IN VITRO STUDY OF THE INHIBITION AND INDUCTION OF HUMAN CYTOCHROMES P450 BY CRYSTALLINE GLUCOSAMINE SULFATE

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SUMMARY

The induction and inhibition of human hepatic cytochrome P450 (CYP) isoforms by crystalline glucosamine sulfate (CGS) was investigated *in vitro*. Inhibition of CYP1A2, CYP2E1, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 by CGS was assessed using recombinant human enzymes incubated with CGS (up to 3 mM expressed as free base). Induction of CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4 by CGS (0.01, 0.3 and 3 mM) was evaluated in cryopreserved human hepatocytes, by determining CYP mRNA expression using quantitative RT-PCR. CGS produced no inhibition or induction of any the CYP enzymes tested at concentrations hundred folds higher than the steady state peak plasma concentrations (approximately 10 μM) observed in man after therapeutic doses of CGS of 1,500 mg once a day. Therefore, no clinically relevant metabolic interactions are expected between CGS and co-administered drugs that are substrates of the CYP enzymes investigated.

KEY WORDS

crystalline glucosamine sulfate, human P450 induction, human P450 inhibition, drug-drug interactions

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INTRODUCTION

Osteoarthritis (OA), previously called degenerative joint disease, is most often characterized by the insidious progressive destruction of articular cartilage and concomitant changes to other articular structures, such as bones, synovium, joint capsules, ligaments, menisci, bursae and peri-articular muscles. It is a complex process which involves biomechanics, accelerated and inadequate tissue repair, and production of inflammatory mediators including degradative enzymes.

The standard classification divides OA into those forms for which the cause is known (secondary OA) and those for which the cause is unknown (primary or idiopathic) /1/.

Several clinical studies have indicated that crystalline glucosamine sulfate (CGS) is effective in controlling knee OA symptoms and disease progression /2-4/. In particular, two randomised, placebo-controlled, double-blind trials of 3 years duration in patients with knee OA showed that this symptom-modifying effect is sustained over long-term treatment courses /2,3/. Moreover, both studies indicated that the drug also has a structure-modifying effect, as assessed by measurement of joint space narrowing using validated techniques on standardized plain radiographs /4-8/. Another, recently completed, trial (the GUIDE study) confirmed the symptomatic results described above and indicated that, at the dose of 1,500 mg/day for six months, crystalline glucosamine sulfate provided an analgesic effect that was significantly superior to that observed after administration of placebo /8/. In the same study and depending on the selected outcome measures, the effect observed after the administration of the currently preferred symptomatic medication in OA (acetaminophen) was not always different from that observed after the administration of placebo /9/.

The *in vivo* metabolism and excretion of CGS has been well investigated. In man, after single bolus intravenous (i.v.) injection of 1,005 mg CGS (628 mg glucosamine) the parent glucosamine disappeared with an apparent half life of 1.11 hours. Investigations with uniformly ^{14}C-labelled glucosamine administered with 502 mg CGS indicated that the disappearance of glucosamine was due to incorporation into plasma globulins. The radioactivity reached a peak after 10 hours and was eliminated with $t_{½}$ of 95 hours /10/. After a single i.v. dose of 502 mg CGS traced with ^{14}C-glucosamine, the

urinary excretion in 120 hours accounted for 29% of the administered dose /10/. After i.v bolus injection of 1,005 mg CGS, the glucosamine urinary excretion determined by ion exchange chromatography was 38% of the administered dose, mostly in the first 8 hours after administration /10/. In addition, in man, a single intramuscular injection of 502 mg CGS traced with ^{14}C-glucosamine gave results similar to those after i.v. administration /10/. Finally, in man, after a single dose of 314 mg CGS traced with ^{14}C-glucosamine, radioactivity appeared in plasma globulins. The peak of radioactivity was reached at the 9th hour after administration and the radioactivity was eliminated with $t_{½}$ of 58 hours /10/. The absolute oral bioavailability evaluated on the globulin-incorporated radioactivity was 44%, whereas fecal excretion in 120 hours was 11.3% of the administered dose, showing that at least 88.7% of the administered dose was absorbed through the gastrointestinal tract /10/. Urinary elimination in man of the parent glucosamine in 24 hours determined by ion exchange chromatography after a single dose of 7.5 g CGS was 1.19% of the administered dose, occurring mostly in the first 8 hours after administration /10/. After administration of 1,884 mg repeated for 7 days the daily urinary excretion of glucosamine increased from 1.60% of the daily dose during the first 24 hours to 2.22% of the daily dose in the last 24 hours. The steady state was reached after the second day /10/.

Knee OA is a chronic disease that affects mainly elderly individuals who are often being treated for other concurrent conditions. Since CGS is marketed in Europe as a prescription drug and thus is usually taken by patients under medical supervision, it is important to evaluate the potential for drug-drug interactions produced by CGS to provide this information to the prescribing physicians, to ensure that the safety and effectiveness of the other medications administered to patients taking CGS are maintained. In addition, it is known that adverse drug reactions are often due to drug-drug reactions /11,12/, and most of the clinically relevant drug-drug interactions are due to either inhibition or induction of drug metabolism /13/. It is therefore important to asses the induction and inhibition potential of a drug to predict and/or exclude clinically relevant metabolic drug-drug interactions and thus adverse drug reactions. Cytochromes P450 (CYP) are involved in the metabolism of several marketed drugs and the *in vitro* investigation of their inhibition and induction by drugs has allowed the prediction of the occurrence of clinically relevant drug-

drug interactions /14-17/. As a result of this the US and European regulatory agencies have released guidance documents for the conduction of *in vitro* and *in vivo* drug metabolism studies to predict drug-drug interactions /18-21/. In a recent document released by the Food and Drug Administration, the Agency recognises the usefulness of this approach not only to increase the safety and effectiveness of marketed drugs but also to reduce the risk of unexpected adverse drug reactions due to metabolic interactions /22/.

The aim of the present investigation was to asses *in vitro* the inhibition and induction effects of CGS on human liver CYP450 enzymes. The drug was incubated with recombinant human enzymes (inhibition) and cryopreserved human hepatocytes (induction) at concentrations up to 3 mM. The selected concentrations were based on previous investigations in healthy volunteers /23/ and patients with OA /24/ that have indicated that after repeated oral doses of CGS of 1,500 mg once a day (the therapeutic dose schedule), the peak plasma concentration of glucosamine at steady state was approximately 10 μM 3 hours after drug administration. Therefore, the selected concentration for the induction and inhibition studies was 300-fold higher than the average maximum glucosamine plasma concentration observed in man. This approach also takes into consideration the extensive liver first pass effect to which glucosamine is subjected after oral administration /10/ that results in much higher drug concentrations around the hepatocyte and liver enzymes compared to those determined in peripheral blood and is thus in line with the requirements of currently adopted USA and European guidelines /16-22/. The present paper describes the results obtained.

MATERIALS AND METHODS

Chemicals, enzymes and hepatocytes

Crystalline glucosamine sulfate was supplied by Rottapharm S.p.A. (Monza, Italy). Phenobarbital, rifampicin, omeprazole, dimethyl sulfoxide (DMSO) and solvents of analytical grade were from Sigma-Aldrich (St. Louis, MO, USA).

BD Gentest™ P450 high throughput inhibitor screening kits were purchased from BD Gentest (Worburn, MD, USA). Each kit included recombinant human P450 cytochrome, its specific fluorescent

substrate, the positive control inhibitor, the fluorescent metabolite standard and all the necessary components for the reaction. The following human cytochromes were tested: CYP1A2, CYP2E1, CYP2C19, CYP2C9, CYP2D6, and CYP3A4.

Ready-to-use Hepatocyte Thawing and Plating Media, Ready-to-use Hepatocyte Incubation Medium, Antibiotic Mix and RNase AWAY™ were from In vitro Technologies (Baltimore, MD, USA). RNA Protect Cell reagent, RNAse free water, QIAshredder homogenizer, Rneasy® mini kit and RNase free DNase Set were from Qiagen Ltd (Crawley, UK). TaqMan® Reverse Transcription Kit, TaqMan® Gene Expression Master Mix Reagents, TaqMan® probes and TaqMan® oligonucleotide primers were obtained from Applied Biosystems (Foster City, CA, USA). Hepatocytes from three human donors (two female and one male) were obtained from a cryopreserved hepatocyte bank maintained at In Vitro Technologies (Baltimore, MD).

Enzyme inhibition

A stock solution was prepared dissolving CGS in a 0.5 M NaCl saline solution; subsequent dilutions followed the kits' recommendations. CGS was incubated with recombinant human P450 cytochrome up to a maximum glucosamine concentration of 3 mM expressed as free base. In each experiment, 0 concentration was used as the no inhibition reference.

The substrates used for each enzyme and the concentrations tested are presented in Table 1. A typical inhibitor for each enzyme included in the kits was also included as a positive control.

Formation of the fluorescent metabolites from each of the CYP substrates and its inhibition produced by CGS, or positive control known inhibitor, was determined using a 96-well plate fluorimetric reader VICTOR^3 from Perkin Elmer (Monza, Italy). In each well a NADPH regenerating system and the enzyme/substrate were added. CGS and the positive control are added to the well at different serial dilutions. Control wells contained no CSG (no inhibition signal) or the known inhibitor. Other wells contained the stopping reagent added prior to the addition of the enzyme and substrate, and represented the background noise signal. Fluorescent signals (see Table 1 for details) were recorded in duplicate according to the indications included in the

TABLE 1

Fluorescent substrates, their metabolites and typical inhibitors used for the enzyme inhibition assays. The concentration at which each substrate was incubated, the concentration range of typical inhibitors, and the excitation and emission wavelengths (λ_{ex} and λ_{em}, respectively) at which the metabolites' formation was monitored are also shown.

Enzyme	Substrate	Concentration (μM)	Metabolite	$\lambda_{ex}/\lambda_{em}$ (nm)	Typical inhibitors	Concentration range (μM)
CYP1A2	CEC	5	CHC	405/460	Furafylline	0.05-100
CYP2C9	MFC	75	HFC	405/535	Sulfaphenazole	0.048-10
CYP2C19	CEC	25	CHC	405/460	Tranylcypromine	0.05-100
CYP2D6	AMMC	1.5	AHMC	380/460	Quinidine	$2.29 \cdot 10^{-4}$-0.5
CYP2E1	MFC	100	HFC	405/535	Diethyldithiocarbamate	0.03-67
CYP3A4	BQ	40	HQ	405/535	Ketoconazole	0.0023-5
CYP3A4	BFC	50	HFC	405/535	Ketoconazole	0.0023-5

CEC = 3-cyano-7-ethoxycoumarin; MFC = 7-methoxy-4-trifluoromethylcoumarın; AMMC, 3-/2-(N,N-diethyl-N-methylamino)ethyl/-7-methoxy-4-methylcoumarin; BQ = 7-benzyloxyquinoline; BFC = 7-benzyloxy-trifluoromethylcoumarin; CHC, 3-cyano-7-hydroxy-coumarin; HFC, 7-hydroxy-4-trifluoromethylcoumarin; AHMC = 3-/2-(N,N-diethylamino)ethyl/-7-methoxy-4-methylcoumarin; HQ = 7-hydroxyquinoline.

kit instruction manuals and were reported as averages of those duplicate determinations.

Cy.otoxicity assessment (neutral red assay) and enzyme induction

CGS stock solutions were prepared by dissolving the compound in 0.5 M NaCl. The application solutions in medium were freshly prepared each day of treatment by diluting the stock solutions 1:200 in the Ready-to-use Hepatocyte Incubation Medium. Typical inducers for each enzyme were also included as positive controls.

Cryopreserved hepatocytes were thawed in Ready-to-use Hepatocyte Thawing Media, transferred in Ready-to-use Hepatocyte Plating Medium and counted for yield. Viability was measured with Trypan Blue. Viability of the cells greater than 70% was assumed acceptable for the determination of the cytotoxic potential of CGS (neutral red assay) and the induction assay.

Treatment groups consisted of three replicates. Each replicate corresponded to a single donor and contained the cells of three wells each.

For the neutral red assay 1.5×10^5 viable cells per well were added to collagen-coated 48-well cluster dishes. After an attachment period of approx. 4 h in a 95% air/5% CO_2 humidified incubator at 37°C the Ready-to-use Plating Medium was renewed, dead cells were removed and the incubation was continued for another 20 h. To adapt the cells to the culture conditions the plating medium was replaced afterwards by Ready-to-use Incubation Medium and the incubation was continued for an additional 24 h. Treatment was initiated by replacing the Ready-to-use Incubation Medium by Ready-to-use Incubation medium containing the test item (CGS 0.01 mM, 0.3 mM and 3 mM expressed as free base) or the control substances, omeprazole (CYP 1A2 inducer, 50 μM), phenobarbital (CYP 2B6 inducer, 750 μM) and rifampicin (CYP 2C9, 2C19 and 3A4 inducer, 25 μM). The incubation was continued for 48 h and the medium was renewed after 24 h. The medium was removed and Ready-to-use Incubation Medium containing 1.5% (v/v) neutral red solution was added. The incubation was continued for an additional 3 h in a 95% air/5% CO_2 humidified incubator at 37°C. Afterwards the solutions were removed and the cells were washed using PBS buffer. For the extraction of the red dye a solution of 50% (v/v) ethanol and 1.0% (v/v) glacial acid in H_2O was added and the cells were incubated for 10 min on a shaker. The

supernatants were then transferred to a 96-well microtiter plate and absorption was measured at 540 nm.

For the induction assay about 3.5 x 10^5 human hepatocytes per well were added to collagen I-coated 24-well cluster dishes using Ready-to-use Plating Medium. After an attachment period of approx. 4 h in a 95% air/5% CO_2 humidified incubator at 37°C the plating medium was renewed and the incubation was continued for approx. 20 h. To allow the cells to adapt to the culture conditions used for the induction the plating medium was changed by Ready-to-use Incubation Medium and the incubation was continued for an additional 24 h in a 95% air/ 5% CO_2 humidified incubator at 37°C. Subsequently treatment was initiated by changing the media by Ready-to-use Incubation Medium (500 µl/well) containing the test item or the positive control substances at the same concentrations of the neutral red assay. The total induction time was 48 h; during this period medium was changed once after 24 h by the same medium (500 µl/well) containing either freshly added test item or control substance. At the end of the treatment period, the medium was aspirated from the wells and replaced with RNAprotect Cell Reagent (Qiagen) (350 µl/well or 200 µl/well for positive controls), and finally the contents of 3 wells were combined in one tube as one replicate.

RNA preparation from human hepatocytes

The RNAprotect Cell Reagent was removed, the cells were homogenized using the QIAshredder homogenizer kit and the total RNA was extracted from the samples using Qiagen Rneasy® mini kit according to the supplier's recommendations.

The quality of the isolated RNA was assessed by the 260/280 nm absorbance ratio (range 1.8-2.0 indicates a high purity sample) /24/.

Reverse transcription

Isolated RNA was reverse-transcribed using TaqMan® Reverse Transcription Kit. The thermal conditions were 25°C 10 min, 37°C 60 min and 95°C 5 min. The efficiency of the reverse transcription was examined by spectrometric absorbance at 260/280 nm.

Real-time quantitative RT-PCR

The levels of CYP mRNAs were determined by real-time quantitative polymerase chain reaction (RT-PCR) methodology, using an ABI 7700 Sequence Detector System (Applied Biosystems) and the TaqMan® Gene Expression Master Mix Reagents, incorporating sequence-specific forward, reverse and fluorescently labelled probes for each cytochrome. The thermal cycle condition was 50°C for 2 min, 95°C for 10 min to activate Amplitaq Gold DNA polymerase, denaturation at 95°C for 15 sec and anneal/extension at 60°C for 1 min (40 cycles).

The Ct values were normalized using the 18S rRNA constitutive endogenous gene values which were obtained after RT-PCR amplification of the eukaryotic 18S rRNA endogenous control (VIC/MGB Probe, Applied Biosystems). The relative expression of each mRNA was calculated by the ΔCt (the value obtained by subtracting the Ct value of 18S rRNA mRNA from the Ct value of the target mRNA) method. The ΔCt average of the 3 replicates in each treatment group was calculated and the inductive effects were evaluated according to the following equation:

$$\text{Expression level} = 2^{-\Delta\Delta Ct} \quad (1)$$

where ΔΔCt = sample ΔCt – control ΔCt. Results were expressed as mean fold induction of the 3 treatment groups (donors) over negative controls incubated with drug free media ± SEM.

Statistical analysis

In the inhibition study, the results obtained with CGS and the reference inhibitors were analyzed by non-linear regression using the software package SigmaPlot 9.0 (Systat Software Inc, San Jose, CA, USA) to calculate the IC_{50} values for each reference inhibitor and for CGS.

RESULTS

Inhibition of human cytochromes P450

The known inhibitors produced significant inhibition of the formation of the fluorescent metabolites. Sigmoidal concentration response curves were obtained for all positive control inhibitors, as shown in Figure 1A. The obtained IC_{50} values for the typical inhibitors were in agreement with the values reported in the kits' specifications and with data in the literature /25,26/. CGS did not interfere with the fluorescent metabolites of the assay substrates and therefore did not lead to false negative results. The formation of fluorescent metabolites from all seven marker substrates tested did not differ significantly when the substrates were incubated with the respective enzyme with or without CGS up to the maximum concentration tested (3 mM). For CGS, due to the lack of any significant inhibition, no IC_{50} values could be calculated, as shown in Figure 1B.

Induction of human cytochromes P450

The cells showed viabilities greater than 79% and were used for the assays. Determination of the cytotoxic potential of CGS with neutral red measurement at 540 nm showed that no significant cytotoxic effect was detected for any of the applied concentrations, thus no CGS cytotoxic potential needs to be taken into account.

The results obtained with the known inducers were in line with the data reported in the literature /27-29/. A clear induction of human CYP1A2 after exposure to omeprazole (about 5-fold) and for human CYP3A4 after exposure to rifampicin (about 55-fold) was observed (data not shown). Rifampicin also induced CYP2C9 (about 4-fold) and CYP2C19 mRNA (about 6-fold), and phenobarbital induced human CYP2B6 mRNA (about 3-fold, data not shown). Treatment of human hepatocytes with CGS did not affect the mRNA expression of human CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4 enzymes. The fold induction produced by CGS for each CYP enzyme is shown in Table 2.

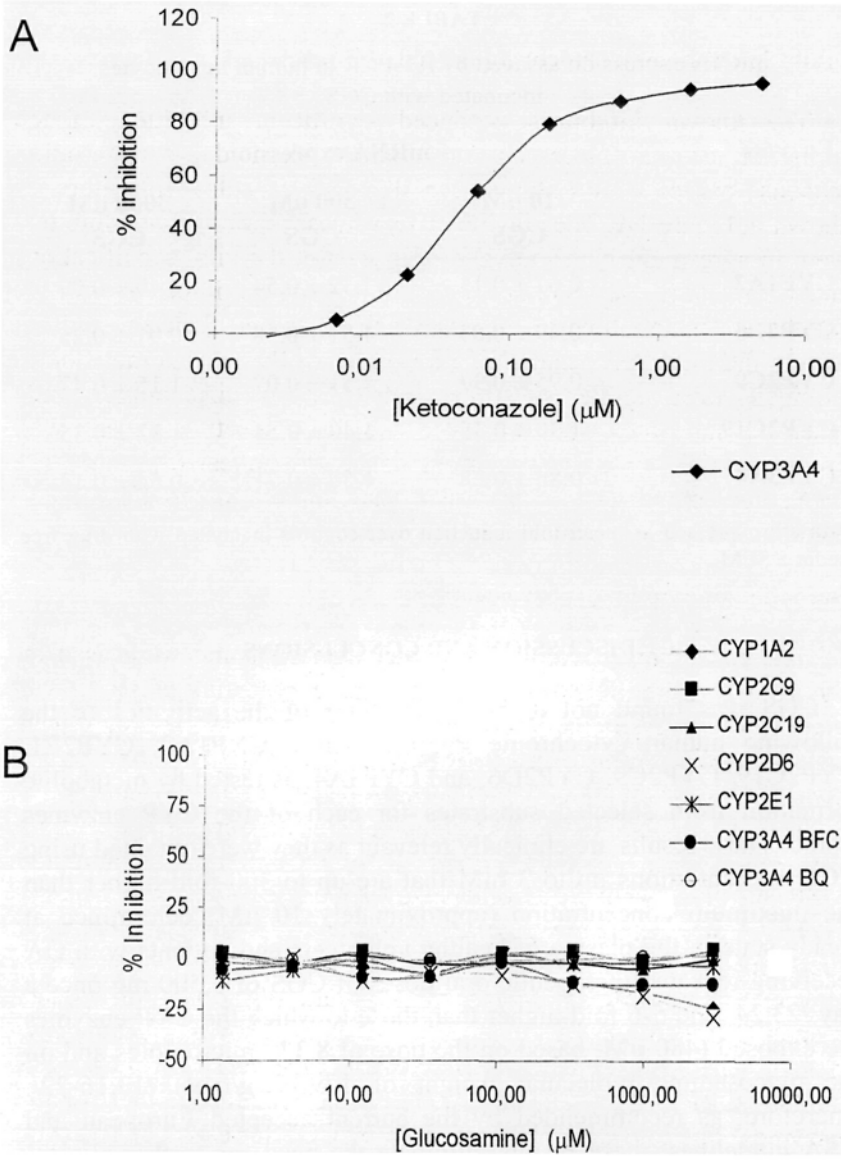

Fig. 1: Effect of (**A**) ketoconazole (a typical positive control inhibitor) and (**B**) crystalline glucosamine sulfate on P450 activities using fluorescence assays. Percentage inhibition of P450 activities is plotted against log molar concentrations of positive control inhibitor or glucosamine. Data shown are taken from a representative experiment.

TABLE 2

mRNA expression assayed by RT-PCR in human hepatocytes incubated with CGS

Enzyme	mRNA expression		
	10 μM CGS	300 μM CGS	3000 μM CGS
CYP1A2	0.67 ± 0.11	1.32 ± 0.54	0.49 ± 0.09
CYP2B6	0.49 ± 0.03	1.51 ± 0.58	0.91 ± 0.25
CYP2C9	0.95 ± 0.37	1.51 ± 0.07	1.15 ± 0.47
CYP2C19	1.30 ± 0.35	1.40 ± 0.54	1.32 ± 0.35
CYP3A4	0.86 ± 0.28	1.50 ± 0.71	0.54 ± 0.13

Data are expressed as mean fold induction over controls incubated with drug free media ± SEM.

DISCUSSION AND CONCLUSIONS

CGS was found not to be an inhibitor of the activities of the following human cytochrome P450 enzymes: CYP1A2, CYP2E1, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, as tested by metabolite formation from selected substrates for each of the CYP enzymes tested. These results are clinically relevant as they were obtained using CGS concentrations up to 3 mM that are up to 300-fold higher than the maximum concentration (approximately 10 μM) determined at steady state in the plasma of healthy volunteers and patients with OA receiving repeated therapeutic oral doses of CGS of 1,500 mg once a day /23,24/ and 5-6 fold higher than those to which the CYP enzymes are exposed (480 μM, based on the dose of 8,370 micromoles and on the glucosamine molecular weight of 179.17 g/mol) /13,16-22/. Therefore, as recommended by the current accepted European and USA guidelines, these results eliminate the need for further clinical investigations for the substrates of these CYPs /16-22/.

Since crystalline glucosamine sulfate is administered chronically for the treatment of OA, induction of CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4 by CGS was also evaluated in cryopreserved human hepatocytes, by determining CYP mRNA expression using

RT-PCR according to the FDA guidelines /21/. The studies indicated the lack of any induction activity toward these enzymes.

In summary, the results obtained in the present study allow the conclusion that CGS should not act as an inhibitor or as an inducer of the human liver CYP enzymes investigated. Therefore, when CGS is administered orally at a dose of 1,500 mg once a day chronically, this drug should produce no clinically relevant metabolic drug interactions with concomitant medications that are substrates for the CYP enzymes investigated.

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