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Biotransformation of Celecoxib Using Microbial Cultures

Keshetty Srisailam · Ciddi Veeresham

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Abstract Microbial transformation studies can be used as models to simulate mammalian drug metabolism. In the present investigation, biotransformation of celecoxib was studied in microbial cultures. Bacterial, fungal, and yeast cultures were employed in the present study to elucidate the metabolism of celecoxib. The results indicate that a number of microorganisms metabolized celecoxib to various levels to yield eight metabolites, which were identified by high-performance liquid chromatography diode array detection and liquid chromatography tandem mass spectrometry analyses. HPLC analysis of biotransformed products indicated that majority of the metabolites are more polar than the substrate celecoxib. The major metabolite was found to be hydroxymethyl metabolite of celecoxib, while the remaining metabolites were produced by carboxylation, methylation, acetylation, or combination of these reactions. The methyl hydroxylation and further conversion to carboxylic acid was known to occur in metabolism by mammals. The results further support the use of microorganisms for simulating mammalian metabolism of drugs.

Keywords Celecoxib · Biotransformation · HPLC · LC–MS/MS · Metabolite · Microorganisms

Introduction

The study of drug metabolism and toxicity of its metabolites are important in the process of drug discovery. Traditionally, drug metabolism studies have used model systems to simulate metabolic pathways of drugs in humans and animals. Microorganisms such as bacteria and fungi have been used as in vitro models for the simulation of mammalian drug metabolism, and successful applications have been reported in the literature [1–6]. Interestingly, some fungi were found to possess cytochrome p450 enzyme system [7] and oxidizing organic compounds in the same way as mammalian hepatic cytochrome p-450. The use of

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

microbial simulation of mammalian metabolism also gives an idea on the mechanism of action, toxicity, and pharmacological activity of the drugs and thus helps in discovery of new drug molecules [8]. This mode of producing metabolites is very convenient and a preparative method for otherwise difficult to obtain metabolites particularly when the structure is complex, and one can neither isolate metabolites from mammals nor synthesize chemically.

Celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl] benzenesulfonamide, a non-steroidal anti-inflammatory drug (NSAID), is the first specific cyclooxygenase-2 (COX-2) inhibitor approved by United States Food and Drug Administration (US-FDA) for the treatment of osteoarthritis, rheumatoid arthritis, and familial adenomatous polyposis. The selective inhibition of COX-2 by celecoxib is thought to reduce unwanted effects in upper gastrointestinal tract (GIT) that are mediated by COX-1 inhibition by conventional NSAIDs. Celecoxib is extensively metabolized by the CYP2C9 isozyme in humans [9] and rats [10] to produce the major metabolites by methyl hydroxylation and its further conversion to carboxylic acid. The metabolic pathway involves oxidation of methyl group to produce hydroxymethyl metabolite, which is further converted to carboxylic acid. Since the drug celecoxib is lipophilic in nature, it should be eliminated predominantly by metabolism, and hence, the study of metabolic pathways is important.

2,5-Dimethyl celecoxib (DMC), a structural analogue of celecoxib, was found to lack COX-2 inhibitory activity but exhibits significant antineoplastic properties in non-small cell lung cancer [11]. Downregulation of survivin expression and concomitant induction of apoptosis by celecoxib and DMC in tumor cells in vitro and in vivo was also reported [12]. Additionally, studies indicate that COX-2 inhibitors show relief to prevent colon cancer and Alzheimer's disease. Celecoxib was also shown to possess inhibitory effect on different carbonic anhydrase isozymes [13]. In 2005, the results of placebo-controlled trials provided new evidence about the increased risk of unwanted cardiovascular effects of celecoxib [14].

The aim of the present work was to study the metabolites of celecoxib formed by microorganisms and to compare them with those produced in mammals. To date, there is no report available for microbial biotransformation of celecoxib. The present work will also help in the production of large quantities of metabolites for further pharmacological and toxicological evaluation apart from structure elucidation. Since celecoxib is metabolized by the CYP group of enzymes and metabolized by oxidative pathway, microbial cultures, particularly fungi, may provide an alternative to produce mammalian metabolites. Moreover, the dimethyl analogue of celecoxib has shown anticancer activity; there are chances of producing new metabolites with fungi that possess COX-2 inhibitory or any other interesting activity. Close structural similarity between the COX-2 inhibitors and possibility of producing new and unusual structures with microorganisms prompted us to investigate the microbial metabolism of celecoxib.

Materials and Methods

Celecoxib used in the study was a kind gift from Unichem Laboratories Ltd., Ahmedabad, India. The microorganisms used in the present study were obtained either from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh or National Collection of Industrial Microorganisms (NCIM), Pune, India. The cultures were revived, subcultured, and stored in refrigerator at 4 °C. The bacterial, fungal, and yeast cultures were maintained on nutrient agar, potato dextrose agar, and MGYP agar slants, respectively.

Culture Procedure

The media used for biotransformation studies were dextrose broth, nutrient broth, and MGYP broth for fungi, bacteria, and yeast, respectively. For screening experiments, a two-stage fermentation protocol was used. The first stage culture was initiated in 50-ml culture flasks containing 10 ml of sterile liquid medium and inoculated with a loop of culture scratched from freshly grown agar slant. The culture flasks were orbitally shaken at 180 rpm, 2.5 cm deflection, and 30 °C in refrigerated shaker incubator (Model Innova 4230, New Brunswick Scientific Co., Inc., NJ, USA). For second stage cultures, 50-ml culture flasks containing 10 ml of the same media were inoculated with 500 μl of 48 h grown first stage culture and incubated under similar conditions for 48 h. Dextrose broth was supplemented with 0.02% Triton X 100 in order to get good dispersion of fungi in the media [15].

Biotransformation

The second stage cultures were added with 2 mg each of celecoxib (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. Drug controls were composed of sterile medium to which the drug was added and incubated without microorganisms. The incubation was continued for 10 days, and the cultures were extracted and analyzed.

Extraction and Sample Preparation of Celecoxib and Metabolites

The cultures, after 10 days of incubation with celecoxib, were extracted with 3 vol. of ethyl acetate. The combined ethyl acetate layers were evaporated, and the dried samples were reconstituted in 1.5 ml each of high-performance liquid chromatography (HPLC) grade methanol. The samples were centrifuged at 12,000×*g* and 20 °C for 20 min, and the supernatants were used for HPLC diode array detection (HPLC-DAD) and liquid chromatography tandem mass spectrometry (LC–MS/MS) analyses.

HPLC-DAD Analysis

The samples were analyzed by an isocratic HPLC method for the presence of metabolites [16]. The HPLC analysis was performed on LC-10AT system by injecting 20 μl of sample. The column used was Luna C18, 5 μ, 250×4.6 mm. The mobile phase consisted of a mixture of acetonitrile/water, pH adjusted to 3.2 with orthophosphoric acid, in 60:40 ratio, and pumped at 1 ml/min. Celecoxib and its metabolites were detected using diode array detector (Shimadzu SPD M10Avp model, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 251 nm. The data analysis was performed by Class M10 software. The UV absorption spectrum of the metabolites was compared with that of celecoxib. 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 *m*/*z* values of drug and metabolites and their fragmentation ions were recorded by LC–MS/MS using Perkin Elmer Sciex API mass spectrometer set in positive mode. The API-

4000 LC–MS/MS was operated under the multiple reaction-monitoring mode. The separation was performed on X-Terra MS C-18 column (4.6×50 mm, 5 μ). The mobile phase, acetonitrile/0.05 M formic acid (50:50), was isocratically 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 fragments 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 a pattern of UV spectra in HPLC-DAD and *m*/*z* values of the fragmentation products obtained in LC–MS/MS analysis.

Results and Discussion

HPLC analysis of the extracts of the cultures showed that 23 out of 39 cultures were able to metabolize celecoxib to produce one or more metabolites (Table 1) evidenced from the absence of these new peaks in drug control as well as culture controls. Representative HPLC chromatograms were depicted in Fig. 1. Most of the organisms studied 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 naturally or induced by the drug or media component(s). Most of the metabolites produced were eluted before the drug indicate that they are polar than celecoxib. The relative polarity of the metabolites also helped in their characterization. In HPLC method development, the retention time of the drug was set at 12 min so that the metabolites, being more polar in general, will elute before the drug. The metabolite peaks were identified in HPLC based on the similarity in 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 celecoxib, indicating that the drug has undergone only minor structural changes in the process of metabolism by microorganisms. This also indicates that only chemical groups have been added or deleted from the structure, and this observation reveals that there was no loss of aromaticity, ring fission, or ring fusion since any of these changes would be expected to lead to a high alteration of the metabolites' UV spectral pattern. Cha et al. [17] reported that the fungi *Cunninghamella elegans* was able to *N*-demethylate malachite green, where the metabolites showed a slight decrease (618–608 nm) in absorption maxima (visible λ_{max}) than the substrate malachite green.

The metabolites were quantified based on their peak areas in HPLC. Zhang et al. [18] and Duhart et al. [19], in their studies on microbial metabolism of cyclobenzaprine and protriptyline, respectively, quantified the metabolites based on the initial substrate concentration added. However, this may produce erroneous results, since one cannot assure 100% extraction of drug and metabolites from the culture. This error may be attributed to the intracellular localization or adsorption to the fungal cells, of some amount of drug and metabolites in the culture samples, which is not the case with drug controls where the substrate is present in freely available and easily extractable form. Hansen et al. [20], after 10 days of incubation, calculated the single metabolite formation based on the initial dose of triprolidine added to the fungal culture and found that the drug and its metabolite were accounted for 23% and 55%, respectively. They assumed that the remaining 22% of the initial dose (may contain both drug and its metabolite) might be adsorbed to the fungal cells.

Celecoxib was eluted at 12 min, and the metabolites produced were designated as M1 (4.5 min), M2 (4.7 min), M3 (6.4 min), M4 (7 min), M5 (8 min), M6 (9 min), M7 (13 min),

Table 1 Metabolites of celecoxib produced by microbial cultures.

Culture	Class	M1 (4.5 min)	M2 (4.7 min)	M3 (6.4 min)	M4 (7 min)	M5 (8 min)	M6 (9 min)	M7 (13 min)	M8 (17 min)
*Absidia coerulea*MTCC 1335	Fungi	30.27	54.92	–	–	–	–	–	–
*Absidia glauca*MTCC 982	Fungi	23.52	45.69	–	–	–	–	–	–
*Aspergillus flavipes*NCIM 1209	Fungi	–	–	–	–	–	–	–	–
*Aspergillus flavus*NCIM 554	Fungi	–	–	–	–	–	1.31	–	–
*Aspergillus flavus*NCIM 557	Fungi	–	–	–	–	–	–	–	–
*Aspergillus niger*NCIM 1006	Fungi	–	–	–	–	–	–	–	–
*Aspergillus niger*NCIM 589	Fungi	–	–	–	–	–	–	–	–
*Aspergillus niger*NCIM 620	Fungi	–	–	–	–	–	–	–	–
*Aspergillus ochraceous*NCIM 1140	Fungi	–	–	–	–	–	–	–	–
*Aspergillus parasiticus*NCIM 898	Fungi	–	–	–	–	–	–	–	–
*Bacillus subtilis*MTCC 619	Bacteria	–	1.82	–	–	–	–	–	0.57
*Beauveria bassiana*NCIM 1216	Fungi	1.1	1.8	–	–	–	–	–	–
*Cunninghamella blakesleana*NCIM 687	Fungi	–	5.57	–	–	–	–	–	–
*Cunninghamella blakesleana*NCIM 688	Fungi	67.94	1.36	0.66	0.16	0.58	–	–	–
*Cunninghamella echinulata*NCIM 691	Fungi	–	80.43	–	–	–	–	–	–
*Cunninghamella echinulata*NCIM 693	Fungi	89.63	2.92	0.43	0.24	1	–	–	–
*Cunninghamella elegans*NCIM 689	Fungi	–	–	–	–	–	–	–	–
*Cunninghamella elegans*NCIM 690	Fungi	54.42	2.71	0.22	0.09	0.44	–	–	–
Cunninghamella sp. NCIM 1184	Fungi	89.53	3.49	0.37	0.83	–	–	–	–
*Curvularia lunata*NCIM 716	Fungi	–	–	–	–	–	–	–	–
*Escherichia coli*MTCC 118	Bacteria	–	–	–	–	–	–	–	0.08
*Fusarium oxysporum*NCIM 1008	Fungi	–	0.15	–	–	–	–	–	–

Table 1 (continued).

Culture	Class	M1 (4.5 min)	M2 (4.7 min)	M3 (6.4 min)	M4 (7 min)	M5 (8 min)	M6 (9 min)	M7 (13 min)	M8 (17 min)
*Mucor plumbeus*NCIM 984	Fungi	–	–	–	–	–	–	–	–
*Mucor rouxii*MTCC 386	Fungi	–	1.34	–	–	–	–	–	–
*Pencillium brevicompactum*MTCC 549	Fungi	–	–	–	–	–	–	–	–
*Pencillium chrysogenum*NCIM 733	Fungi	–	0.38	–	–	–	–	–	–
*Pencillium chrysogenum*NCIM 738	Fungi	–	0.66	–	–	–	–	–	–
*Pseudomonas putida*NCIM 2782	Bacteria	–	–	–	–	–	–	–	–
*Rhizopus arrhizus*NCIM 997	Fungi	–	11.86	–	–	–	–	–	–
*Rhizopus stolonifer*NCIM 880	Fungi	–	0.99	–	–	–	–	–	–
*Rhodotorula rubra*NCIM 3172	Yeast	–	–	–	–	–	–	–	–
*Saccharomyces cerevesiae*NCIM 3090	Yeast	–	–	–	–	–	–	–	–
*Streptomyces griseus*NCIM 2622	Yeast	58.5	32.16	0.08	0.08	–	–	–	0.08
*Streptomyces griseus*NCIM 2623	Yeast	6.29	2.24	–	–	–	–	–	0.34
*Streptomyces lavendulae*NCIM 2827	Yeast	–	–	–	–	–	–	–	–
*Streptomyces rimosus*NCIM 2213	Yeast	55.61	21.79	0.41	0.56	8.79	2.1	3.45	0.43
Streptomyces sp. NCIM 2214	Yeast	–	0.46	–	–	–	–	–	0.53
*Thamnostylum piriforme*NCIM 974	Fungi	–	0.3	–	–	–	–	–	–
*Trichothecium roseum*NCIM 1147	Fungi	–	–	–	–	–	–	–	–

Values indicate the percentage of metabolites' area in HPLC analysis. The value is the percentage of a metabolite with respect to the total area of drug and all the metabolites formed

M1 methyl carboxylation, *M2* methyl hydroxylation, *M3* methyl carboxylation and N4 acetylation, *M4* methyl hydroxylation and N4 acetylation, *M5* methyl carboxylation and N4 methylation, *M6* methyl hydroxylation and N4 methylation, *M7* ring-*o*-hydroxylation and N4 acetylation, *M8* N4 acetylation

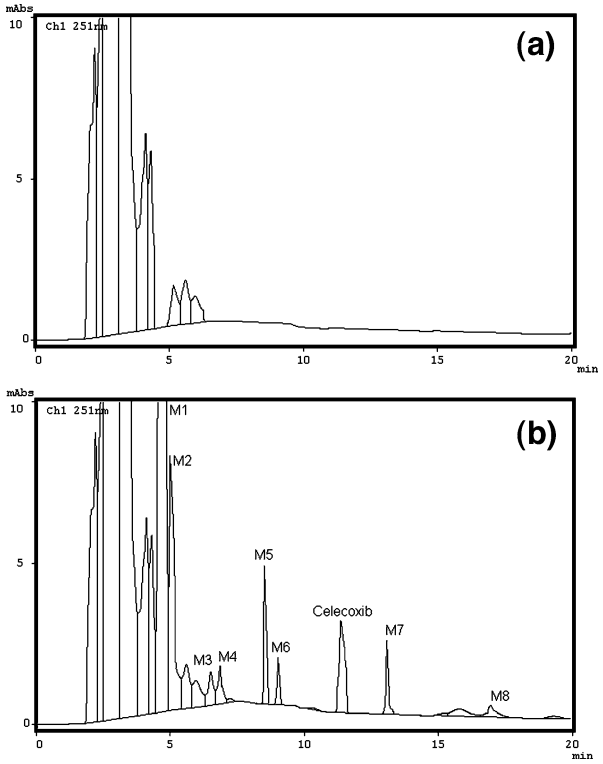

Fig. 1 HPLC chromatogram showing **a** culture control and **b** celecoxib and its metabolites' peaks (M1, M2, M3, M4, M5, M6, M7, and M8) detected in *Streptomyces rimosus* NCIM 2213

and M8 (17 min). Control culture incubations without celecoxib added showed no coincidental peaks eluting with identified metabolites. The results pertaining to the metabolite production is shown in Table 1. A large variation was observed in the quantitative metabolite production among various cultures. M2 was the metabolite found in most of the cultures. *Cunninghamella echinulata* NCIM 691 produced 80.43% of this metabolite, whereas *Fusarium oxysporum* NCIM 1008 produced only 0.15% of this metabolite. The low production of M2 in *F. oxysporum* may be due to several reasons, viz., low levels of enzyme expression required for this reaction, substrate/product inhibited biotransformation, etc.

The structure elucidation of the metabolites was carried out from the *m*/*z* values of the fragmentation ions in LC–MS/MS analysis and HPLC retention times. Fragments provided an idea about the structure, and retention time has given about the relative polarity of the transformed compound. LC–MS spectra of all these metabolites are shown in Fig. 2a–c. Fragmentation pattern for celecoxib and its major metabolites M1 and M2 are shown in Fig. 3a–c. Table 2 gives the retention times, *m*/*z* values of parent ions $(M+H)^+$, and predicted molecular formulae and metabolic reactions for various metabolites. Celecoxib and its metabolites M1, M2, M3, M4, M5, M6, M7, and M8 had shown the protonated molecular ions at *m*/*z* values 382, 412, 398, 454, 440, 426, 412, 440, and 424, respectively.

M2 gave the protonated molecular ion at *m*/*z* 398, that is, 16 units higher than celecoxib. This suggests that M2 might be a hydroxylation product of celecoxib. Based on the mass

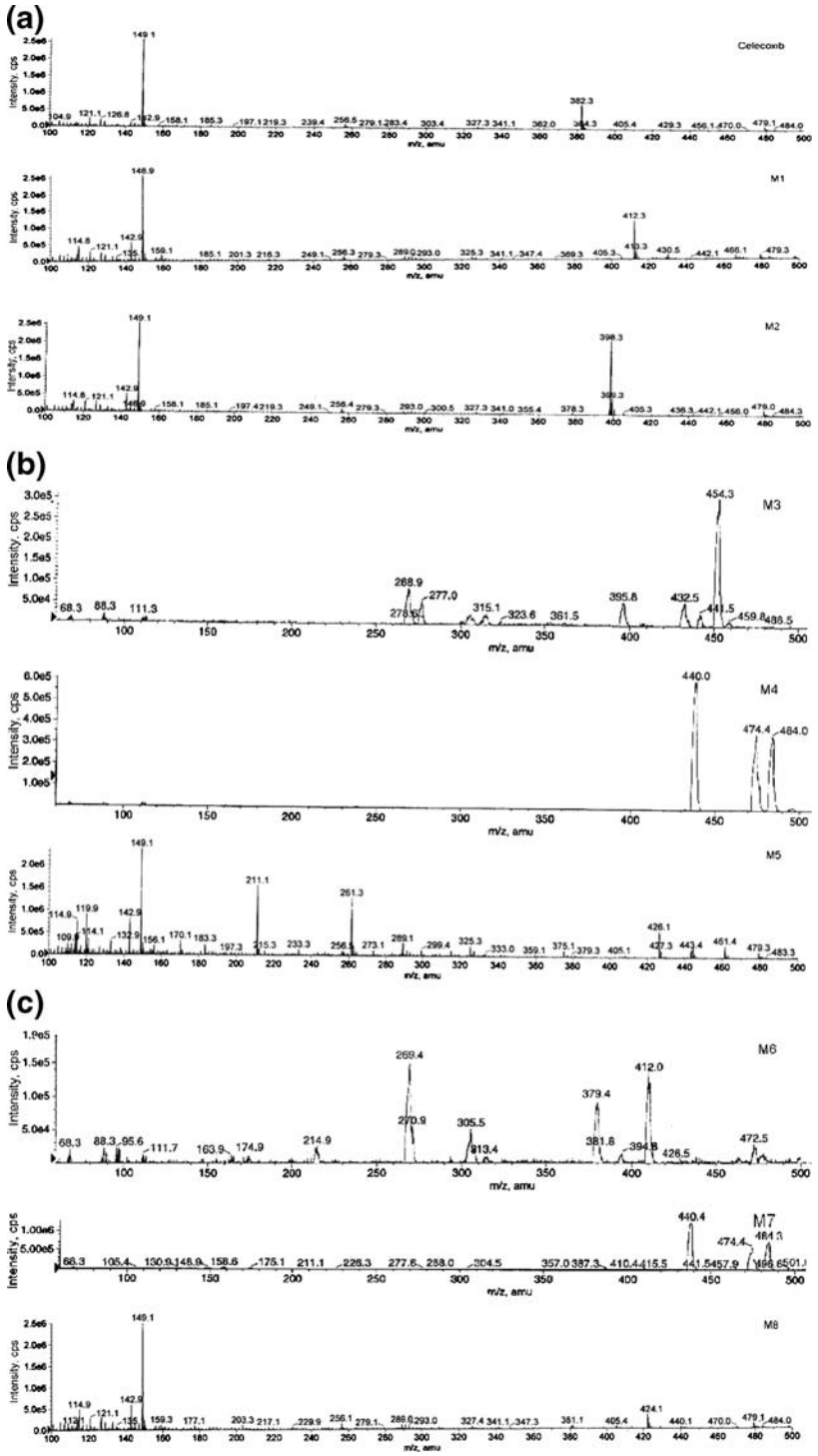

Fig. 2 **a**–**c** LC–MS spectra of celecoxib and its metabolites (M1, M2, M3, M4, M5, M6, M7, and M8)

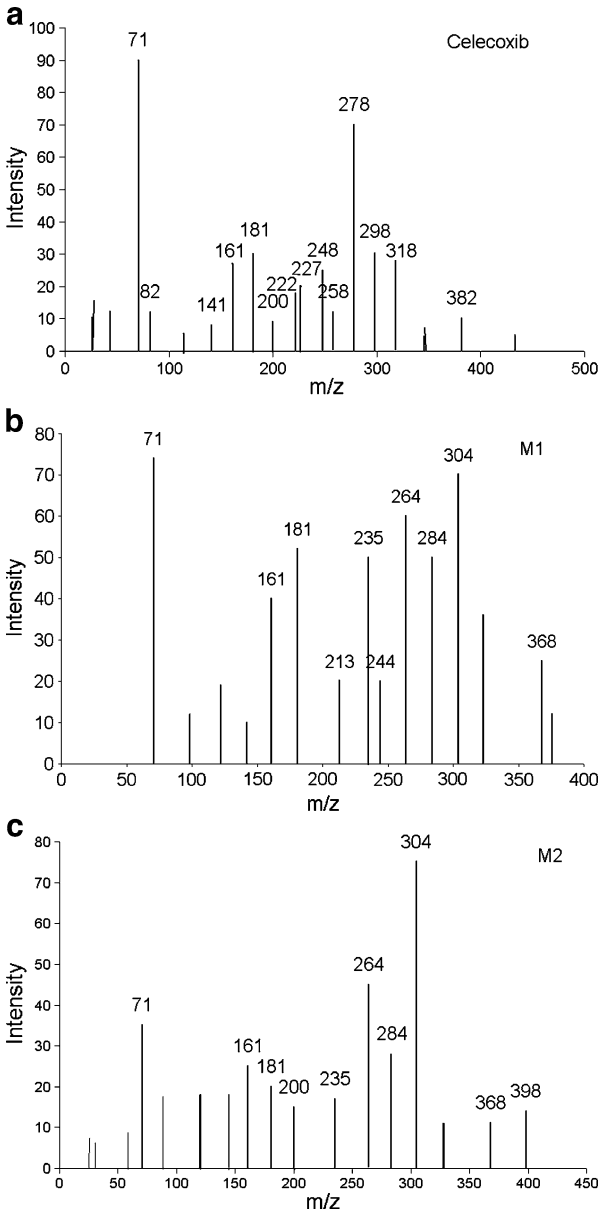

Fig. 3 **a**–**c** Mass spectral fragmentation of celecoxib and its major metabolites (M1 and M2)

fragmentation and HPLC retention time, M2 was assumed as a hydroxymethyl metabolite of celecoxib, where the hydroxylation took place on the methyl group of 5-(4-methyl) phenyl moiety. This metabolite was also formed in rabbits [21], which was found to be mediated by CYP2C9. Hansen et al. [20], Otten and Rosazza [22], Sariaslani and Rosazza [23], Hufford et al. [24], Freitag et al. [25], and Huang et al. [26] reported this type of terminal methyl hydroxylation by microorganisms. In the present study, 21 cultures have

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

Metabolite	Rt	*m/z*value	Molecular formulae	Predicted reaction
Celecoxib	11.5	382	$C_{17}H_{14}F_3N_3O_2S$	–
M1	4.5	412	$C_{17}H_{12}F_3N_3O_4S$	Methyl carboxylation
M2	4.7	398	$C_{17}H_{14}F_3N_3O_3S$	Methyl hydroxylation
M3	6.4	454	$C_{19}H_{14}F_3N_3O_5S$	Methyl carboxylation and N^4acetylation
M4	7	440	$C_{19}H_{16}F_3N_3O_4S$	Methyl hydroxylation and N^4acetylation
M5	8	426	$C_{18}H_{14}F_3N_3O_4S$	Methyl carboxylation and N^4methylation
M6	9	412	$C_{18}H_{16}F_3N_3O_3S$	Methyl hydroxylation and N^4methylation
M7	13	440	$C_{19}H_{16}F_3N_3O_4S$	Aromatic ring hydroxylation and N^4acetylation
M8	17	424	$C_{19}H_{16}F_3N_3O_3S$	N^4acetylation

produced the metabolite M2. Penning et al. [27], in their structure–activity relationship (SAR) studies, reported that the hydroxymethyl metabolite of celecoxib exhibit no COX-1 and poor COX-2 inhibitory activity.

M1 and M6 both gave the protonated molecular ion at *m/z* 412, i.e., 30 higher than celecoxib. However, M1 was eluted before M2, and M6 was eluted after M2. This indicates that M2 has undergone structural modification to a more polar metabolite M1. This polarity difference and a mass increment of 30 from celecoxib or 14 from M2 suggest that M1 might be a carboxylic acid metabolite of celecoxib. Based on the mass fragmentation and HPLC retention time, M1 was assumed to be the carboxylic acid of celecoxib where the carboxylic acid group replaced the methyl group of 5-(4-methyl) phenyl moiety. Furthermore, it was assumed that this metabolite was obtained from a second hydroxylation of the metabolite M2, i.e., $–CH_3$ to $–CH_2OH$ to –COOH. The carboxylic acid metabolite was also formed in rabbits [21]. The formation of carboxylic acid metabolite in mammals was thought to be mediated by cytosolic alcohol dehydrogenase instead of CYP2C9 [28]. Similar sequential reactions, i.e., $–CH_3$ to $–CH_2OH$ to –COOH, were found in microbial oxidation of ebastine and terfenadine using *Cunninghamella blakesleana*, *C. elegans*, *Cunninghamella echinulata*, *Streptomyces rimosus*, *Streptomyces platensis*, and *Absidia corymbifera* [29, 30]. In the study by Mazier et al. [30], it was found that the enzymes responsible for oxidation of alcohol to carboxylic acid were induced by the presence of soybean peptone in the media. The present study was also performed in the media containing peptone as the nitrogen source. Hence, it can be said that, in microbial cultures, the enzymes responsible for the formation of alcohol and carboxylic acid metabolites may or may not be the same. In general, oxidation of alcohols into carboxylic acids takes place in two steps, via the formation of aldehydes, and are catalyzed by alcohol dehydrogenase and aldehyde oxidoreductase. All these reports together suggest that the formation of carboxylic acid metabolite in the present study was mediated by alcohol dehydrogenase type of enzyme instead of CYP. This type of sequential oxidation reactions by microorganisms was reported previously with many substrates [20, 31–34]. In the present study, ten cultures have produced this metabolite M1. This metabolite was being produced from M2 by successive oxidation on alcoholic group. Penning et al. [27], in their SAR studies, reported that the carboxylic acid metabolite of celecoxib exhibit no COX-1 and poor COX-2 inhibitory activity.

M8 gave the protonated molecular ion at *m*/*z* 424, that is, 42 higher than the parent drug celecoxib, indicating that this might be an acetylated metabolite. This metabolite was eluted at 17 min in HPLC, indicating that the compound is less polar than the parent drug. Hence, it is assumed that the acetylation occurred on the sulfonamido group of the molecule. The fragmentation of this metabolite has shown a characteristic product ion peak at *m*/*z* 303, indicating a loss of 121 units ($SO_2NHCOCH_3$) and that the acetylation has occurred on the sulfonamido group of celecoxib. The *N*-acetylation of various organic compounds by microorganisms was extensively reported [19, 35–41]. In the present investigation, few microbial cultures produced N-acetylation of the sulfonamido group. Acetylation of the para amino group of the sulfonamides is a prime example of acetylation in mammalian metabolism reactions. In mammals, the N-acetylation takes place in many tissues, including liver, GIT, etc. However, the acetylated metabolites were not detected in any of the mammalian system for the metabolism of celecoxib. Since N-acetylated metabolites are more hydrophobic, they might reabsorb back in the mammalian system to undergo further metabolism. In the present celecoxib N^4-acetylation, the microbial N-acetyltransferase enzyme transfers the acetyl group to NH_2 in the sulfonamide moiety to form N-acetylated metabolite. The acetylation enzymes utilize acetyl-coenzyme A to form an acetylated enzyme intermediate that transfers the acetyl group to an acceptor, usually an amine or hydrazine group. The amine group is thought to be the acceptor of acetyl group in the celecoxib metabolism by microorganisms. In the present study, six cultures have produced this metabolite. Interestingly, two bacterial and four yeast cultures have produced this reaction, and none of the fungi studied had shown this reaction. The pathway for the formation of this metabolite from celecoxib might involve direct acetylation of amino group. This metabolite was reported to be 3.3 times more potent inhibitor toward COX-1 than COX-2 [42].

M4 and M7 both gave the protonated molecular ion at *m*/*z* 440, that is, 16 higher than M8, indicating that these might be hydroxylated products of the acetylated metabolite M8 or acetylated products of the hydroxylated metabolite M2. M7 was eluted at 13 min in HPLC, indicating that the compound is less polar than the parent drug and more polar than M8. M4 was eluted at 7 min, indicating that the compound is more polar than both the celecoxib and M8. This polarity differences may be attributed to the site of hydroxylation of M8. Usually, ring hydroxylated products are less polar than the side chain hydroxylated products. Hence, it is assumed that M4 is the methyl hydroxylated product of M8, and M7 is the ring hydroxylated product of M8. Both the metabolites M4 and M7 were assumed to be formed via M8. The formation of M4 might also take place from M2; however, this is a rare event, since in the process of metabolism, less polar molecules will be converted to more polar metabolites. This M4 metabolite was found to be produced by six cultures in the present study, out of which four cultures produced M2 but not M8. Two out of these six cultures produced both M2 and M8.

The ionization spectrum of M7 produced product ions at *m*/*z* 319 and 304, 299, 279, and 180. M7 has shown a characteristic fragment ion at *m*/*z* 319, with a loss of $SO_2NHCOCH_3$ (121) from *m*/*z* 440, indicating that the hydroxylation did not occur on the sulfonamido group of the molecule. The product ions with *m*/*z* 304, 299, and 279 were assumed to produced from M7 by further sequential losses (from *m*/*z* 319) of CH_3 (15), HF (20) and HF (20), respectively. The product ion of *m*/*z* 180 was formed by the loss of $CH_3C_6H_4OC$ (119) from *m*/*z* 299, suggesting that the hydroxyl group was located on the phenyl moiety of the 5-(4-methyl)phenyl group. Dirikolu et al. [43] reported the possibility of involving ring hydroxylation of the celecoxib benzyl group ortho to the methyl group. Based on these data, M7 was identified as the celecoxib metabolite with N-

acetylation and phenyl-*o*-hydroxylation with the hydroxylation occurring on the 5-(4-methyl) phenyl group. This M7 metabolite was produced in only one culture *Streptomyces rimosus* NCIM 2213, which also produced M8. Hence, M7 was predicted to be formed via M8.

M3 gave its protonated molecular ion peak at m/z 454, i.e., 14 higher than M4 and M7. M4 is the methyl hydroxylated and N-acetylated metabolite of celecoxib, and there is a possibility that the hydroxymethyl group is undergoing further metabolism to produce carboxylic acid derivative of acetylated metabolite. The fragmentation pattern of this metabolite is in agreement with this. Hence, M3 can be predicted as N-acetylated and carboxylic acid metabolite of celecoxib, where the carboxylic acid is formed on the pyrazole ring of the substrate. This assumption is in agreement with the polarity of M3, which was eluted before celecoxib, M4, M7, and M8. This M3 metabolite was produced in six cultures, out of which four cultures produced M1 and two cultures produced both M1 and M8. The pathway for the formation of M3 might include acetylation of M1 or successive double hydroxylation of M8 on its methyl group.

M6 showed a protonated molecular ion peak at *m*/*z* 412, i.e., 30 higher than the parent drug. Another 412 peak was already assigned with carboxylic acid metabolite of celecoxib. This M6 metabolite was eluted at 9 min, i.e., before celecoxib and N-acetylated metabolite but after M1, M2, M3, and M4. Based on the increment in *m*/*z* value and polarity difference, M6 was assumed to be of N-methylated and methyl hydroxylated metabolite of celecoxib. M5 showed protonated molecular ion peak at *m*/*z* 426, i.e., 14 higher than M6. This was eluted just before M6. It is assumed that the metabolite may be carboxylic acid derivative of N-methylated metabolite of celecoxib; that is, M5 is the N-methylated and carboxylic acid metabolite of celecoxib, where the carboxylic acid is being formed on the pyrazole ring. However, there is no N-methylated metabolite detected in any of the cultures, which indicates that the metabolites M5 and M6 were formed either from M1 and M2 or instable N-methylated metabolite. This unstable N-methylated metabolite might be instantaneously converting to M6 and then to M5.

Many amines are methylated in the mammalian metabolism, where the products are usually biologically active, sometimes more so than the parent compound. In the present celecoxib N^4-methylation, the microbial enzymes transfer the methyl group to NH_2 in the sulfonamide moiety to form N-methylated metabolite. Usually, the cofactor S-adenosyl methionine (SAM) serves as a methyl donor in mammalian drug metabolism studies. The methylation is a two-step process whereby the cofactor SAM that transfers a methyl group is first biosynthesized from methionine. Once available, SAM is utilized by a methyltransferase to transfer an activated methyl group to an acceptor molecule, viz., alcohol, amine, or thiol. The methyltransferases are primarily cytosolic in origin, although some microsomal forms have also been found. Penning et al. [27], in their SAR studies, reported that the N-methylation of celecoxib at the sulfonamide moiety resulted in compounds with no COX-2 inhibitory activity.

All the reactions involved in the biotransformation of celecoxib by various cultures are depicted in Fig. 4. Some of the cultures produced interesting results in the formation of metabolites. *Aspergillus flavus* NCIM 554 produced M6 as the only metabolite. This methyl carboxylation and N-methylation metabolite might be formed from the carboxylic acid metabolite M1 or unstable N-methylated metabolite, which was not detected in any of the cultures. In this case, N-methylated metabolite might be undergoing instantaneous conversion to M5 and then to M6. However, M5 was not detected in this culture. Hence, it can be assumed that M6 is forming from M1 by N-methylation.

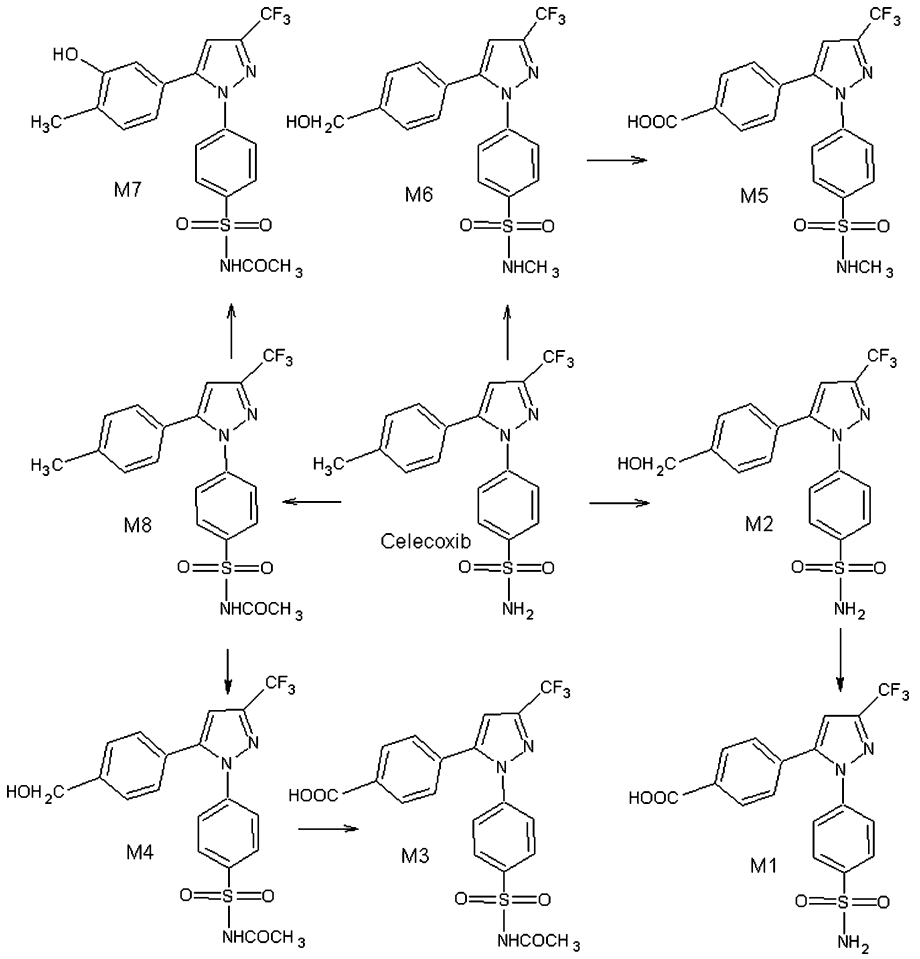

Fig. 4 Scheme showing the biotransformation of celecoxib in microbial cultures

Some of the cultures employed in the present study did not produce any metabolites for celecoxib. This may be attributed to the absence of substrate specific enzymes required for biotransformation. It may also be due to the substrate not reaching the site of biotransformation, i.e., lack of membrane transport processes involved to get the substrate into the site where the enzymes are known to be located.

Conclusion

From the present investigation, it could be observed that microbial transformation studies can be used as models to simulate mammalian drug metabolism. Celecoxib was metabolized by the studied microorganisms into reported human and animal metabolites. Few novel metabolites were also produced, which might be promising to develop new drug entities with interesting pharmacological activities.

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References

1. Smith, R. V., & Rosazza, J. P. (1975). *Journal of Pharmaceutical Sciences*, *11*, 1737–1759.
2. Smith, R.V. and Rosazza, J.P. (1982) In J. P. Rosazza (Ed.), Microbial transformations of bioactive compounds (pp.1–42). Boca Raton: CRC
3. Smith, R. V., & Rosazza, J. P. (1983). *Journal of Natural Products*, *46*, 79–91.
4. Clark, A. M., McChesney, J. D., & Hufford, C. D. (1985). *Medicinal Research Reviews*, *5*, 231–253.
5. Clark, A. M., & Hufford, C. D. (1991). *Medicinal Research Reviews*, *11*, 473–501.
6. Abourashed, E. A., Clark, A. M., & Hufford, C. D. (1999). *Current Medicinal Chemistry*, *6*, 359–374.
7. Ferris, J. P., MacDonald, L. H., Patrie, M. A., & Martin, M. A. (1976). *Archives of Biochemistry and Biophysics*, *175*, 443–452.
8. Venisetty, R. K., & Ciddi, V. (2003). *Current Pharmaceutical Biotechnology*, *4*, 123–140.
9. Paulson, S. K., Hribar, J. D., Liu, N. W. K., Hajdu, E., Bible, R. H., Jr., Piergies, A., et al. (2000). *Drug Metabolism and Disposition*, *28*, 308–314.
10. Paulson, S. K., Zhang, J. Y., Breau, A. P., Hribar, J. D., Liu, N. W. K., Jessen, S. M., et al. (2000). *Drug Metabolism and Disposition*, *28*, 514–521.
11. Backhus, L. M., Petasis, N. A., Uddin, J., Schonthal, A. H., Bart, R. D., Lin, Y., et al. (2005). J. *Journal of Thoracic and Cardiovascular Surgery*, *130*, 1406–1412.
12. Pyrko, P., Soriano, N., Kardosh, A., Liu, Y. T., Uddin, J., Petasis, N. A., et al. (2006). Mol. *Cancer*, *5*, 19.
13. Abbate, F., Coetzee, A., Casini, A., Ciattini, S., Scozzafava, A., & Supuran, C. T. (2004). *Bioorganic & Medicinal Chemistry Letters*, *14*, 337–341.
14. Solomon, S.D., McMurray, J.J.V., Pfeffer, M.A., Wittes, J., Fowler, R., Finn, P., Anderson, W.F., Zauber, A., Hawk, E. and Bertagnolli, M. (2005) *New Eng. J. Med. 352*, 1071–1080.
15. Venisetty, R. K., Keshetty, S., & Ciddi, V. (2004) Abstract 64th International Pharmaceutical Federation Congress, New Orleans, p. 16.
16. Jayasagar, G., Kumar, M. K., Chandrasekhar, K., Prasad, P. S., & Rao, Y. M. (2002). *Pharmazie*, *57*, 619–621.
17. Cha, C. J., Doerge, D. R., & Cerniglia, C. E. (2001). *Applied and Environmental Microbiology*, *67*, 4358–4360.
18. Zhang, D., Evans, F. E., Freeman, J. P., Yang, Y., Deck, J., & Cerniglia, C. E. (1996). *Chemico-Biological Interactions*, *102*, 79–92.
19. Duhart, B. T., Zhang, D., Deck, J., Freeman, J. P., & Cerniglia, C. E. (1999). *Xenobiotica*, *29*, 733–746.
20. Hansen, E. B., Jr., Heflich, R. H., Korfmacher, W. A., Miller, D. W., & Cerniglia, C. E. (1988). *Journal of Pharmaceutical Sciences*, *77*, 259–264.
21. Zhang, J. Y., Wang, Y. F., Dudkowski, C., Yang, D., Chang, M., Yuan, J., et al. (2000). *Journal of Mass Spectrometry*, *35*, 1259–1270.
22. Otten, S., & Rosazza, J. P. (1981). *Journal of Natural Products*, *44*, 562–568.
23. Sariaslani, F. S., & Rosazza, J. P. (1985). *Applied and Environmental Microbiology*, *49*, 451–452.
24. Hufford, C. D., Lee, I. S., ElSohly, H. N., Chi, H. T., & Baker, K. T. (1990). *Pharmaceutical Research*, *7*, 923–967.
25. Freitag, D. G., Foster, R. T., Coutts, R. T., Pickard, M. A., & Pasutto, F. M. (1997). *Drug Metabolism and Disposition*, *25*, 685–692.
26. Huang, H., Yang, X., Li, Q., Sun, L., & Zhong, D. (2006). *Applied Microbiology and Biotechnology*, *72*, 486–491.
27. Penning, T. D., Talley, J. T., Bertenshaw, S. R., Carter, J. S., Collins, P. W., Docter, S., et al. (1997). *Journal of Medicinal Chemistry*, *40*, 1347–1365.
28. Sandberg, M., Yasar, U., Stromberg, P., Hoog, J. O., & Eliasson, E. (2002). *British Journal of Clinical Pharmacology*, *54*, 423–429.
29. Schwartz, H., Liebig-Weber, A., Hochstätter, H., & Böttcher, H. (1996). *Applied Microbiology and Biotechnology*, *44*, 731–735.
30. Mazier, C., Jaouen, M., Sari, M., & Buisson, D. (2004). *Bioorganic & Medicinal Chemistry Letters*, *14*, 5423–5426.

31. Mountfield, R. J., & Hopper, D. J. (1998). *Applied Microbiology and Biotechnology*, *50*, 379–383.
32. Rosi, D., Peruzotti, G., Dennis, E. W., Berberian, D. A., Freele, H., Tullar, B. F., et al. (1967). *Journal of Medicinal Chemistry*, *10*, 867–876.
33. Schwartz, H., Licht, R. E., & Radunz, H. E. (1993). *Applied Microbiology and Biotechnology*, *40*, 382–385.
34. Zhang, D., Zhang, H., Aranibar, N., Hanson, R., Huang, Y., Cheng, P. T., et al. (2006). *Drug Metabolism and Disposition*, *34*, 267–280.
35. Clark, A. M., Hufford, C. D., & McChesney, J. D. (1981). *Antimicrobial Agents and Chemotherapy*, *19*, 337–341.
36. Clark, A. M., Evans, S. L., Hufford, C. D., & McChesney, J. D. (1982). *Journal of Natural Products*, *45*, 574–581.
37. Foster, G. R., Coutts, R. T., Pasutto, F. M., & Mozayani, A. (1988). *Life Sciences*, *42*, 285–292.
38. Foster, B. C., Wilson, D. L., & McGilveray, I. J. (1989). *Xenobiotica*, *19*, 445–452.
39. Foster, G. R., Lister, D. L., Zamecnic, J., & Coutts, R. T. (1991). *Canadian Journal of Microbiology*, *37*, 791–795.
40. Wetzstein, H. G., Stadler, M., Tichy, H. V., Dalhoff, A., & Karl, W. (1999). *Applied and Environmental Microbiology*, *65*, 1556–1563.
41. Parshikov, I. A., Freeman, J. P., Lay, J. O., Jr., Beger, R. D., Williams, A. J., & Sutherland, J. B. (1999). *FEMS Microbiology Letters*, *177*, 131–135.
42. Pal, M., Madan, M., Padakanti, S., Pattabiraman, V. R., Kalleda, S., Vanguri, A., et al. (2003). *Journal of Medicinal Chemistry*, *46*, 3975–3984.
43. Dirikolu, L., Lehner, A. F., Jacobs, J., Woods, W. E., Karpiesiuk, W., Harkins, J. D., Carter, W. G., Boyles, J., Hughes, C. G., Bosken, J. M., Holtz, C., Natrass, C., Fisher, M., Tobin, T. (2000) Proceedings of the 13th International Conference of Racing Analysts and Veterinarians, Cambridge, pp. 162–170.