Appl Biochem Biotechnol (2010) 160:1508–1516
DOI 10.1007/s12010-009-8605-0

Predicting Drug Interaction of Clopidogrel on Microbial Metabolism of Diclofenac

K. Srisailam · V. Raj Kumar · C. Veeresham

Received: 22 January 2009 / Accepted: 3 March 2009 /
Published online: 4 April 2009

Abstract Seven fungal cultures were studied for the metabolism of diclofenac in order to elucidate the nature of enzymes involved in biotransformation, as diclofenac is a specific substrate to cytochrome P450 (CYP) 2C9 isozyme in mammals. The metabolites were identified by high-performance liquid chromatography–diode array detection and liquid chromatography–tandem mass spectroscopy analysis. The study included clopidogrel, a selective inhibitor of CYP2C9 isozyme, to inhibit the metabolism of diclofenac. Two-stage fermentation protocol was used to study the diclofenac metabolism and its inhibition by clopidogrel. Among the cultures studied, four have shown positive indication for drug interaction, since clopidogrel inhibited the metabolism of diclofenac in a dose-dependent manner. The results indicate that microbial cultures possess enzyme systems similar to mammals and they can be used to predict drug interactions in mammalian systems.

Keywords Biotransformation · HPLC · LC–MS–MS · Metabolites · Microorganisms · Diclofenac · Clopidogrel · Drug interaction

Introduction

Once the metabolic routes of a drug are characterized, the in vivo prediction and correlation requires elucidation of drug-metabolizing enzymes that are involved in the in vitro biotransformation of the drug. This is usually studied using substrates and inhibitors selective or specific for the respective cytochrome P450s (CYPs). Some in vitro methods were reported for identifying the CYP(s) involved in the metabolism of a new chemical entity, and these studies will be conducted using human liver microsomes, purified enzymes, cDNA expressed enzymes, etc., and investigators use different probe reactions to determine the CYP involved. The evaluation should primarily focus on the specificity, selectivity, and sensitivity of the marker reaction for the enzyme. To date, no ideal probe

K. Srisailam · V. Raj Kumar · C. Veeresham (✉)
University College of Pharmaceutical Sciences, Kakatiya University,
Warangal 506009 Andra Pradesh, India
e-mail: ciddiveeresham@yahoo.co.in

substrate compound was found for any of the CYP isozyme. The use of specific or selective marker compounds to inhibit or induce the enzymes will greatly help in predicting enzyme-specific drug–drug interactions, since majority of the drug interactions are mediated through CYP.

Diclofenac is extensively metabolized in humans, and the main metabolite in plasma and urine is 4′-hydroxy diclofenac. The other metabolites of minor quantities include 3′-hydroxy and 5-hydroxy diclofenac [1]. The formation of 4′- and 3′-hydroxy metabolites is mediated by CYP2C9, whereas 5-hydroxy metabolite is formed due to CYP3A4, CYP2C8, CYP2C18, and CYP2C19 enzymes in in vitro studies [2–5]. 4′-Hydroxylation of diclofenac has been used as the marker reaction for CYP2C9 activity for the past few years, and many studies were reported [2].

Ibrahim and El-Feraly [6] reported that *Aspergillus niger*, *Aspergillus alliaceous*, *Aspergillus ochraceus*, *Cunninghamella elegans*, *Cunninghamella echinulata*, *Cunninghamella blakesleana*, *Pencillium chrysogenum*, *Pencillium vermiculatum*, *Rhizopus nigricans*, and *Streptomyces fulvissimus* were able to produce 4′-hydroxy metabolite of diclofenac. They also reported that *A. niger*, *S. fulvissimus*, and *C. blakesleana* produced 5-hydroxy metabolite, and *A. niger*, *A. alliaceous*, and *C. elegans* produced 4′-hydroxy diclofenac lactam metabolites. Webster et al. [7] reported the production of 3′-hydroxy, 4′-hydroxy, and 5′-hydroxy metabolites of diclofenac using the microbial cultures *Paecilomyces farinosus*, *Arthrinium phaeospermum*, *Mucor plumbeus*, *Scytalidium* sp., *Pestalotiopsis* sp., and *Epiccocum nigrum* with 4′-hydroxy diclofenac as the major metabolite in the majority of the cultures.

In all the diclofenac metabolism studies conducted either in mammalian or microbial systems, the major metabolite produced was 4′-hydroxy diclofenac. In the present investigation, characterization of the enzymes involved in the CYP2C9 marker reaction, diclofenac 4′-hydroxylation, was studied in selected fungal cultures: *Absidia coerulea* MTCC 1335, *C. blakesleana* NCIM 687, *C. blakesleana* NCIM 688, *C. echinulata* NCIM 691, *C. echinulata* NCIM 693, *C. elegans* NCIM 690, and *Cunninghamella* sp. NCIM 1184. The study also included clopidogrel, a known CYP2C9 selective inhibitor from which the inhibition of 4′-hydroxy diclofenac formation was studied. The study reveals the microbial metabolism of diclofenac and its inhibition by clopidogrel, a known enzyme inhibitor.

Material and Methods

Chemicals

Diclofenac sodium and clopidogrel bisulphate were kindly gifted by Dr. Reddy's Laboratories, Hyderabad. The microbial cultures used in the study were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India or National Collection of Industrial Microorganisms (NCIM), Pune, India.

Culture Procedure

A two-stage fermentation protocol was used in the present study. The fungal cultures were grown in liquid medium containing (per liter) dextrose 20 g, yeast extract 5 g, peptone 5 g, sodium chloride 5 g, and KH_2PO_4 5 g supplemented with 0.02% Triton X 100. Fifty-milliliter conical flasks containing 10 ml of sterile liquid medium were inoculated with

50 μl of culture obtained from a freshly grown agar slant and orbital shaken at 180 rpm and 30 °C in a refrigerated shaker incubator. The cultures were allowed to grow for 48 h to obtain second-stage cultures.

Biotransformation

The second-stage cultures (10 ml culture in 50-ml capacity culture flask) were added with 2 mg each of diclofenac (in 100 μl methanol) to obtain a final drug concentration of 0.2 g/l. Each culture was studied in quadruplicate while running suitable controls. Culture controls consisted of culture blanks in which the organisms were grown under identical conditions but without adding the substrate. The culture controls were added with 100 μl methanol, the solvent used to dissolve the drug. Drug controls were composed of the sterile medium to which same amount of the drug was added and incubated without microorganisms. The incubation was continued under similar conditions for 10 days, the flasks were taken out, extracted, and analyzed.

Extraction and Sample Preparation

The cultures, after 10 days of incubation with diclofenac, were taken out and extracted with three volumes of dicloromethane. The combined organic phases (lower dichloromethane layers) were evaporated under reduced pressure and the dried samples were reconstituted in 1.5 ml each of high performance liquid chromatography (HPLC) grade methanol. The samples were then centrifuged at 12,000×*g* and 20°°C for 20 min in a micro-refrigerated centrifuge. The supernatants were used for HPLC–diode array detection (HPLC-DAD) and liquid chromatography–tandem mass spectroscopy (LC–MS–MS) analysis.

HPLC-DAD Analysis

The samples were analyzed by an isocratic HPLC method for the presence of metabolites. The HPLC analysis was performed on LC-10AT system (Shimadzu, Japan) by injecting 20 μl of the sample into a syringe loading sample injector. The column used was Wakosil II C18, 5 μ and 250×4.6-mm i.d. (SGE, USA), and the mobile phase consisted of a mixture of acetonitrile/methanol/water, pH adjusted to 3.2 with orthophosphoric acid, in 40:20:40 ratio. The analysis was performed at a flow rate of 1.2 ml/min, and diclofenac and its metabolites were detected using diode array detector (SPD-M10Avp, Shimadzu) at a wavelength of 274 nm. The data analysis was performed by Class M10 software. The UV absorption spectrum of the metabolites was compared with that of diclofenac. The metabolites were quantified based on the peak areas and expressed as percentages of metabolites formed. The calculations were performed with respect to the total area of drug and metabolites together taken as 100%.

LC–MS–MS Analysis

The mass/charge (*m*/*z*) values of diclofenac and its metabolites and their fragmentation ions were recorded by LC–MS–MS using Perkin Elmer SCIEX API mass spectrometer set in positive mode. The column used was Inertsil ODS-2, RP, C18 of 250×4.6 mm, and the mobile phase, acetonitrile/water (pH adjusted to 3.2 with formic acid; 50:50), was pumped at a flow rate of 1 ml/min. Fifty microliters of the sample was injected using an autosampler device. The vaporizer temperature and the discharge current were set at 300°°C and 10 μA,

respectively. The fragment ions were scanned in the *m*/*z* range of 100–500, and the data were processed with Sciex Analyst software. The metabolites were identified basing on pattern of UV spectra in HPLC-DAD and *m*/*z* values of the fragment ions obtained in LC–MS–MS analysis.

Inhibition of Metabolism by Clopidogrel

The inhibition of 4′-hydroxy metabolite formation by clopidogrel was studied in all the seven fungal cultures. The fungal cultures were grown in the same medium that is used for the biotransformation, and a two-stage fermentation protocol was used for the study. Each 10 ml of the second-stage cultures was added with 0.5, 1, 1.5, and 2 mg of clopidogrel (in 100 μl of methanol) to get the inhibitor concentrations of 0.05, 0.1, 0.15, and 0.2 g/l culture and incubated at 180°°C for 24 h to inhibit the enzymes. Each culture was studied in quadruplicate while running suitable controls. The incubation time was high enough to achieve maximal inhibition of enzymes, since inhibition of enzymes is a quick process when compared to induction.

The clopidogrel-inhibited second-stage cultures were added with 2 mg each of diclofenac (in 100 μl methanol) to get a final drug concentration of 0.2 g/l. Each culture was studied in quadruplicate while running suitable controls, and the incubation was continued under similar conditions for 10 days. The contents of the flasks were extracted and analyzed using the procedure similar to that employed for diclofenac metabolism by microbial cultures. The percentages of 4′-hydroxy metabolite in the cultures were calculated.

Results and Discussion

The drug control has not shown any additional peaks in HPLC analysis, which indicates that the substrate diclofenac was stable in the media. Upon extraction with dichloromethane, the recovery of diclofenac from the dextrose broth was >90.2%. HPLC analysis of the extracts of the cultures showed that all the cultures studied were able to metabolize diclofenac to produce two or more metabolites (Table 1). Representative HPLC chromatograms were shown in Fig. 1. Many organisms produced the same range of metabolites, but in different relative amounts. The production of metabolites by microbial cultures may be due to the presence of enzymes expressed in the microbial cells. All the metabolites produced were eluted before the drug, indicating that they are polar than diclofenac. The metabolite peaks were identified in HPLC basing on the similarity in the UV spectra in the diode array detector. All these new peaks had shown similar UV spectral pattern (from 190 to 370 nm) with that of diclofenac, which indicates that the drug has undergone only minor structural changes. The UV λ_{max} of the metabolites were within a narrow range with that of diclofenac, which indicates that their extinction coefficient at 274 nm was not significantly different from that of the parent drug.

The metabolites were quantified basing on their areas and expressed as percentages of metabolites formed with respect to the total area of drug and metabolites together taken as 100%. Diclofenac was eluted at 17 min and the metabolites produced were designated as M_1 (4.8 min), M_2 (7 min), M_3 (7.4 min), and M_4 (8.2 min). Control incubations without diclofenac added showed no coincidental peaks eluting with identified metabolites. From Table 1, a significant variation was observed in the quantitative metabolite production among various cultures. M_3 was the major metabolite found in all the cultures studied. *C.*

Table 1 Biotransformation of diclofenac by microbial cultures.

Culture	$\%M_1$	$\%M_2$	$\%M_3$	$\%M_4$
Absidia coerulea MTCC 1335	5.15	5.74	48.87	0.00
Cunninghamella blakesleana NCIM 687	9.18	24.59	39.05	0.00
Cunninghamella blakesleana NCIM 688	3.79	0.00	59.33	0.00
Cunninghamella echinulata NCIM 691	6.89	1.36	35.65	0.00
Cunninghamella echinulata NCIM 693	4.33	2.32	40.52	0.58
Cunninghamella elegans NCIM 690	5.33	0.00	14.83	0.00
Cunninghamella sp. NCIM 1184	13.63	0.00	67.29	0.00

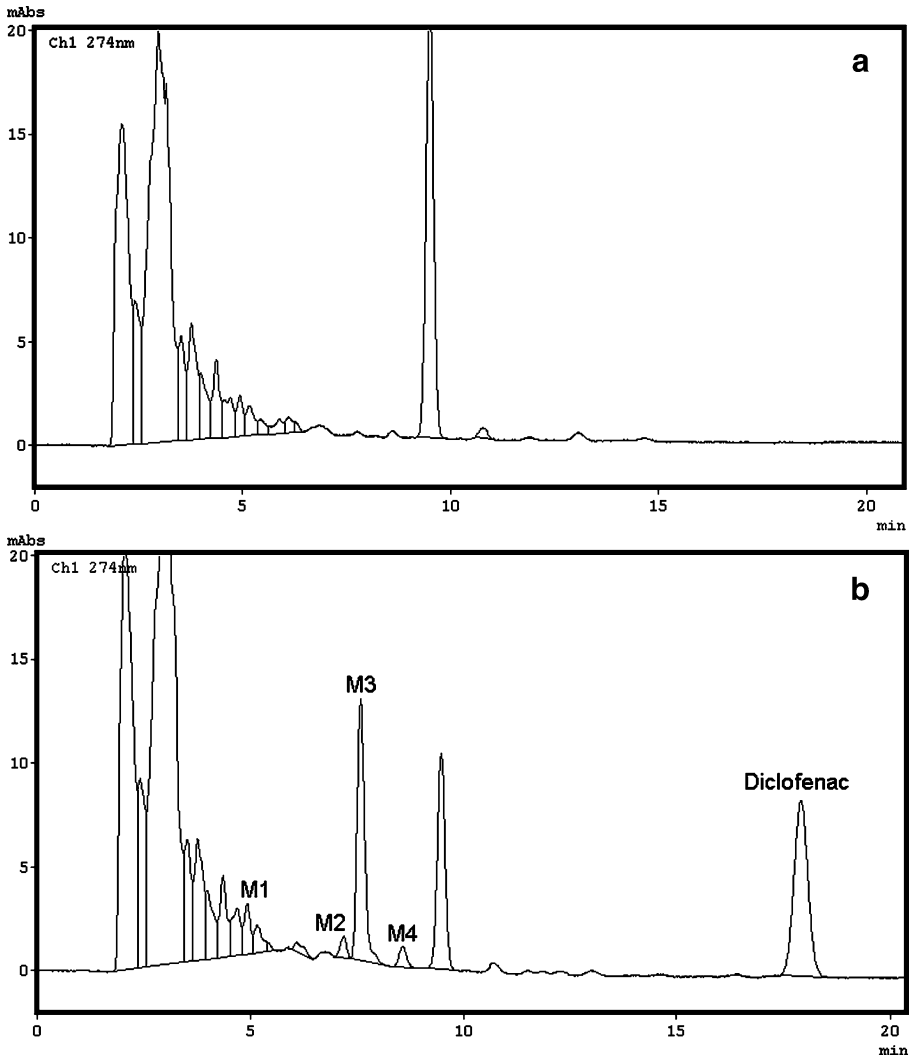

Fig. 1 HPLC chromatogram of culture control (**a**) and metabolites (**b**) obtained with *C. echinulata* NCIM 693

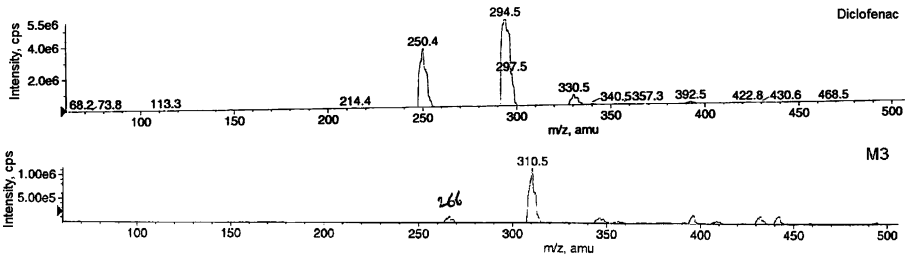

Fig. 2 LC-MS spectra of diclofenac and its 4′-hydroxy metabolite (M_3)

blakesleana NCIM 688 produced 67.29% of this metabolite, whereas *C. elegans* NCIM 690 produced 14.83% of this metabolite. The varying production may be attributed to many reasons, viz., expression of varying levels of enzymes required for this reaction, substrate/product inhibited biotransformation, non-optimal conditions (media and incubation) for biotransformation, etc.

The structure elucidation of the metabolites was carried out from the *m*/*z* values of the protonated molecular ion peaks and fragment ions obtained in LC–MS–MS analysis. The structures of the metabolites were proposed from the *m*/*z* values of the protonated molecular ions and fragment ions, HPLC retention times, chromatographic elution order, and comparison with previous reports.

In the positive mode of LC–MS–MS analysis, diclofenac M_1, M_2, M_3, and M_4 gave their protonated molecular ions at *m*/*z* values of 297, 329, 313, 313, and 313 respectively. LC–MS spectrum of M_3 was shown in Fig. 2. M_2, M_3, and M_4 gave the same protonated molecular ion at *m*/*z* 313, that is, 16 higher than diclofenac. This suggests that these might be hydroxylation products of diclofenac. Since these three metabolites produced the same protonated molecular ion, they might be positional isomers having the same molecular formula. The structures of these three metabolites were assigned from previous reports [6, 7]. The structure elucidation was carried out using retention times and elution order of metabolites in HPLC. M_1 gave protonated molecular ion peak at *m*/*z* 329, which is 32 higher than the parent drug and 16 higher than the hydroxylated metabolites. This indicates that it might be the dihydroxylated metabolite of diclofenac.

Based on the previous reports and the *m*/*z* values of protonated molecular ions, the metabolites M_2, M_3, and M_4 were found to be 3′-hydroxy, 4′-hydroxy, and 5-hydroxy metabolites of diclofenac, respectively. M_1 was a dihydroxylated metabolite of diclofenac,

Table 2 Retention times, *m*/*z* values, and predicted reactions involved in the biotransformation of diclofenac by microbial cultures.

Metabolite	R_t	$[M+H]^+$	Predicted molecular formulae	Predicted reaction
Diclofenac	17	297	$C_{14}H_{11}Cl_2NO_2$	–
M_1	4.7	329	$C_{14}H_{11}Cl_2NO_4$	Dihydroxylation
M_2	7	313	$C_{14}H_{11}Cl_2NO_3$	3′-Hydroxylation
M_3	7.4	313	$C_{14}H_{11}Cl_2NO_3$	4′-Hydroxylation
M_4	8.2	313	$C_{14}H_{11}Cl_2NO_3$	5-Hydroxylation

Table 3 Effect of clopidogrel on diclofenac metabolism.

Treatment	$\%M_3$ produced						
	Absidia coerulea MTCC 1335	*Cunninghamella blakesleana* NCIM 687	*Cunninghamella blakesleana* NCIM 688	*Cunninghamella echinulata* NCIM 691	*Cunninghamella echinulata* NCIM 693	*Cunninghamella elegans* NCIM 690	*Cunninghamella* sp. NCIM 1184
Control	48.87	39.05	67.29	59.33	40.52	14.83	35.65
+ Clopidogrel (0.005%)	45.97	6.52	60.59	50.19	37.69	11.44	34.56
+ Clopidogrel (0.01%)	40.08	4.75	51.82	42.29	33.44	8.36	35.98
+ Clopidogrel (0.015%)	37.42	1.91	43.29	34.35	29.55	5.59	34.69
+ Clopidogrel (0.02%)	35.42	0.52	35.68	28.52	26.56	3.52	33.22

the structure of which was not elucidated. Table 2 gives the retention times, *m*/*z* values of parent ions $(M+H)^+$, and predicted molecular formulae and metabolic reactions for various metabolites. From Table 1, it was found that M_3 was the major metabolite produced in all the cultures studied. Hence, it can be concluded that 4′-hydroxy metabolite, which was eluting at 7.4 min in HPLC analysis, is the major metabolite. In the present investigation, seven fungi were screened for their ability to metabolize diclofenac. The results of the present investigation show that 4′-hydroxy diclofenac was the major metabolite produced by all the microorganisms investigated.

Clopidogrel was found to be a potent inhibitor of CYP2C9 responsible for the metabolism of diclofenac. In the present study, clopidogrel was incubated with all the microbial cultures expressing diclofenac-metabolizing enzymes. All the seven cultures were employed in the inhibition study because all these cultures produced good amounts of metabolite M_3. Among the seven cultures studied, some of them have shown potential for inhibition of diclofenac metabolism by clopidogrel. Clopidogrel is a CYP2C9 specific inhibitor, and the present study reveals that the evidence for the functional role of the enzymes in these cultures was similar to CYP2C9 in their metabolic nature. Few cultures did not show any decrease in diclofenac metabolism, since those enzymes responsible for metabolism were not inhibited by clopidogrel, which indicates that the enzymes are functionally different from CYP2C9. The results pertaining to this investigation were shown in Table 3.

Among the cultures tested, *C. blakesleana* NCIM 687, *C. blakesleana* NCIM 688, *C. echinulata* NCIM 691, and *C. elegans* NCIM 690 showed positive for drug interaction. Clopidogrel has shown clear a concentration-dependent inhibition of diclofenac metabolism in these cultures. Figure 3 shows the percentages of M_3 formed in the presence of various concentrations of clopidogrel in these four cultures.

Conclusion

In vitro studies have become a means of rapid identification of potential drug–drug interactions. The in vitro methods are useful in predicting the inhibition of drug-metabolizing enzymes, but may not predict the magnitude of interactions in vivo. The same may be applicable to microbial cultures for drug metabolism as well as drug interactions.

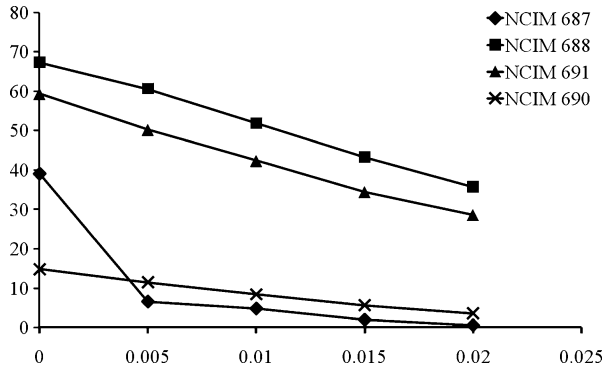

Fig. 3 Effect of clopidogrel on the formation of M_3 in various fungal cultures

References

1. Davies, N. M., & Anderson, K. E. (1997). *Clinical Pharmacokinetics*, *33*, 184–213. doi:10.2165/00003088-199733030-00003.
2. Leeman, T., Transon, C., & Dayer, P. (1993). *Life Sciences*, *52*, 29–34. doi:10.1016/0024-3205(93)90285-B.
3. Bort, R., Mace, K., Boobis, A., Gomez-Lechon, M. J., Pfeifer, A., & Castell, J. (1999). *Biochemical Pharmacology*, *58*, 787–796. doi:10.1016/S0006-2952(99)00167-7.
4. Shen, S., Marchick, M. R., Davis, M. R., Doss, G. A., & Pohl, L. R. (1999). *Chemical Research in Toxicology*, *12*, 214–222. doi:10.1021/tx9802365.
5. Tang, W., Stearns, R. A., Wang, R. W., Chiu, S. H., & Baillie, T. A. (1999). *Chemical Research in Toxicology*, *12*, 192–199. doi:10.1021/tx9802217.
6. Ibrahim, A. R. S., & El-Feraly, F. S. (1996). *Saudi Pharmaceutical Journal*, *4*, 165–169.
7. Webster, R., Pacey, M., Winchester, T., Johnson, P., & Jezequel, S. (1998). *Applied Microbiology and Biotechnology*, *49*, 371–376. doi:10.1007/s002530051184.