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Effects of repeated allopurinol administration on rat cytochrome P450 activity

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Allopurinol is a popular and widely-prescribed anti-hyperuricemic agent that has been implicated in drug interactions with substrates of several cytochrome P450 (CYP) enzymes. The effect of repeated allopurinol administration (20 mg/kg, once daily for 14 days) on metabolic activity of CYP was assessed in rats. This was a randomized, double-blind, two-way crossover study with a 4-week washout period between phases. The substrates used in this study were phenacetin (CYP1A2), tolbutamide (CYP2C9), omeprazole (CYP2C19) and dextromethorphan (CYP2D6). Validated HPLC-MS/MS was used to quantify all compounds. Our study showed that allopurinol administration inhibited CYP1A2 activity, causing a significant increase in $AUC_{(0\text{-}\infty)}$ ($P<0.01$) and $t_{1/2}$($P<0.05$) of phenacetin, and a distinct decline in CL($P<0.01$). However, there were no significant differences of another three probe drugs in plasma concentrations and the corresponding pharmacokinetic parameters between the allopurinol-treated and normal saline-treated rats. The findings in this study suggested that allopurinol could inhibit CYP1A2 but did not influence CYP2C9, CYP2C19 and CYP2D6 enzymes.

1. Introduction

The Cytochrome P450 (CYP) superfamily, one of the most important drug-metabolizing enzyme systems in humans, is responsible for the oxidative metabolism of numerous xenobiotic substances as well as endogenous substrates (Kim and Novak 2007). It is also generally conceded that over 90% of drug metabolism in humans are mediated by CYP enzymes. Specific probe drugs have been widely used for evaluating various individual CYP enzymes activities (Streetman et al. 2000). Prior to more definitive single-probe substrate studies, a "cocktail" approach has been widely used *in vitro* (Gomez-Lechon et al. 2012; Lee and Kim 2011; Rhodes et al. 2011) but also *in vivo* (Ardjomand-Woelkart et al. 2011; Flaherty et al. 2011; Han et al. 2012; Zadoyan et al. 2012). As is well known, two greatest advantages of this so-called "cocktail" approach are predicting the effect of a New Chemical Entity (NCE) on the CYP activities in a single experiment (Tanaka et al. 2003) and minimizing the inter- and intra-subject variability (Breimer 1983), respectively.

Gout is a common disease, affecting at least 1% of human in the West (Kramer and Curhan 2002). Allopurinol (1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one), a competitive inhibitor of the enzyme xanthine oxidase at low doses and a noncompetitive inhibitor at high levels, is an effective and widely-prescribed anti-hyperuricemic agent (Terkeltaub 2003). Recently, allopurinol is not only known for its ability to treat gout and hyperuricemia, but has also an effect on cardiovascular diseases (Kelkar et al. 2011). To date, no study has investigated the effect of allopurinol on the activity of the principal CYP enzymes involved in drug metabolism.

In this study, phenacetin (PHE), tolbutamide (TB), omeprazole (OME), dextromethorphan (DXM), respectively, as probe drugs of CYP1A2, CYP2C9, CYP2C19, CYP2D6 for profiling the Phase I metabolism were employed to evaluate the potential effect of allopurinol on the status of hepatic oxidative metabolism in rats.

2. Investigations and results

2.1. *Validation of the cocktail approach*

Under the conditions described in the experimental section, a reliable liquid chromatography-mass spectrometry has been developed for simultaneous evaluation of the activities of four CYP enzymes (CYP1A2, CYP2C9, CYP2C19 and CYP2D6) in rats. The ion transitions monitored were as follows: m/z 180 for PHE, m/z 271 for TB, m/z 346 for OME, m/z 272 for DXM and m/z 237 for IS. The lower limit of quantification for PHE, TB, OME and DXM were all 5.0 ng/mL, and the assay ranges used were all 5.0–1000 ng/mL in rat plasma. The plasma samples with analyte concentration above the upper limit of quantitation were diluted with blank rat plasma. Correlation coefficient of the calibration curves for PHE was 0.9978, for TB 0.9963, for OME 0.9980 and for DXM 0.9976. The intra-day and inter-day CVs for the low-, medium- and high-quality control samples were less than 15%. The result of the chromatographic validation showed that assay methods were suitable for this study.

2.2. *Effects of allopurinol on rat CYP activities*

The primary pharmacokinetic objective of this study was to evaluate the effect of allopurinol on the pharmacokinetics of

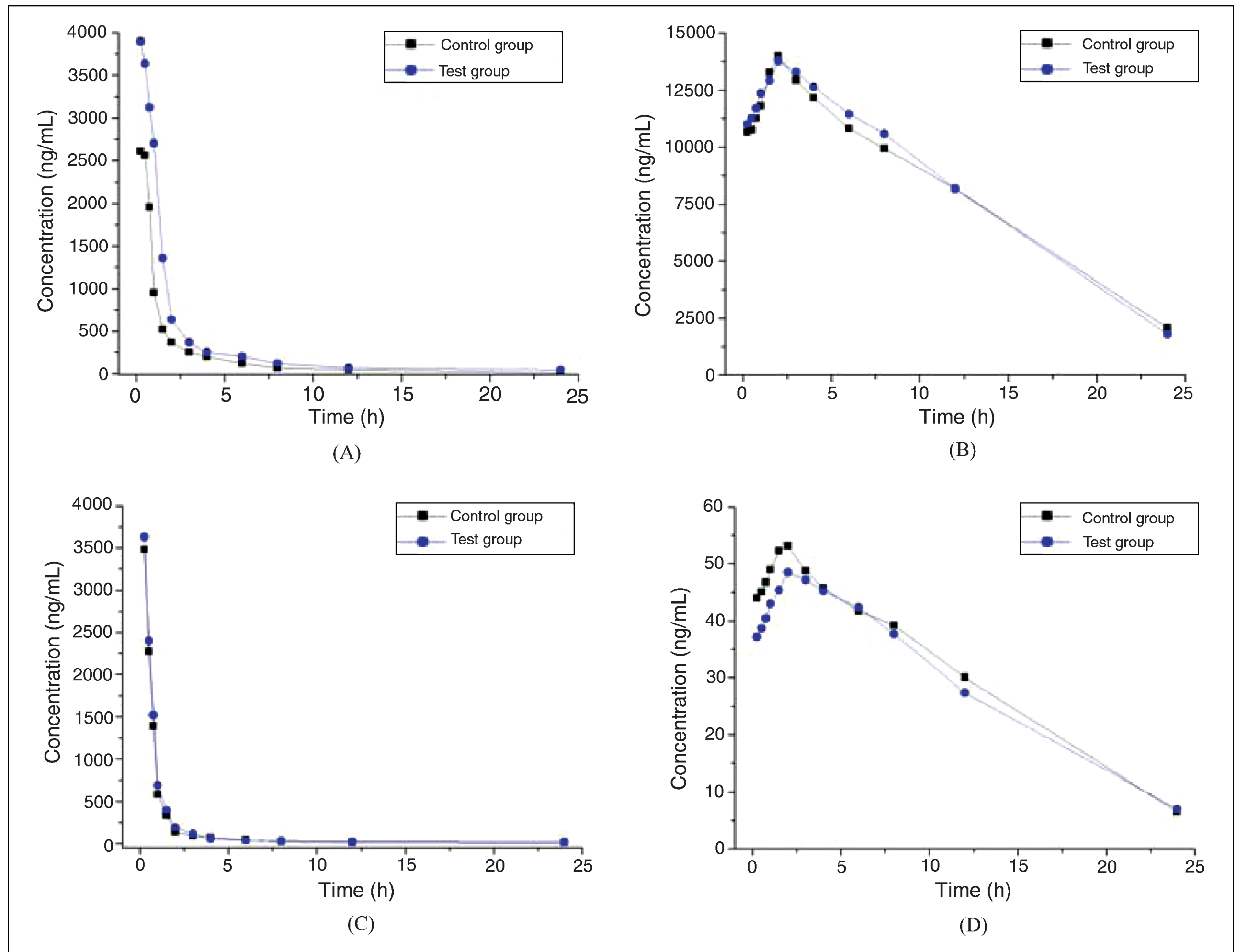

Fig.: Time-concentration curves of the four probe drugs after oral administration with allopurinol 20 mg/kg in rats. A: Phenacetin; B: Tolbutamide; C: Omeprazole; D: Dextromethorphan

PHE, TB, OME and DXM. After blood sample processing, the pharmacokinetics of four probe drugs were simultaneously determined by HPLC-MS/MS. All pharmacokinetic analyses of the four probe drugs were performed on pharmacokinetic data both for the control group (14 days of normal saline treatment) and the test group (14 days of allopurinol treatment). The mean

Table: Effects of allopurinol on pharmacokinetic parameters of the four probe drugs (mean ± SD, n = 14)

Probe Drug	Parameter	Normal saline	Allopurinol
Phenacetin	$AUC_{(0-\infty)}$(μg·h/L)	4128.932 ± 698.329	7781.030 ± 958.621[b]
	C_{max} (ng/mL)	2749.640 ± 935.553	4078.2 ± 881.747[a]
	$t_{1/2}$ (h)	4.975 ± 0.355	10.897 ± 5.955[a]
	T_{max}(h)	0.393 ± 0.197	0.357 ± 0.134
	CL (L/h/kg)	3.741 ± 0.752	1.956 ± 0.268[b]
Tolbutamide	$AUC_{(0-\infty)}$(μg·h/L)	198733.286 ± 33635.258	201655.747 ± 36402.020
	C_{max} (ng/mL)	14697.300 ± 1175.841	14508.072 ± 1040.106
	$t_{1/2}$ (h)	6.397 ± 1.297	5.758 ± 0.896
	T_{max}(h)	2.200 ± 1.095	2.250 ± 0.612
	CL (L/h/kg)	0.015 ± 0.003	0.016 ± 0.003
Omeprazole	$AUC_{(0-\infty)}$(μg·h/L)	2973.148 ± 264.148	2887.644 ± 347.139
	C_{max} (ng/mL)	3542.714 ± 790.558	3525.500 ± 739.000
	$t_{1/2}$ (h)	4.792 ± 1.171	4.328 ± 0.172
	T_{max}(h)	0.286 ± 0.094	0.250 ± 0.086
	CL (L/h/kg)	5.082 ± 0.481	5.259 ± 0.719
Dextromethorphan	$AUC_{(0-\infty)}$(μg·h/L)	784.614 ± 125.755	739.564 ± 133.463
	C_{max} (ng/mL)	55.826 ± 3.523	53.161 ± 2.927
	$t_{1/2}$ (h)	6.296 ± 1.921	6.190 ± 1.545
	T_{max}(h)	1.607 ± 0.453	2.500 ± 0.866
	CL (L/h/kg)	19.590 ± 3.496	20.896 ± 4.038

[a] $P < 0.05$; [b] $P < 0.01$ vs control

plasma concentration-time curves for four probes were illustrated in the Fig. and pharmacokinetic parameters were shown in the Table. As shown in the Fig., the plasma concentrations of PHE were significantly higher in the allopurinol-treated group than in the normal saline-treated control group. The values of the area under the plasma concentration-time curve from 0 time to infinity ($AUC_{(0-\infty)}$) in allopurinol-treated rats were significantly larger for PHE than that in normal saline-treated rats. The values of total clearance (CL) of PHE in test rats were significantly smaller than those in control rats. Allopurinol increased elimination half-time $t_{1/2}$ and $AUC_{(0-\infty)}$ of PHE, and the corresponding decrease in CL suggested that allopurinol could inhibit CYP1A2 in rats at a dose of 20 mg/kg every day for 14 days. The pharmacokinetic parameters ($t_{1/2}$, C_{max}, $AUC_{(0-\infty)}$, and CL) of another three probe drugs in the allopurinol-treated rats showed no statistically significant difference from the normal saline-treated control rats. This demonstrated that allopurinol did not influence CYP2C9, CYP2C19 and CYP2D6.

3. Discussion

Allopurinol, an inhibitor of xanthine oxidase, is widely used for the treatment of hyperuricemia associated with chronic gout, acute uric acid nephropathy, recurrent uric acid stone formation, certain enzyme/blood disorders, and cancer chemotherapy (Chohan and Becker 2009). A recent multinational case-control study showed that allopurinol is the most common cause of Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) in Europe, Israel (Halevy et al. 2008), and in some Asian countries (Lee et al. 2008; Tassaneeyakul et al. 2009). Given that 5% of the general population has hyperuricemia, there is a significant risk of allopurinol-induced severe cutaneous adverse reactions (SCARs) (Shalom et al. 2008). It is increasingly important to elucidate the potential effect of drugs on the CYP activities so that they can be used safely and effectively. Hence, in the present study, we investigated the effect of allopurinol on rat CYP1A2, CYP2C9, CYP2C19 and CYP2D6 activity. Phenotyping for drug-metabolizing enzymes is defined as measuring its actual activity in an individual and is performed by administering a special substrate for this enzyme and subsequently determining appropriate pharmacokinetic parameters closely reflecting enzyme activity (Fuhr et al. 2007). Compared to the individual administration of specific probes in multiple studies, simultaneous administration of multiple probes of CYP enzymes offers several advantages. The most obvious advantage is to minimize the confounding influence of inter-individual and intra-individual variability. Therefore, a new four-drug cocktail including PHE, TB, OME and DXM to study the influence of natural products and new chemical drugs on the metabolizing activity of CYP1A2, CYP2C9, CYP2C19 and CYP2D6, respectively, had been established in our laboratory. As far as is known, the probe drugs within a cocktail should not interfere with metabolism of other drugs in the cocktail and may be considered as best validated (Turpault et al., 2009).
In our present study, the potential effect of allopurinol on CYP activities including CYP1A2, CYP2C9, CYP2C19 and CYP2D6 in rats were assessed. The study is also providing the opportunity to help us to find the metabolites of allopurinol. In our study, $AUC_{(0-\infty)}$ ($P < 0.01$) and $t_{1/2}$($P < 0.05$) of PHE significantly increased, and the corresponding CL markedly decreased ($P < 0.01$), which illustrated that the activity of CYP1A2 tended to be inhibited. The complicated mechanism of this effect of allopurinol on the activity of CYP1A2 remains to be further studied. Another possibility is that the concentration of allopurinol in our study might not be high enough to induce the activity of CYP1A2. The study focusing on the effect of allopurinol on the CYP enzymes *in vitro* is so critical that it helps us to make the mechanism of the metabolism of allopurinol understood completely. So, *in vitro* study of allopurinol impacts on CYP1A2 activity needs to be discussed in the future. In addition, the dose dependency and kinetics of allopurinol on the activity of CYP1A2 *in vitro* and *in vivo* also remain to be studied. However, we find that allopurinol did not influence the plasma concentration of another three probe drugs (TB, OME and DXM) as well as their associated pharmacokinetic parameters, and had no affect on the CYP activities of CYP2C9, CYP2C19 and CYP2D6. In previous research, allopurinol could potentially inhibit CYP2C9 (Wang et al. 2009). But our present study showed that allopurinol had no or little effect on CYP2C9, which suggested that our *in vivo* study result was not in good agreement with above result. It is well known that a major contributing factor of the drug-drug interaction is the inhibition of CYP enzyme-mediated activities, of which human CYP1A2 accounts for about 13% of the total CYP content in human liver (Shimada et al. 1994). CYP1A2 is mainly responsible for metabolizing a variety of clinically important drugs, such as clozapine (Bertilsson et al. 1994), ropivacaine (Oda et al. 1995), olanzapine (Ring et al. 1996) and theophylline (Ha et al. 1995). In addition to this, CYP1A2 also metabolizes a number of procarcinogens and endogenous substrates (Zhou et al. 2009). People should pay more attention to the side effects of allopurinol caused by drug-drug interactions when administered with other drugs, especially with substrates of CYP1A2.
In conclusion, the inconspicuous effects of allopurinol *in vivo* on probes of CYP2C9, CYP2C19 and CYP2D6 metabolism suggest that there are no clinically relevant drug-drug interactions between the drugs metabolized by these enzymes and allopurinol when used concomitantly. However, from our present results, we cannot exclude that comedication of allopurinol with drugs metabolized by human CYP1A2 may inhibit metabolism of these drugs and increase plasma concentrations of these drugs, which will result in relevant drug-drug interactions. Further clinical studies are required to fully assess the safety of allopurinol in terms of CYP1A2 inhibition.

4. Experimental

4.1. Chemicals and reagents

PHE (purity > 98.0%), TB (purity > 98.0%), OME (purity > 98.0%), DXM (purity > 98.0%) and the internal standard carbamazepine (IS, purity > 98.0%) were purchased from Sigma-Aldrich Company (St. Louis, USA). HPLC grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). All other chemicals were of analytical grade and used without further purification. Ultra-pure water (resistance > 18.2 mΩ) prepared by a Millipore Milli-Q purification system (Bedford, USA).
Stock solutions of 1.0 mg/mL each of PHE, TB, OME, DXM and IS were prepared in methanol. The working standard solutions of each analyte were prepared by serial dilution of the stock solution with methanol. And IS was diluted to 500 ng/mL in acetonitrile as the precipitant.

4.2. Animals

Fourteen male Sprague-Dawley rats with body weights of 220 ± 30 g were provided by the Animal Care and Use Committee of Wenzhou Medical College. They were housed in cages at 23–25 °C and allowed free access to regular rodent diet and water. After the 1-week acclimatization period, the rats were used for experiments and all efforts were made to minimize any animal suffering. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals.

4.3. Apparatus and chromatographic conditions

All analysis was performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software.

Chromatographic separation was achieved on a 150 mm × 2.1 mm, 3.5 μm particle, Agilent Zorbax SB-C18 column at 30 °C. A gradient elution programme was conducted for chromatographic separation with mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) as follows: 0–1.5 min (10–85% B), 1.5–6.0 min (85–85% B), 6.0–7.0 min (85–10% B), 7.0–10.0 min (10–10% B). The flow rate was 0.4 mL/min.
The quantification was performed by the peak-area method. The determination of target ions were performed in SIM mode (m/z 180 for PHE, m/z 271 for TB, m/z 346 for OME, m/z 272 for DXM and m/z 237 for IS) and positive ion electrospray ionization interface. Drying gas flow was set to 6 L/min and temperature to 350 °C. Nebuliser pressure and capillary voltage of the system were adjusted to 20 psi and 3,500 V, respectively.

4.4. Sample preparation

In a 1.5 mL centrifuge tube, aliquot of 0.2 mL acetonitrile with carbamazepine (500 ng/mL) as the internal standard was added to 0.1 mL of collected plasma sample. After the tube was vortex-mixed for 1.0 min, the sample was centrifuged at 13,000 rmp for 10 min. Next, the supernatant (10 μL) was injected into the HPLC-MS/MS system for analysis. The standards were prepared in the same way.

4.5. Effects of allopurinol on the CYP activities

This was a randomized, double-blind, two-way crossover study with a 4-week washout period between phases. In each phase 14 male SD rats were administered by gastric irrigation with normal saline or allopurinol for 14 days. A dose of allopurinol was 20 mg/kg for the allopurinol group and normal saline was given in the same way for the control group. On the 15th day, after an overnight fast, rats of the two groups simultaneously received an oral dose of 15, 3, 15 and 15 mg/kg for PHE, TB, OME and DXM, respectively.
Blood samples (0.15–0.2 mL) were collected through the tail vein into heparinized 1.5 mL polythene tubes before (0 min) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h after probe drugs administration and immediately separated by centrifugation at 13,000 rpm for 10 min to obtain plasma. The total volume of blood taken from each animal did not exceed 2.2 mL. 100 μL plasma samples were transferred to another tube and stored frozen at −80 °C until analyzed.

4.6. Statistical analysis

The concentration-time profile of each probe drug was analyzed by DAS software (Version 3.0, Wenzhou Medical College, China) and statistic analyses were tested by t-test using SPSS (Version 13.0, Wenzhou Medical College, China). A value of $P < 0.05$ was considered to be statistically significant.

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