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CYP-dependent Metabolism of Antitumor Pyrazolo[3,4-d]pyrimidine Derivatives Is Characterized by an Oxidative Dechlorination Reaction

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Summary: The aim of this study is to investigate the metabolic (cytochrome P450-dependent) behaviour of pyrazolo[3,4-d]pyrimidines 1–10 dual Abl/Src kinase inhibitors. All the compounds demonstrate good metabolic stability both in human liver (HLM) and in rat liver (RLM) microsomes. Moreover, all the tested molecules undergo the same metabolic CYP-dependent reactions, namely oxidative dechlorination and N-dealkylation. These metabolic pathways are fully characterized for compound 1. In HLM, the dehalogenated metabolite accounts for about 87% of the full 1 metabolism, while the N-dealkylated metabolite accounts for 12%. Inhibition studies performed using different CYP-inhibitors indicate that the 3A family is the isoenzyme family most involved in pyrazolo[3,4-d]pyrimidine metabolism. This observation is confirmed by studies performed by using CYP3A selective substrates. Furthermore kinetic analysis performed in RLM, HLM and cDNA CYP3A4 shows that the affinity of CYPs towards compound 1 is similar in all the tested preparations (Km = 32.7, 21.8, and 48.7 µM, respectively).

Keywords: anticancer agents; cytochrome P450; CYP3A4; liver microsomes; metabolite kinetics; metabolite identification

Introduction

In recent years, attention has been focused on the importance of studies on the absorption, distribution, metabolism, excretion and toxicity (ADMET) of drug candidates. These investigations are performed simultaneously with the evaluation of compound activity, with the aim of speeding up the drug discovery process and advancing high quality candidates to clinical studies. The ideal candidate must possess the satisfactory pharmacokinetic and toxicological features identified by the ADMET properties. Non-compliance with these optimal requirements by a drug candidate accounts for the failure of about 50% of new potential therapeutic agents in late clinical studies. The goal of a successful drug discovery program is therefore not only the identification of a new molecule highly active and selective towards a suitable molecular target, but also the early assessment of its ADMET properties, in order to discard those compounds that are unlikely to be successful drug candidates.1)

In this context, the cytochrome P450 family (CYP450) plays a crucial role both in metabolism and in the toxicity of a drug. CYP450 enzymes can be inhibited or induced by drugs, resulting in clinically significant drug-drug interactions that can cause unpredictable adverse reactions or therapeutic failures. Therefore, the knowledge of the CYP-dependent metabolism of a drug candidate can help to minimize the possibility of adverse drug reactions and interactions. In the last few years our research group synthesized a library of 4-amino substituted pyrazolo[3,4-d]pyrimidines (consisting of about 400 structurally characterized compounds with purity >98%), which proved to be potent Bcr-Abl/Src inhibitors. Briefly, Bcr-Abl and Src are two cytoplasmic tyrosine kinases involved in different pathologies, first of all in cancer. In fact, Bcr-Abl is the causative agent of chronic myeloid leukemia (CML),2) and Src is overexpressed or hyperactivated in both solid and hematologic malignancies. For these reasons, dual Bcr-Abl/Src

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inhibitors represent an attractive field of research aimed at the discovery of new anticancer drugs, as demonstrated by the approval for clinical use of the dual Bcr-Abl/Src inhibitor dasatinib for the treatment of CML.\(^3\)

Our pyrazolo[3,4-d]pyrimidines inhibit both Src and Ab1 in assays on the isolated enzymes, with IC\(_{50}\) values in the sub-micromolar and more frequently nanomolar ranges. The different potency toward one or the other tyrosine kinase depends on the substitution pattern of the pyrazolo[3,4-d]pyrimidine scaffold.\(^4,5\) At the cellular level, these inhibitors induce apoptosis and reduce cell proliferation in different solid tumor cell lines which over-express Src, such as epidermoid carcinoma A431 and breast cancer 8701-BC cells,\(^6,7\) osteosarcoma SaOS-2 cells,\(^8\) prostate cancer PC3 cells,\(^8\) HT22 medulloblastoma\(^9\) and SH-SY5Y neuroblastoma cells.\(^9\) In addition, this new class of inhibitors is well tolerated in engraftment experiments with different tumor cell lines.\(^9,11,12\) Other studies showed that selected derivatives are potent inhibitors of angiogenesis in vivo on a zebrafish model.\(^3\) A number of C6-unsubstituted pyrazolo-pyrimidines are able to inhibit the proliferation of three Bcr-Abl-positive human leukemia cell lines (K-562, KU-812, and MEG-01), to reduce Bcr-Abl tyrosine phosphorylation and to promote apoptosis of Bcr-Abl expressing cells.\(^14\)

As a continuation of our preliminary investigation on the metabolism of pyrazolo[3,4-d]pyrimidines and on the basis of computational predictions that demonstrated their high metabolic stability,\(^15\) we herein report the characterization of the metabolic pathway of pyrazolo[3,4-d]pyrimidine compounds 1–10 (Table 1), in order to support their further development as promising antitumor agents and in vivo pharmacokinetic study subjects.

The CYP-dependent reactions were studied in detail for test compound 1, analyzing the kinetic parameters, metabolites and CYP isozymes involved.

Compound 1 is particularly interesting because it is a promising antileukemia lead. Indeed, it is active on Ba/F3 cell lines transducing both the wild type Bcr-Abl construct and Y253F, E255K and T315I Bcr-Abl mutated forms, with a LC\(_{50}\) range of 0.7–4.3 \(\mu\)M.\(^16\) The activity toward T315I is particularly important, since this mutation confers resistance to the available kinase inhibitors for CML therapy, including imatinib, dasatinib and nilotinib.

Moreover, 1 also shows antiproliferative effects on CD34\(^+\) cells, collected from IM-resistant patients bearing the T315I mutation, with LC\(_{50}\) values in the range 0.14–0.38 \(\mu\)M. Conversely, this derivative exhibited a much lower toxicity against CD34\(^+\) cells obtained from healthy people.\(^17\)

### Materials and Methods

**Chemicals and reagents:** NADP, NADPH, D-glucose-6-phosphate, glucose-6-phosphate-dehydrogenase benzyllox-resorufin (BZR), oxidized glutathione, glutathione reductase, and tris[hydroxymethyl]amionemethane (TRIZMA-base) were all purchased from Sigma-Aldrich (Milan, Italy). Resorufin, KCl, EDTA, [3-[3,4-difluorobenzyloxy]-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(SH)-one] (DFB) and [3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(SH)-one] (DFH) were provided by Merck Canada. Pooled human liver microsomes (HLM) and cDNA-expressed human CYP3A4, 3A5, 2C9, 2D6 (supersomes) were obtained from BD Biosciences (Woburn, MA). All other chemicals and solvents were of the highest grade available and obtained from common commercial sources.

**Synthesis of compounds 1–10, M1, M2 and M5:**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R(_1)</th>
<th>R(_2)</th>
<th>M1 N-dealkylation</th>
<th>M2 N-dealkylation</th>
<th>M3 or M4 oxidative dechlorination</th>
<th>M5 oxidative dechlorination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bn</td>
<td>H</td>
<td>13.57</td>
<td>9.67</td>
<td>3.95</td>
<td>72.16</td>
</tr>
<tr>
<td>2</td>
<td>4-F-Bn</td>
<td>Cl</td>
<td>5.02</td>
<td>4.81</td>
<td>0.1</td>
<td>90.14</td>
</tr>
<tr>
<td>3</td>
<td>4-F-Bn</td>
<td>H</td>
<td>16.90</td>
<td>8.60</td>
<td>0.1</td>
<td>74.42</td>
</tr>
<tr>
<td>4</td>
<td>4-F-Bn</td>
<td>F</td>
<td>10.96</td>
<td>6.96</td>
<td>0.1</td>
<td>82.03</td>
</tr>
<tr>
<td>5</td>
<td>4-F-Bn</td>
<td>Br</td>
<td>11.77</td>
<td>14.36</td>
<td>0.1</td>
<td>73.84</td>
</tr>
<tr>
<td>6</td>
<td>4-Cl-Bn</td>
<td>H</td>
<td>9.14</td>
<td>6.43</td>
<td>0.1</td>
<td>84.41</td>
</tr>
<tr>
<td>7</td>
<td>2-F-Bn</td>
<td>Cl</td>
<td>6.76</td>
<td>7.90</td>
<td>0.1</td>
<td>85.29</td>
</tr>
<tr>
<td>8</td>
<td>3-F-Bn</td>
<td>Cl</td>
<td>6.00</td>
<td>7.02</td>
<td>0.1</td>
<td>86.96</td>
</tr>
<tr>
<td>9</td>
<td>CH2-Bn</td>
<td>Cl</td>
<td>3.94</td>
<td>5.43</td>
<td>0.1</td>
<td>85.28</td>
</tr>
<tr>
<td>10</td>
<td>N</td>
<td>Cl</td>
<td>0.1</td>
<td>15.29</td>
<td>0.1</td>
<td>84.69</td>
</tr>
</tbody>
</table>

**Supplemental Table 1s in Supplemental materials.**

**HPLC-UV-MS analysis:** Quantitative analysis, and metabolite recognition and separation were performed by means of the LC-UV and MS method. LC analysis were performed with the Agilent 1100 LC/MSD VL system (G1946C) (Agilent Technologies, Palo Alto, CA) constituted by a vacuum solvent degassing unit, a binary LC system, an autosampler, an Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument with LC-ESI-UV and MS method. LC analysis were performed with the Agilent 1100 LC/MSD VL system (G1946C) (Agilent Technologies, Palo Alto, CA) constituted by a vacuum solvent degassing unit, a binary LC system, an autosampler, an Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument with LC-ESI-UV and MS method. LC analysis were performed with the Agilent 1100 LC/MSD VL system (G1946C) (Agilent Technologies, Palo Alto, CA) constituted by a vacuum solvent degassing unit, a binary LC system, an autosampler, an Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument with LC-ESI-UV and MS method. LC-UV-MS determination was performed by operating the MSD in the positive ion mode. Spectra were acquired over the scan range m/z 50–1,500 using a step size of 0.1 u.

Chromatographic analysis was performed using a Varian Polaris...
using basis of calibration curves realized with reference compounds and volume was 20 µL. Quantitative analysis was performed on the synthetic time, MS spectra and fragmentation behavior with those of M1 lites (the dehydrochlorinated-oxidate UV analysis). The fractions containing the two principal metabolites (M1 and M2) were characterized by LC-MS, NMR and IR analysis and all the spectra were compared with pure compounds synthesized in our lab.

**CYP-dependent metabolism of pyrazolo[3,4-d]pyrimidine derivatives:** The animal protocols used were reviewed and approved by the Animal Care and Ethics Committee of the Università degli Studi di Siena, Italy. The rat liver microsomes (RLM) were prepared as previously reported.\(^\text{19}\) The pellets, containing the microsomal fraction, were suspended in 2 mL of Tris–HCl buffer (10 mM, pH 7.6) containing 0.154 M KCl and 1 mM EDTA and stored at −80°C until their use. The protein content of RLM was determined according to Lowry et al.\(^\text{20}\) using serum albumin as a standard.

To investigate the CYP-dependent metabolism of compounds 1–10, an incubation mixture (final volume 500 µL) was prepared, consisting of 125 mM phosphate buffer, pH 7.4, containing 0.2 mg·mL\(^{-1}\) of HLM or 0.4 mg·mL\(^{-1}\) RLM protein, and the tested compounds (at a final concentration of 50 µM). The reaction was started by adding NADPH-GS. After incubation at 37°C in a shaking incubator up to 60 min or 15 min for HLM or RLM, respectively, the reaction was terminated by adding 1 mL of cold ACN. Blanks containing boiled microsomes were incubated under the same conditions.

Kinetic analysis of CYP-dependent metabolism of compound 1 both in humans and rats was performed by incubating microsome or supersome preparations expressing human CYPs in the presence of variable concentrations of substrate (0.1–100 µM). The quantification of the parent drug and metabolites was subsequently achieved by HPLC-UV-MS analysis. In different experiments, performed under the same experimental conditions, CYP2C9, 2D6, 3A4 and 3A5 were tested in the presence of compound 1 or M5 or M1.

The quantification of the parent drug and metabolites was subsequently determined by HPLC-UV-MS analysis.

**In vitro inhibition of compound 1 metabolism in human liver microsomes:** Preliminary inhibition experiments were carried out by incubating HLM with 50 µM of the following inhibitors: α-naphthoflavone (ANF), 8-methoxypsoralen (8-MP), sulfaphenazole (SPZ), quinidine (QND), 4-methylpyrazole (4-MPY) and ketoconazole (KTZ).\(^\text{21}\) These inhibitors are considered to be relatively specific for some CYP enzymes (CYPs 1A, 2A6, 2C9, 2D6, 2E1, 3A respectively). The reaction mixture contained 0.2 mg·mL\(^{-1}\) of human microsomal protein and 50 µM of inhibitor in 125 mM phosphate buffer, pH 7.4. The CYP inhibitor was added to microsomes and preincubated for 5 min. The reaction was then started by adding 50 µM of compound 1 and NADPH-GS. After 60 min the reaction was stopped by adding 1 mL of cold ACN containing compound 12 as an internal standard and analyzed as described above. In a second series of experiments CYP-dependent metabolism of compound 1 was studied in the presence of variable concentrations of ketoconazole (0.03–100 µM).

In the successive experiments the inhibition effect of compound 1 on CYP 3A family was studied by using a marker substrate such as DFB ([3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5H)-one]) for the human 3A4 isoform and BZR (benzoxylresorufin) for rat CYP, as described by D’Elia et al.\(^\text{21}\) The studies were performed at different concentrations of marker substrate (12.5–50.0 µM for DFB and 1.0–2.5 µM for BZR) in the presence of (0–100 µM) compound 1, in order to calculate the kinetic parameter (K). To assess the reversibility of the inhibition, HLMs (2 mg

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Fig. 1. Metabolic pathway obtained for compound 1 with HLM experiments

5 C18-A column (150 x 4.6 mm, 5 µm particle size) at room temperature. Analysis was carried out using gradient elution of a binary solution; eluent A was ACN (acetonitrile), while eluent B consisted of an aqueous solution of formic acid (0.1%). The analysis started at 0% A for 3 min, then rapidly increased to 98% in 12 min and finally remained at 98% A until 18 min. The analysis was performed at a flow rate of 0.8 mL min\(^{-1}\) and the injection volume was 20 µL. Quantitative analysis was performed on the basis of calibration curves realized with reference compounds and using 12 (Supplemental Fig. 1s),\(^\text{20}\) an analogue of the compounds 1–10, as an internal standard.

Details on validation of the chromatographic method are reported in Supplemental materials.

**Structure determination of compound 1 metabolites:** A suitable amount of metabolites necessary for their full characterization was obtained by incubation of RLM (2 mg protein) in the presence of 1 mM compound 1 and a NADPH-generating system, (NADPH-GS) containing 1 mM NADPH, 4 mM glucose-6-phosphate and 1 U/mL glucose-6-phosphate-dehydrogenase in 48 mM MgCl\(_2\). The isolation of each single metabolite was performed using the above reported chromatographic separation method (LC-UV analysis). The fractions containing the two principal metabolites (the dehydrochlorinated-oxidate M1 and the N-dealkylated M2) were collected separately and evaporated to dryness prior to the spectroscopic analysis.

The structure of the third most abundant metabolite (the dechlorinated-oxidate M5), which is formed only with human microsomes, was further confirmed by comparison of its retention time, MS spectra and fragmentation behavior with those of synthetic M5\(^\text{40}\) available in our laboratory (see Supplemental Table 1s).

All metabolites were characterized by LC-MS, NMR and IR analysis and all the spectra were compared with pure compounds synthesized in our lab.
protein·mL⁻¹) were incubated in the presence of compound 1 (at a concentration twice the $K_v$ value) and NADPH. After 15 min, samples were diluted tenfold with buffer and the enzyme activity was assayed by adding DFB. The decrease of the concentration of a reversible inhibitor was expected to give rise to a decrease of inhibition, whereas no restoration of activity occurs for an irreversible inhibitor.²¹

Data analysis: Data were reported as means ± S.E.M. of at least three different experiments. Kinetic parameter ($V_{\text{max}}$ and $K_v$) were calculated according to the Michaelis-Menten equation for one or two enzymes by nonlinear regression analysis while $K_v$ was calculated from the Dixon Plot by using GraphPad Prism version 4.03 (GraphPad Software, La Jolla, CA).

Statistical analyses and determination of significance as measured by ANOVA (followed by Dunnett’s post test)²² or by the One-sample t test, were performed by means of GraphPad InStat version 3.06 (GraphPad Software). In all comparisons, $p < 0.05$ was considered significant.

Results

Metabolic stability and metabolite identification of pyrazolo[3,4-d]pyrimidine derivatives: The study performed on RLM preparations showed that compound 1, beside its potent in vivo biological activity, represents a good substrate for CYP-dependent metabolism (Table 1). In fact, at a 50 µM concentration, about 30% of compound 1 was metabolized after 15 min of incubation while, under the same experimental conditions, most of the tested compounds gave a lower percentage of metabolites (the only exceptions being compounds 3 and 5).

A typical chromatogram, obtained after 15 min of incubation of compound 1 with RLM, is reported in Supplemental Figure 1s-A, where compound 12 was used as an internal reference. The HPLC profile showed the presence of different metabolites identified by UV and MS analysis. A similar chromatogram was obtained for the other derivatives tested. The uncommon dehydrohalogenated-oxidized product M1 resulted as the major metabolite of several tested compounds, while the other abundant metabolite was represented by the N-dealkylated species M2. Two minor peaks characterized by $m/z$ value and MS spectra corresponding to the hydroxylated derivatives (metabolites M3 and M4) were observed, mainly for compounds 1 and 9 when incubated in presence of RLM. Those peaks, corresponding to the identified metabolites, were not observed when the compound 1 was incubated in the presence of either boiled RLM or HLM, or in the absence of NADPH-GS, indicating that the formation of all the products is a result of a microsomal enzymatic reaction.

Similarly to the previous results obtained with RLM, compound 1 was the best pyrazolo[3,4-d]pyrimidine substrate for HLM. The analysis showed the formation of the two metabolites M1 and M2 after 1 h of incubation (Table 1 and Supplemental Fig. 1s-B) and of an additional metabolite M5, corresponding to an alcohol derivative, produced by a dechlorination reaction (Table 1).

The main metabolites of compounds 2–10 were separated by HPLC (using the method reported in the experimental section for compound 1) and characterized by MS and fragmentation analysis (data not reported). Metabolites M1, M2, M3, M4 and M5 cited hereafter refer to compound 1 (Fig. 1).

The identification of the CYP-dependent dechlorinated metabolites M1 and M5 was confirmed by comparison of their MS spectra with those of the same synthetic compounds obtained in our lab (see Supplemental materials).

Kinetic study of compound 1 metabolism: The CYP-dependent metabolism of compound 1 was characterized in terms of the kinetic parameters and CYP isoenzyme involvement.

Preliminary assays of the CYP-dependent metabolism of 1 in HLM and RLM showed that the amount of metabolites increased linearly with time, up to 60 min, and that the initial rates were proportional to the amount of microsomal protein added to the reaction mixture (up to 0.4 mg·mL⁻¹ for HLM and up to 2 mg·mL⁻¹ for RLM). These experiments allowed us to define the optimum parameters regarding the incubation time and the protein concentration: i) 60 min and 0.2 mg·mL⁻¹ for HLM; ii) 40 min and 0.4 mg·mL⁻¹ for RLM. In all the experiments with HLM, M1 was the principal metabolite, with a concentration about 2.5 fold greater than M2 and M5 (Fig. 2B, the inset represents an Eadie-Hofstee plot that showed monophasic kinetics). In the presence of RLM, M1 and M2 were formed at a similar rate, while M5 was not detected (Fig. 2A, the inset represents an Eadie-Hofstee plot that showed monophasic kinetics). Furthermore, the hydroxylated metabolites M3 and M4 were observed when compound 1 was incubated at concentrations higher than 50 µM and their formation was inhibited when ketoconazole was present in the medium (data not shown).

As reported in Figure 2, the kinetics of the interaction of compound 1 with CYP followed a Michaelis-Menten hyperbolic curve. In HLM, the apparent $K_v$ values for M2 and M5 formation were comparable, while M1 formation presented a $K_v$ value of one order of magnitude higher (Table 2). The apparent $V_{\text{max}}$ value was

![Fig. 2. Michaelis-Menten plots showing the metabolism of compound 1 to M1(▲), M2(●) or M5(○)](image)

Compound 1 (0.1–100 µM) was incubated in the presence of RLM (A: 0.4 mg protein·mL⁻¹) or HLM (B: 0.2 mg protein·mL⁻¹), and the metabolites were determined by HPLC-UV-MS as described in Materials and Methods. The inset represents the Eadie-Hofstee plot of the same data. $V$ is expressed as mmol·min⁻¹·mg protein⁻¹ and $V/S$ as mmol·min⁻¹·mg protein⁻¹·µM⁻¹; (A: $K_v = 32.7$; 25.5 and $V_{\text{max}}$ = 1.004; 0.850 for M1 and M2, respectively; B: $K_v = 21.8$; 4.2; 4.9 and $V_{\text{max}}$ = 0.228; 0.086; 0.101 for M1, M2 and M5, respectively). Data are expressed as mean ± SEM of 3 different experiments.
about three fold higher for M1 formation compared to M2 and M5 formation.

In RLM preparations, the apparent \( V_{\text{max}} \) and \( K_m \) values for M1 and M2 formation were similar to each other, and higher than those observed in HLM preparations (Table 2). As shown in Table 2, the ratio \( V_{\text{max}}/K_m \), as an index of intrinsic clearance (CL\(_{\text{int}}\)), indicated that the metabolism of compound 1 was lower in human than in rat preparations; in fact the ratios \( V_{\text{max}}/K_m \) for M1 and M2 were 1.6 to 2.3 fold greater in RLM.

**Inhibition of compound 1 metabolism in HLM:** In order to define the type of CYPs involved in the metabolism of compound 1, an inhibition study was performed by incubating compound 1 at 50 \( \mu \)M concentration, in the presence of CYP selective inhibitors (Fig. 3). Ketoconazole (KTZ in Fig. 3, selective for CYP3A) promoted a significant inhibition of metabolite formation (97%, 90%, and 80% for M1, M2, and M5, respectively). In the presence of sulphaphenazole (50 \( \mu \)M SPZ in Fig. 3), only M2 formation was inhibited, although to a lesser extent (–42%) (Fig. 3B). It is important to underline that ketoconazole showed a strong concentration-dependent inhibition of compound 1 metabolism with an IC\(_{50}\) value of 9.9 \( \times \) 10\(^{-8}\), 4.5 \( \times \) 10\(^{-8}\) and 8.9 \( \times \) 10\(^{-8}\)M for the M1, M2 and M5 formation, respectively (Fig. 4). At the highest ketoconazole concentration (100 \( \mu \)M), M1 and M2 formation was almost abolished, while M5 formation was still present at 25%, compared to the control (i.e. without ketoconazole). These results indicated that the CYP3A family plays the most important role in the metabolism of compound 1.

Further experiments were performed to evaluate a possible inhibition of CYP3A4 by compound 1 and if this inhibition is competitive or noncompetitive.

For this scope an experiment was performed with a DFB probe substrate of CYP3A4 and 1 in the presence of HLM (see experimental section). Compound 1 inhibited the metabolism of DFB in a dose-dependent manner. A kinetic analysis performed following the Dixon procedure (Supplemental Fig. 2s) suggested that such an inhibition is competitive, with a \( K_i \) value of 28 \( \mu \)M.

In a second set of experiments, HLMs were pre-incubated with compound 1, at two fold the \( K_i \) value, in the presence of NADPH at different times up to 60 min. After this pre-incubation, the DFB substrate was added and the substrate concentration was measured after 60 min of incubation.\(^{19}\) In all the experiments, the inhibition of DFB metabolism exerted by 1 did not prove to be dependent on pre-incubation time (data not shown).

Reversible inhibition of DFB metabolism exerted by compound 1 was assessed by dilution experiments. Reaction mixtures containing microsomes, compound 1 and NADPH were pre-incubated for 15 min and then diluted 10 fold. The observed residual DFB-oxidase activities exhibited values close to those of control con-

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**Table 2. Kinetic parameters obtained for the metabolites of 1 with human and rat microsomes**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Human microsomes</th>
<th>CYP 3A4 supersomes</th>
<th>Rat microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1</td>
<td>M2</td>
<td>M5</td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>0.288 ± 0.086</td>
<td>0.101</td>
<td>1.745 ± 0.258</td>
</tr>
<tr>
<td>( K_m )</td>
<td>21.8 ± 4.2</td>
<td>4.9</td>
<td>48.7 ± 15.3</td>
</tr>
<tr>
<td>CL(<em>{\text{int}}) (( V</em>{\text{max}}/K_m ))</td>
<td>0.013 ± 0.020</td>
<td>0.020</td>
<td>0.036 ± 0.017</td>
</tr>
</tbody>
</table>

\( V_{\text{max}} \) is mmol min\(^{-1}\)mg protein\(^{-1}\) in the case of HLMs and RLMs; pmol min\(^{-1}\)pmol CYP3A4\(^{-1}\) for supersomes. \( K_m \) value is reported as \( \mu \)M.

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**Fig. 3. Inhibition of metabolite M1 (panel A), M2 (panel B) and M5 (panel C) formation by different CYP inhibitors α-naphthoflavone (ANF), 8-methoxypsoralen (8-MP), sulphaphenazole (SPZ), quinidine (QND), 4-methylpyrazole (4-MPY) and ketoconazole (KTZ) at a concentration of 50 \( \mu \)M for each inhibitor.** Compound 1 was 50 \( \mu \)M. The mixture was incubated in the presence of HLM (0.2 mg protein-L\(^{-1}\)) at 37°C for 1 h. The metabolites formed were determined by HPLC-UV-MS as described in Materials and Methods. The comparison between metabolite formation in the absence (control) and in the presence of inhibitors was performed by using the ANOVA test followed by Dunnett’s post test. Data are expressed as mean ± S.E.M. of 3 different experiments (**p < 0.01 vs. control**).

**Fig. 4. Concentration-dependent inhibition of compound 1 metabolism in the presence of ketoconazole.** HLMs (0.2 mg protein-L\(^{-1}\)) were incubated in the presence of 1 (50 \( \mu \)M) and variable concentrations of ketoconazole (0.03–100 \( \mu \)M). Data are expressed as mean ± S.E.M. of 3 different experiments.
ditions (no compound 1 added), suggesting that the inhibition of DFB metabolism was reversible (Supplemental Fig. 3s). A similar experiment was performed on RLM using BZR (benzoxoxyresorufin) as the CYP substrate. The $K_i$ value, calculated as reported above, was 2.8 µM and the inhibition was not time-dependent (data not shown).

**Kinetic analysis of compound 1 metabolism in supersomes expressing human CYP3A4:** In order to confirm the role of CYP3A4 in the metabolism of compound 1, a series of experiments was performed using cDNA-CYP3A4. The kinetic analysis of the metabolism of compound 1 is reported in Figure 5. According to the previous observations in HLM, M1 was the major metabolite with a $K_m$ value comparable to that obtained with RLM. On the other hand, the apparent $K_m$ value for M2 formation was 3.6 fold higher in supersomes than in HLM preparations. The calculated $V_{max}$ were 145.5 ± 19.8 and 21.50 ± 2.2 pmol-min⁻¹·mg protein⁻¹, corresponding to 1.74 ± 0.2 and 0.258 ± 0.026 pmol-min⁻¹·pmol CYP3A4⁻¹ for M1 and M2 formation, respectively ($K_m$ M1 = 48.74 ± 1.65; $K_m$ M2 = 15.25 ± 0.59). Moreover it should be underlined that M5 was not formed when 1 was incubated in the presence of supersomes.

The possible involvement of other CYP isozymes in promoting the metabolism of compound 1 was studied by incubating compound 1 with a sup persome preparation expressing CYP3A5, 2C9 and 2D6. As reported in Table 3 only CYP3A4 and 3A5 were able to promote M1 formation. Furthermore, 1 µM ketoconazole was able to abolish almost entirely the formation of M1 promoted by CYP3A4, while the CYP3A5 activity was only inhibited by 30%.

Detailed studies were performed with the aim of verifying the reaction mechanism that leads to the formation of the metabolites M1 and M5, using compounds M5 and M1 respectively as substrates, incubated in the presence of HLM, RLM, c-DNA-expressed CYP2C9, 2D6, 3A4 and 3A5.

Experiments were performed by incubating pure M5 (synthesized in our lab) with HLM, RLM and c-DNA-expressed human CYP2C9, 2D6, 3A4 and 3A5 in separate tests. M5 was used at a concentration of 50 µM (equivalent to the $K_m$ value for M1 formation; Table 2).

Under these experimental conditions M1 formation was observed when M5 was incubated in the presence of RLM, HLM or c-DNA-CYP3A4 and 3A5, while in supersomes expressing 2C9 and 2D6 the M1 formation was not detectable. It is interesting to note that the amount of M1 formed by incubation with RLM or c-DNA-CYP3A4 and 3A5 was higher than that formed by HLM (Table 4).

In order to verify which enzymatic reactions are involved in the alcohol M5 formation, M1 (synthesized in our lab) was incubated in the presence of HLM and NADPH. Under our experimental conditions M1 was metabolized, forming the alcohol metabolite M5. Furthermore, this reaction was not observed when HLM was incubated in the presence of a mixture of NAD⁺ and NADP⁺, or in the absence of NADPH. Addition of dicumarol (50 µM), a keto-redirectase inhibitor, resulted in slight inhibition of this enzymatic activity (Table 5). This reaction was observed only using HLM, while with RLM, cDNA CYP3A4 and 3A5 the alcohol M5 formation was not detectable (data not shown).

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**Table 3. Formation rate of alcohol (M5) and ketogenic (M1) metabolites from compound 1 in different microsomal or cDNA CYP preparations**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite</th>
<th>RLM</th>
<th>HLM</th>
<th>2C9</th>
<th>2D6</th>
<th>3A4</th>
<th>3A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M1</td>
<td>241.2 ± 2.6</td>
<td>110.3 ± 1.2</td>
<td>ND</td>
<td>ND</td>
<td>66.2 ± 1.7</td>
<td>73.0 ± 2.9</td>
</tr>
<tr>
<td>1 + ketoconazole</td>
<td>M1</td>
<td>22.0 ± 0.3</td>
<td>12.8 ± 0.5</td>
<td>ND</td>
<td>ND</td>
<td>1.8 ± 0.5</td>
<td>51.7 ± 1.8</td>
</tr>
<tr>
<td>1</td>
<td>M5</td>
<td>ND</td>
<td>43.5 ± 0.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1 + ketoconazole</td>
<td>M5</td>
<td>ND</td>
<td>8.1 ± 0.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. of 3 different experiments.

+ Fifty µM of compound 1, alone or in the presence of 1 µM ketoconazole, was incubated in the presence of different CYP preparations as reported in Materials and Methods.
+ Rat liver microsomes. Human liver microsomes.
+ Supersome expressing human CYP2C9.
+ Supersome expressing human CYP2D6.
+ Supersome expressing human CYP3A4.
+ Supersome expressing human CYP3A5.
+ Not detected.

**Table 4. Formation rate of ketogenic (M1) metabolite from M5 in different microsomal or cDNA CYP preparations**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite</th>
<th>RLM</th>
<th>HLM</th>
<th>2C9</th>
<th>2D6</th>
<th>3A4</th>
<th>3A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>M5</td>
<td>M1</td>
<td>70.6 ± 1.2</td>
<td>14.4 ± 0.9</td>
<td>ND</td>
<td>ND</td>
<td>19.7 ± 2.3</td>
<td>44.6 ± 0.2</td>
</tr>
<tr>
<td>M5 + ketoconazole</td>
<td>M1</td>
<td>1.2 ± 0.6</td>
<td>2.9 ± 0.6</td>
<td>ND</td>
<td>ND</td>
<td>1.3 ± 1.0</td>
<td>13.5 ± 1.9</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. of 3 different experiments.

+ Fifty µM of compound M5, alone or in the presence of 1 µM ketoconazole, was incubated in the presence of different CYP preparations as reported in Materials and Methods.
+ Rat liver microsomes. Human liver microsomes.
+ Supersome expressing human CYP2C9.
+ Supersome expressing human CYP2D6.
+ Supersome expressing human CYP3A4.
+ Supersome expressing human CYP3A5.
+ Not detected.

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Table 5. Formation rate of alcohol metabolite (M5) from M1 in human liver microsomes (HLMs)

<table>
<thead>
<tr>
<th>Substratea</th>
<th>M5 formation (HLM)</th>
<th>% formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (M1+ NADPH)</td>
<td>43.6 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td>M1+ dicumarol + NADPHb</td>
<td>38.7 ± 1.5</td>
<td>88.3</td>
</tr>
<tr>
<td>M1 + NADPH+</td>
<td>2.4 ± 0.1</td>
<td>4.6</td>
</tr>
<tr>
<td>M1 – NADPHc</td>
<td>1.4 ± 0.1</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. of 3 different experiments.

a Fifty µM of compound M1, alone or in the presence of 50 µM ketoconazole, was incubated in HLM preparations. b NADPH 200 µM in final volume. c Dicumarol 50 µM in final volume. d NADPH 200 µM in final volume. e NADP 200 µM in final volume. /Without NADPH.

Discussion

In the drug discovery process, the early characterization of the metabolic profile of a new biologically active compound gives information that might be used to guide further modifications of its chemical structure, in order to obtain other derivatives endowed with more favorable metabolic properties. In this context, the determination of enzyme kinetic data regarding metabolic reactions can be a tool for the estimation of the pharmacokinetic parameters. Furthermore, the use of human enzymes, cells and liver fractions gives additional insights for a more accurate prediction of the human in vivo behavior.

Among the enzymes involved in the xenobiotic metabolism, CYP enzymes are the most versatile oxidative proteins. The basic CYP reactions include C-hydroxylation, heteroatom oxygenation, heteroatom release (dealkylation), and epoxide formation. However, in some instances the CYP isoenzymes promote more complex reactions that include reduction, desaturation, ester cleavage, ring expansion, ring formation, aldehyde scission, dehydroxylation, one-electron oxidation, and coupling reactions. A surprising datum from our study was the identification of a metabolic reaction for the CYP-mediated metabolism of pyrazolo[3,4-d]pyrimidine derivatives, involving the oxidation of the substituent on the N1 phenylalkyl chain. In fact, the incubation of compound 1 with HLM allowed us to identify two metabolites, M1 and M5, a ketone and an alcohol respectively formed by an oxidative dechlorination reaction. In these microsomal preparations the dehalogenated metabolites M1 and M5 accounted for about 87% of compound 1 metabolism under our experimental conditions, while the N-dealkylated metabolite M2 resulted in another 12%. A similar metabolic pathway involving the oxidative dechlorination reaction was observed using RLM. In this case, however, both the N-dealkylation (leading to M2) and dechlorination (leading to M1) pathways presented a similar reaction rate, with M1 as the only dechlorinated metabolite observed.

He et al.240 reported the oxidation of 12-halododecanoic acid to 12-hydroxydodecanoic acid promoted via ω-hydroxylation by CYP4A1. In this case the authors evidenced two types of oxidative reactions. The first is a conventional reaction where the enzyme hydroxylates the halogen-substituted carbon to give 12-halo-12-hydroxydodecanoic acid, which undergoes intramolecular elimination of HX (X = halide ion) to yield the corresponding aldehyde. The latter is then partially oxidized to the acid in a second step by a well-established reaction mediated by CYP4A1. The second mechanism involves the direct oxidation of the chlorine by the P450 ferryl intermediate, producing the corresponding oxochloronium species that is displaced by water to give the alcohol.

From the present results we can speculate that compound 1 was metabolized to M5, and rapidly oxidized to M1, probably in the same enzymatic active site of CYP3A, which could be able to allocate more than one molecule of substrate.25 M5 formation exclusively in HLM could be ascribed to a lower rate of M1 formation from M5 compared to the other CYP preparations (RLM, cDNA-CYP3A4 and 3A5; Table 4). However, it is not possible to exclude the possibility that other enzymatic reactions could be involved in the alcohol M5 formation; in fact, the alcohol metabolite M5 was found after incubation of M1 in the presence of HLM and NADPH and slightly inhibited in presence of 50 µM dicumarol (Table 5). From this evidence we can conclude that a NADPH-dependent ketone-reductase, able to reduce M1, was only present in HLM preparations. The formation rate of M5 from M1 was much lower (~68%) than the one observed when compound 1 was incubated in the presence of HLM under similar experimental conditions, demonstrating that this reaction is a secondary pathway, compared to the CYP’s oxidations and could partially contribute to M5 formation.

The contribution of CYP3A5 was also tested. The CYP3A5-dependent M1 formation rate was similar to that observed in the presence of CYP3A4 (73.0 pmol min⁻¹ mg protein⁻¹ and 66.2 pmol min⁻¹ mg protein⁻¹ for CYP3A5 and CYP3A4, respectively). The presence of ketoconazole (1 µM) in CYP3A5 experiments inhibited by 30% the M1 formation rate. On the other hand, ketoconazole inhibited M1 formation more than 90% when CYP3A4 or HLM was used (Table 3). All these data suggest that CYP3A5 partially contributes to the overall metabolism of compound 1, while CYP3A4 represents the isozyme most involved in the metabolism, this being in accordance to the evidence that ketoconazole inhibited this metabolic pathway in HLM with an IC₅₀ lower than 1 µM (Fig. 4).26,27 To summarize, the study suggests that the dehalogenation-oxidation reaction on our derivatives occurs following the second mechanism proposed by He et al.240 The chlorine substituent is directly oxidized by the heme group of P450, forming the oxochloronium species. The latter is displaced by water, forming the alcohol, which is oxidized to form the ketone.

The results of this study indicate that CYP3A4 is the CYP isozyme most involved in the metabolism of compound 1. In fact, according to the application note of the producer (130 pmol-min-mg protein⁻¹ and 83.3 pmol-min-mg protein⁻¹ in HLM and cDNA-CYP3A4 supersome, of P450, respectively), CYP3A4 accounts for 66% and 37% of the overall formation of M1 and M2 promoted by HLM at the maximum concentration (100 µM) of compound 1.

Furthermore, in accordance to the “well stirred” model, the intrinsic clearance can be converted in hepatic clearance using the following equation as reported by Mohutsky et al.28

\[
CL_H = \frac{Q \times CL_{int}}{Q + CL_{int}}
\]

where Q is the liver blood flow of 21 mL·min⁻¹·kg⁻¹. According to our data the CL_H was 8.02 mL·min⁻¹·kg⁻¹ for M1 and 10.2 mL·min⁻¹·kg⁻¹ for M2 and M5, respectively, suggesting that the liver presents a similar capacity for all three identified metabolites.

In this paper we reported the metabolic behaviour of compound 1, which is characterized by an oxidative dehalogenation reaction. Although other experiments are needed to fully characterize the ADME properties of the studied molecules, this work demonstrates
that compound 1 shows a very high metabolic stability, being metabolized by liver microsomes at low capacity and without promoting an irreversible inhibition of CYP3A4 enzymes. The present study, given the different behaviour of compound 1 in experiments with HLM and RLM, also points out the importance of performing a metabolic study using human microsomes before drawing a conclusion.

References


Claudio Zamperini, *et al.*