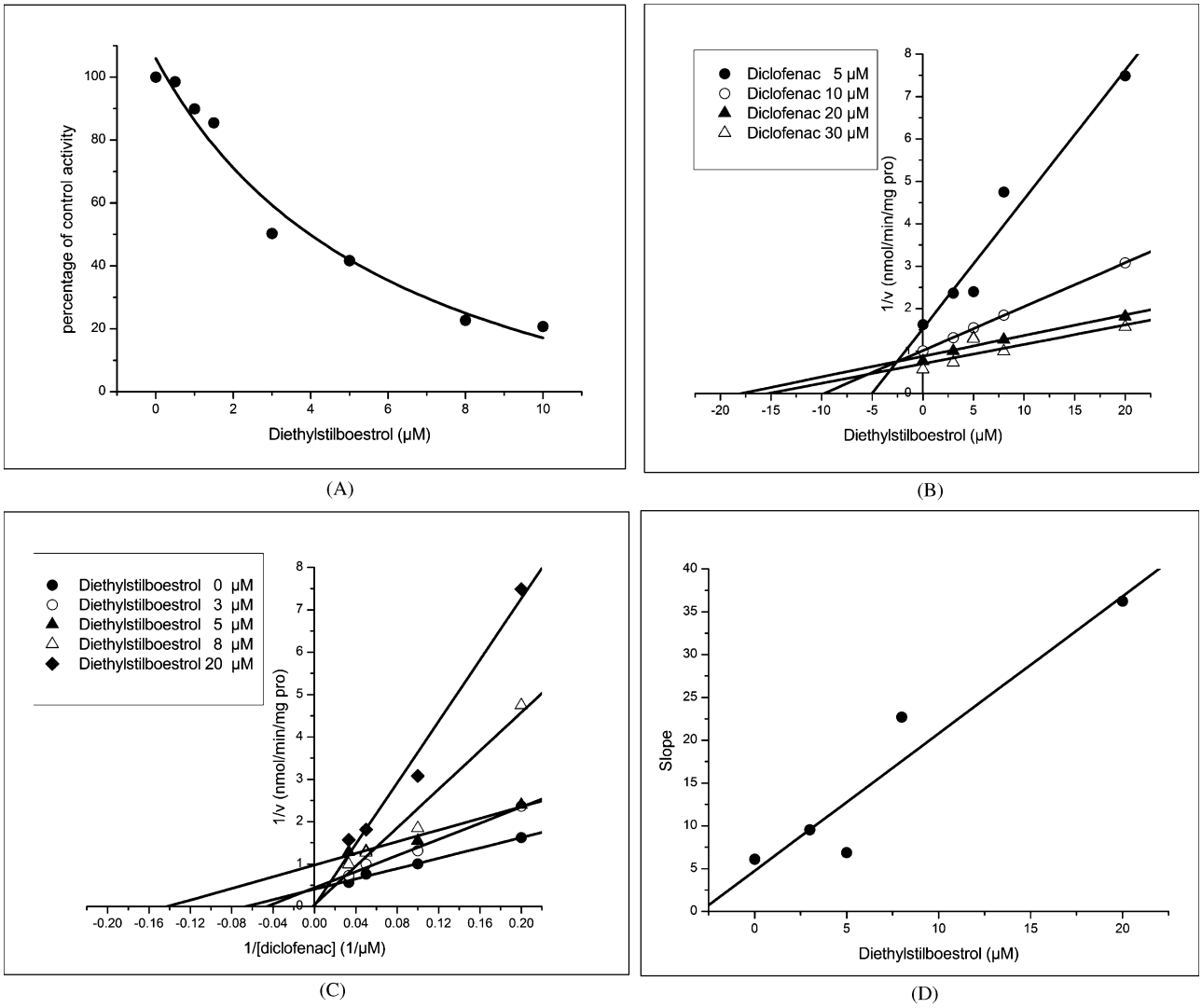


Fig. 4: A: Inhibitory effect of DES on diclofenac 4'-hydroxylation (CYP2C9). B:Dixon plot of inhibitory effect of DES on diclofenac 4'-hydroxylation (CYP2C9). C: Lineweaver-Burk plot of inhibitory effect of DES pm diclofenac 4'-hydroxylation (CYP2C9). D: Second plot of slopes from Lineweaver-Burk plot versus DES concentrations

(Zhang et al. 2009). Microsomal protein concentrations were determined by the Lowry method with bovine serum albumin as standard (Lowry et al. 1951). Total CYP concentration was determined according to Omura & Sato with the use of molar extinction coefficient 91 mM^{-1} cm^{-1} (Omura et al. 1964). Pooled human microsomes (n = 12) were used to reflect the average CYP activities in humans.

4.3. CYP probe substrate reactions

Human liver microsomal phenacetin O-deethylation, diclofenac 4'-hydroxylation, paclitaxel 6a-hydroxylation, chlorzoxazone 6-hydroxylation and testosterone 6β-hydroxylation activities were used as selective markers for CYP1A2, CYP2C9, CYP2C8, CYP2E1 and CYP3A4 as previously reported (Fang et al. 2010).

4.4. Enzyme inhibition experiments

To investigate the inhibitory effects of DES on five different human CYP isoforms (CYP1A2, CYP2C8, CYP2C9, CYP2E1, CYP3A4), marker assays for each CYP isoform were performed in the presence of 100 μM DES. The concentrations of positive inhibitors used were as previously reported (Fang et al. 2010). For CYP isoforms that were strongly inhibited, IC_{50} values were determined using various concentrations of DES. To determine the K_i value for CYP3A4, 2C8, 2E1 and 2C9, various concentrations of DES was added to the reaction mixture containing different concentrations of probe substrate (30, 50, 80, 100 μM testosterone for CYP3A4; 5, 10, 20, 50 μM paclitaxel for CYP2C8; 5, 10, 20, 50 μM diclofenac for CYP2C9; 40, 60, 120, 240 μM chlorzoxazone for CYP2E1). Dixon and Lineweaver-Burk plots were employed to evaluate the reversible inhibitory type, and second plot of slopes from Lineweaver-Burk plot over the concentrations of DES was utilized to calculate K_i value.

To investigate whether DES inhibits CYP isoforms in a mechanism-based manner, single-point inactivation experiments were used to determine time- and NADPH- dependent inhibition as previously reported. For CYP3A4, 2C8, 2E1 and 2C9, the concentration of DES utilized was 10-fold that gave 25% inhibition under reversible inhibition situations. For CYP1A2, 50 μM of DES tiliroside was selected. 1 mg/ml of pool HLMs were utilized to pre-incubate with DES tiliroside. After incubation, an aliquot (20 μl) was transferred to another tube (final volume 200 μl) containing an NADPH-generating system and probe substrates which concentration was proximal to K_m values. Further incubations were performed to measure residual activity.

4.5. Prediction of in vivo DDI magnitude from in vitro data

Eq. (1) was employed to predict *in vivo* DDI potential caused by inhibition of CYPs by DES (Bachmann et al. 2005).

$$\frac{AUC_i}{AUC} = 1 + \frac{[I]}{K_i} \quad (1)$$

The terms are defined as follows: AUC_i/AUC is the predicted ratio of *in vivo* exposure of victim drugs with coadministration of DES curcumenol versus that in control situation, K_i is the reversible inhibition constant of CYP3A4 and [I] is *in vivo* concentration of DES. Based on the ration of $[I]/K_i$, DDI potential is divided into three categories: 1) likely, $[I]/Ki > 1$; 2) possible, $0.1 < [I]/Ki < 1$; 3) remote, $[I]/Ki < 0.1$ (Tucker et al. 2001).

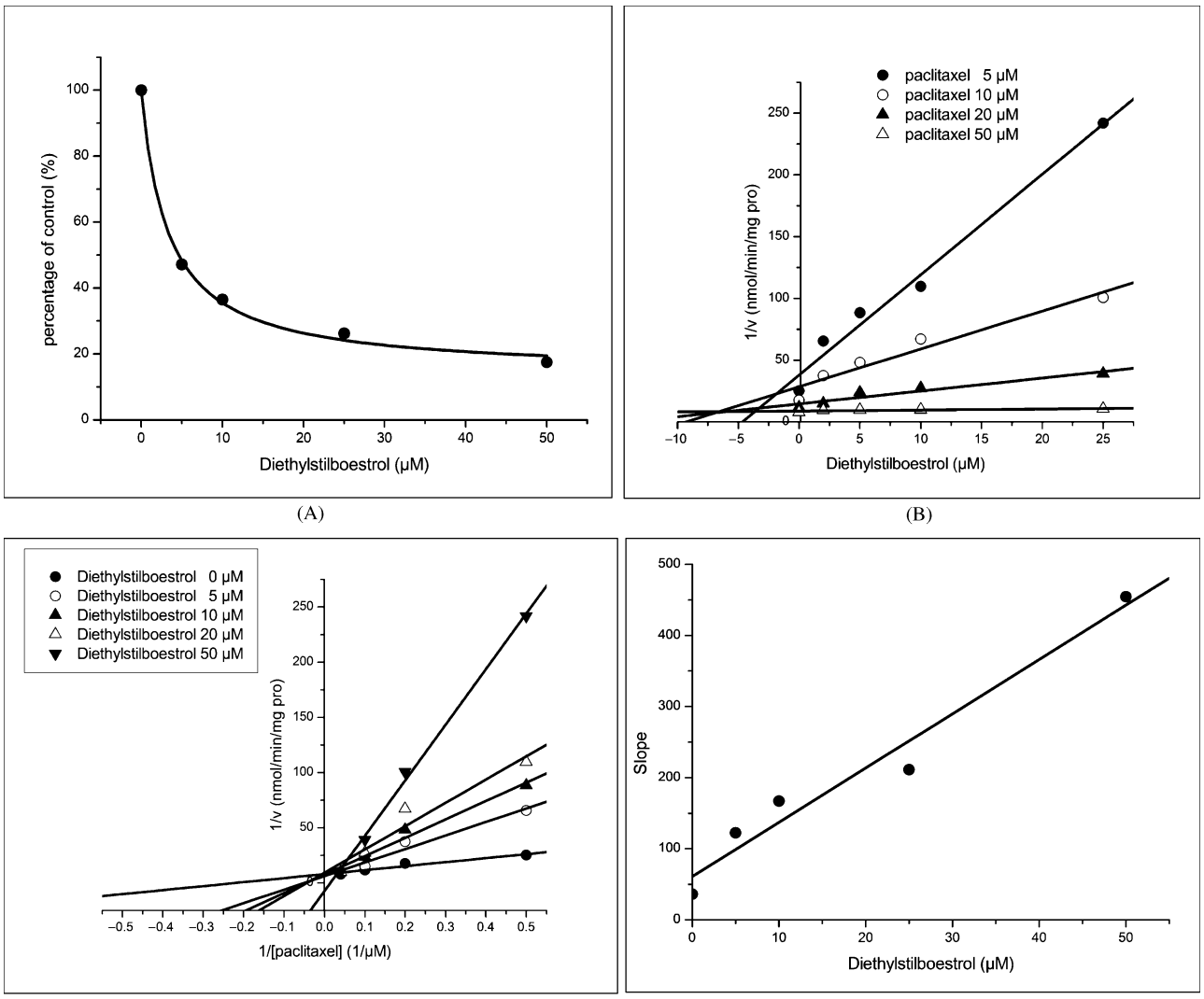


Fig. 5: A: Inhibitory effect of DES on paclitaxel 6a-hydroxlation activity (CYP2C8). B: Dixon plot of inhibitory effect of DES on paclitaxel 6a-hydroxlation activity (CYP2C8). C: Lineweaver-Burk plot of inhibitory effect of DES on paclitaxel 6a-hydroxlation activity (CYP2C8). D: Second plot of slopes from Lineweaver-Burk plot versus DES concentrations

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