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Detection and Identification of Reactive Drug Metabolites Leading to Idiosyncratic Toxicity: Lapatinib as a Case Example

Rayane Mohamed[1,2], Flavia Storelli[2], Jonathan Sidibe[1], Nasim Bararpour[1], Jules Desmeules[2,3], Marc Ausburger[1], Youssef Daali[2,3*] and A Thomas[1,4]

[1]Unit of Toxicology, CURML, Lausanne University Hospital, Geneva University Hospitals, Ch. de la Vulliette 4, 1000 Lausanne 25, Switzerland
[2]Clinical Pharmacology and Toxicology Service, Anesthesiology, Pharmacology and Intensive Care Department, Geneva University Hospitals, Geneva, Switzerland
[3]Faculty of Medicine, University of Geneva, Switzerland
[4]Faculty of Biology and Medicine, University of Lausanne, Ch. de la Vulliette 4, 1000 Lausanne 25, Switzerland

Abstract

New pharmaceutical drug development requires more than a billion dollars and can take 12 years of research effort. New chemical entities or NCEs can be dropped from development for many reasons during this process, two major reasons being efficacy and toxicity. Lapatinib was approved in 2007 by FDA as an orally active drug against breast cancer. While some advantages were observed over chemotherapy, clinical evidences of idiosyncratic hepatoxicity were reported for this compound. Numerous studies showed that lapatinib was extensively metabolized and the formation of reactive metabolites could be the origin of the observed toxicity. It has been shown that CYP3A are the main drug metabolizing enzymes involved in the metabolism pathway of lapatinib. This study aims to identify and detect potential reactive metabolites of lapatinib formed after *in vitro* microsomal incubation using a comprehensive workflow for drug metabolism studies. Using micro ultra-high performance liquid chromatography coupled to high resolution mass spectrometry, we were successful to identify a new quinoneiminine reactive metabolite, which upon conjugation with gluthatione (GSH) formed a GSH related product. This product was further characterized by tandem mass spectrometry, using data-dependent and neutral loss scans. This workflow could be successfully applied to other drugs and pharmaceuticals in order to allow the comprehensive mapping of metabolism pathways and the potential identification of reactive species.

Keywords: Lapatinib; Reactive metabolites; Hepatoxicity; High resolution mass spectrometry

Introduction

Lapatinib is an orally active drug against breast cancer and other solid tumors [1]. Briefly, its activity results from the reversible inhibition of both epidermal growth factor receptor and human epidermal growth factor receptor-2 tyrosine kinases [2], hence, belonging to the so-called tyrosine kinase inhibitors (TKI) drug family. Since, this targeted anticancer drug specifically inhibits cellular processes that are deregulated in tumor cells, it was initially considered as less toxic than conventional chemotherapy [3]. Existing clinical evidence indicates that TKI are associated with idiosyncratic hepatoxicity [4]. Different mechanisms were proposed to explain the mechanism of toxicity of TKI; one hypothesis is the formation of reactive metabolites (RM) as they are often involved in idiosyncratic hepatoxicity [5]. Lapatinib is metabolized mainly by cytochrome P450 3A4/3A5 to form O- and N-dealkylated metabolites [6-8]. A number of the metabolites generated from the metabolism by CYP3A4 and CYP3A5 could potentially form reactive electrophilic intermediates that could contribute to hepatoxicity [6]. The O-dealkylated metabolites have the structural feature that is susceptible to oxidation leading to reactive quinoneimine species [8]. Formation of such reactive species can have also a toxicological significance because modifications of biological macromolecules by reactive metabolites are associated with various drug toxicities [9]. In the case of lapatinib the CYP3A4 enzyme catalyzes the formation of the quinoneimine reactive metabolite [8]; it was demonstrated that this enzyme can be inactivated by another lapatinib metabolite probably a nitroso intermediate compound [7]. A similar process can be encountered with CYP3A5 [10]. CYP3A being the main metabolizing enzyme present in liver, the reactivity of lapatinib metabolites can be related to the risk of idiosyncratic hepatoxicity.

The increasing need of incorporating metabolite profiling for ADME properties, in early drug development, lead to the use of different tools to predict *in vitro* toxicity [11,12]. New strategies for performing *in vitro* metabolism studies have been developed recently [13,14]. These strategies are often based on the hyphenation of liquid chromatography (LC) and mass spectrometry-based techniques (MS). Those techniques provide numerous advantages such as high separation capabilities especially when using ultra performance liquid chromatography (UPLC), high detector sensitivity, specificity and resolution [15,16]. Since its introduction in the early 2000s, Orbitrap technology was used in many fields of application [15,17,18]. Their use in drug metabolism field has tremendously increased due to the new acceptably priced instruments and their capacity to provide high resolution tools for quantitative/qualitative applications [15]. Monitoring of isobaric species remains challenging even using state-of-the art HRMS. Therefore, a proper chromatographic set-up must be considered to separate such species but also to prevent ion suppression effect commonly observed using ESI-MS [19]. In this work, we used a microflow ultra high performance liquid chromatography system to enhance separation and

***Corresponding author:** Youssef Daali, Faculty of Medicine, Hopitaux Universitaires de Geneve APSI Rue Gabrielle-Perret-Gentil 4 Geneva, 1205 Switzerland, E-mail: youssef.daali@unige.ch

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sensitivity [20,21]. We take advantage of the features of both a hybrid quadrupole Orbitrap and a hybrid Quadrupole linear ion trap to detect and identify potential drug metabolites through the example of lapatinib as a case model. A comprehensive strategy for drug metabolism studies is presented for the characterization of lapatinib metabolism. *In vitro* microsomal incubations are used to generate lapatinib metabolites, UHPLC-HRMS is then performed to identify potential metabolites and data processing are used to exploit the large dataset generated by HRMS instrumentation. Tandem mass spectrometry was used through data dependent and neutral loss scans for the structural elucidation of a new quinoneimine reactive metabolite detected in UHPLC-HRMS and confirmed by targeted MRM analysis. This workflow could be successfully applied to other drugs and pharmaceuticals in order to allow the comprehensive mapping of metabolism pathways and the potential identification of reactive species.

Materials and Methods

Standard and reagents

Lapatinib ditosylate was provided by Toronto Research Chemicals (Toronto, ON, Canada), acetonitrile (ACN), methanol (MeOH) were HPLC-MS grade and were provided by Merck (Darmstadt, Germany), as well as magnesium chloride. Dimethyl sulfoxide (DMSO) was provided by Applichem (Darmstadt, Germany). Ultra-pure water was obtained through our Millipore system (Bedford, MA, USA). Formic acid 99% HPLC-MS grade, as well as nicotinamide adenine dinucleotide phosphate disodium salt (NADP), isocitric deshydrogenase, isocitric acid, alamethicin, uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS) and gluthatione were obtained through Sigma-Aldrich (Buchs, Switzerland). For preparation of the phosphate buffer, potassium dihydrogenophosphate and potassium hydroxide were provided by BDH Laboratory Supplies (Poole, England) and Sigma Aldrich, respectively. Pooled human microsomes from 20 donors were obtained from BD Biosciences (Allschwill, Switzerland). Human recombinant cytochromes P450 obtained from insect cells infected with recombinant baculovirus (Baculosomes®) as well as S9 fractions were obtained from Life technologies (Carlsbad, CA, USA). For cell culture, Williams medium E, fetal bovine serum (FBS) trypsin, Dulbecco's Phosphate-Buffer Saline and GlutamaxTM were purchased from Invitrogen (Grand Island, NY, USA). Penicillin/streptomycin and hydrocortisone were supplied from Sigma-Aldrich (Buchs, Switzerland). Insuline (Humalog®) was purchased from Lilly (Indianapolis, IN, USA).

Lapatinib was dissolved in methanol to reach a stock solution concentration of 2 mg/mL. Different incubation solutions were prepared from the stock solution by evaporation using a Mivac concentrator (Genevac Ltd, Suffolk, United Kingdom) followed dissolution and sterilization through a Millex filter with pore size of 0.22 µM (Merck, Darmstadt, Germany). Lapatinib was dissolved in the same medium as the proliferation medium but deprived of fetal bovine serum in order to avoid drug binding to serum proteins. In addition, DMSO was added in the incubation medium at a final concentration of 0.25% DMSO to ensure solubility of the test substance. All incubations were performed in triplicates.

Incubations with human liver microsomes

Lapatinib at a final concentration of 20 µM was preincubated with pooled human liver microsomes (final concentration 0.5 mg/ml) in phosphate buffer 0.1 M (pH 7.4) for 3 min in a 37°C water bath and reaction was initiated by addition of a NADPH-generating system solution (NADP 0.4 mM, isocitrate 2 mM, $MgCl_2$ 2 mM, and isocitrate dehydrogenase 0.4 IU/mL in phosphate buffer 0.1 M pH 7.4). The final methanol concentration in the reaction mixture (250 µl) was 1%. After one h at 37°C, the reaction was quenched by addition of 125 µl ice-cold acetonitrile. The mix was then centrifuged 3 min at 10'000 g (Eppendorf 5417C, Eppendorf, Hamburg, Germany) and the supernatant was stored at -20°C until further analyses. The same workflow using the trapping agent gluthatione at a final concentration of 10 mM was performed in triplicates.

In order to study glucuronidation, alamethicin (final concentration 25 µg/ml) and $MgCl_2$ (final concentration 1 mM) were added in the reaction mixtures and similar incubations as described above were performed in triplicates with UDPGA as a cofactor to initiate the reaction (final concentration 5 mM).

Incubations with human recombinant CYP isoenzymes

Baculosomes® expressing CYP3A4, 3A5, 1A2, 2E1, 2C9, 2C8, 2D6 and 2J2 at final concentrations of 0.1 pmol/µl were incubated with lapatinib 20 µM and NADPH-generating system in phosphate buffer 0.1 M pH 7.4 (final volume 50 µl) for 1 h à 37°C. Final methanol concentration was 1%. Reaction was stopped by addition of 50 µl ice-cold methanol. After centrifugation (3 min, 10,000 g) supernatants were recovered and stored until further analyses. All analyses with the different CYP isoenzymes were performed in triplicates.

Incubations with S9 fractions

In order to study the involvement of sulfotransferases, lapatinib at a final concentration of 20 µM was incubated with S9 fraction (final concentration 0.5 mg/ml) in a phosphate buffer 0.1 M pH 7.4. The reaction mixture was preincubated 3 min at 37°C and then PAPS was added as cofactor to initiate the reaction. After 1 h incubation at 37°C, the reaction was quenched by addition of 125 µl methanol to the reaction mixture. Then the supernatant was recovered by the same procedure described for microsomal incubations.

Similar procedures were applied with the addition of GSH (final concentration 1 mM) in order to observe GSH adducts and with addition of recombinant CYP3A5 baculosomes (0.1 pmol/µl) and GSH 1 mM to study the involvement of CYP3A5 in the formation of GSH adducts.

Liquid chromatography and mass spectrometry

HRMS analysis of lapatinib was performed on a Dionex Ultimate 3000 RSLC nano (Dionex, Thermo Scientific, Reinach) system coupled to a Q Exactive Plus mass spectrometer (Thermo Scientific, Reinach). The chromatographic column was an Acquity UPLC BEH C18 (1.0×100 mm, 1.7 µm). The mobile phases were constituted by A: H_2O with 0.1% FA and B: ACN+0.1% FA. The linear gradient program was 0-0.5 min 2%B, 0.5-8 min up to 95%B, 8-9 min held at 95% B, 9-9.5 min down to 2%B and 9.5-13 min held at 2%B at a flow rate of 45 µL/min. The column was heated at 45°C using the Dionex column oven and a volume of 2 µL was injected onto the column. The Qexactive Plus Orbitrap mass spectrometer was operated in positive electrospray ionization mode with an electrospray voltage of 4.0 kV. The sample was sprayed in the mass spectrometer with the sheath, auxiliary and sweep gas set at 10, 3 and 0 arbitrary units, respectively. In full scan mode a resolution R=70000 was set up. Single ion monitoring (SIM) data dependence was enabled to get MS2 data of metabolites and structural elucidation. Triple quadrupole experiment was performed on a 5500 QTRAP (AB Sciex, Concord, Canada) equipped with a TurboIonSpray

ionization source in positive mode and coupled to a Dionex Ultimate 3000 liquid chromatography system. HPLC analyses were run on a Chromolith Performance RP-18e (3 × 100 mm) at a flow rate of 600 µL/min.

Data treatment

Data processing was done by using the Metworks LC-MS software version 1.3 SP4 provided by Thermo Scientific (Waltham, MA, USA). LC-MS datasets were analysed using the online available platform XCMS version 3.01.12 [22] (http://metlin.scripps.edu/xcms/) as a data preprocessing tool for peak detection, chromatogram alignment, and isotope annotation. After the MS acquisition, mUHPLC-MS data were analyzed to identify potential discriminatory signals. The raw mUHPLC-MS data are first convert to a common format. mzXML using the software MSConvert. This online platform was used for the alignment of non-linear data and the extraction of peak intensity. The next step was to differentiate the variable pair (retention time (RT)-m/z) between the control groups and the HLM groups. In this matter, we used the following filtering conditions: RT< 7 min., fold change between group >25, p-value <0.05, number of present peak in the control dataset=0, number of peak in the sample dataset=3 or 4, up/down regulation set on «UP» regulation. This way we could reduce the data from more than 7000 RT/m/z pairs found to 112. Isotopic clusters were identified visually and the subsequent +1, +2 and +3 isotopes were removed. By sorting this list by fold change, we obtained a list of potential hits (Table 1) for the first 10 values.

Results and Discussion

Optimization of the LC-MS(MS) conditions

Lapatinib is a fairly hydrophobic compound with a reported log P value at 5.1 in addition its structure (Fig.1) is composed of two primary amino groups that should bring it amenable to electrospray positive ionization. Using a standard solution of lapatinib, the high-resolution power of the mass spectrometer enabled to identify the protonated ion of lapatinib at m/z value =581.1420 representing a measurement error of less than 1 ppm. This high-resolution power and mass accuracy are key factors to perform metabolism studies and to try to identify new metabolites, but sensitivity is also a key parameter that needs to be optimized. Therefore a column with a reduced internal diameter (i.e. 1.0 mm was chosen over a conventional 2.1 mm resulting in less chromatographic dilution and consequently, increased concentration of the injected sample on the HPLC system (data not shown). Using the micro UHPLC system coupled to our HRMS we had analyzed the HLM extract by directly injecting 2 µL of the extract into our system, this way four known major metabolites of Lapatinib were separated and identified (Figure 1) with a good sensitivity in less than 10 min run analysis as described in the literature [20, 21] .

These compounds were further characterized by setting up a data dependent experiment in MS2 mode triggered by a SIM scan. The m/z values found in full scan were used to trigger the subsequent MS2 scan when a pre-fixed intensity threshold is reached. The daughter ions generated are then evaluated and compared with the ones in the literature to fully confirm the identity of the analyte as shown, as an example, for lapatinib in Figure 2 [6-8].

Differential analysis: HLM incubation group versus control group

Preliminary analysis of HLM incubations provided satisfactory results and in accordance to the current literature state regarding the obtained metabolites [6-8]. Therefore, we decided to add in our experimental design a differential analysis between control samples (all the reagents except lapatinib are present in the incubation procedure) and incubated extract. The sample extracts were injected in full HRMS scan in a random fashion to reduce the analytical bias. Two independent extracts of each control and HLM group were analysed in duplicate. Data treatment was subsequently performed *via* the online platform XCMS (http://metlin.scripps.edu/xcms/).The data obtained were quite difficult to interpret, based on the exact masses the ions at m/z values of 489.0996 and 473.1046 were respectively identified as the O-dealkylated and O-dealkylated hydroxy metabolites. The characteristic fragment of lapatinib at m/z 350 is also present but besides those 3 ions not much could be inferred from this table. In addition, one can question the relevance of sorting our data based on the change fold and then the possibility to lead to misinterpretation.

Isotopic pattern based search

In order to take full benefit of our high resolving power instrument we decided to sort out our data based on the characteristic isotopic pattern of lapatinib. Lapatinib contains a chlorine and a sulfur atoms its isotopic pattern should then include both the contribution of Cl37 and also of S34 in terms of both intensity ratio between the [M+H] + and the isotope [M+2+H]+ and exact masses differences. For instance, lapatinib that contains both chlorine and sulphur atoms shows an intensity ratio of 0.35 and for the ion 350 that should not contain sulfur (Figures 2 and 3) the intensity ratio is 0.30. We could also use the exact mass difference between an entity containing both chlorine and sulphur atoms versus only chlorine as a criterion but the difference is so small than even our high resolution instrument could not differentiate them (i.e. 1.9964 and 1.9970 respectively for lapatinib and the ion 350). Therefore we modified the data filtering parameters for our XCMS dataset according the following: p-value <0.05, UP regulation, peak not detected in control Dataset, and peak present in extract Dataset

Fold Change	m/z	p-value	Regulation	Retention time (min)	Number of peak present in control sample	Number of peak present in extract sample (NADPH)
1531.0364	350.0692	0.01637	UP	3.54	0	3
1296.2327	508.2512	0.01115	UP	3.42	0	3
819.8937	242.5578	0.02226	UP	3.54	0	3
788.2271	536.2825	0.01968	UP	3.42	0	3
722.8462	290.0669	0.02529	UP	5.58	0	3
714.8276	489.0996	0.01656	UP	4.5	0	3
652.4646	473.1046	0.01314	UP	3.54	0	3
583.1026	319.2051	0.0293	UP	3.42	0	3
548.327	395.0684	0.01179	UP	3.42	0	3
429.4822	175.5385	0.01331	UP	3.41	0	3

Table 1: XCMS differential analysis and metabolites identification.

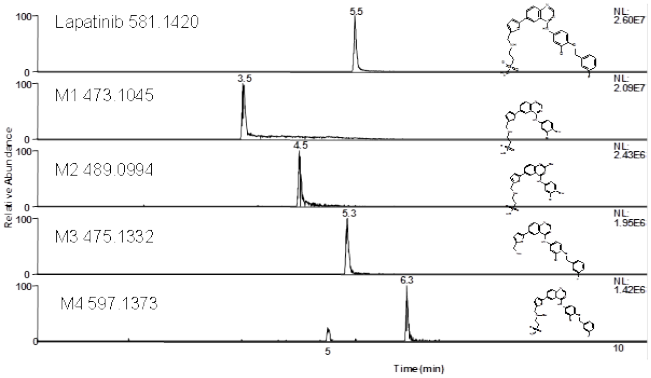

Figure 1: Micro LC-ESI/HRMS spectra of a HLM extract: Positive extract ion chromatogram of Lapatinib and four known major metabolites.

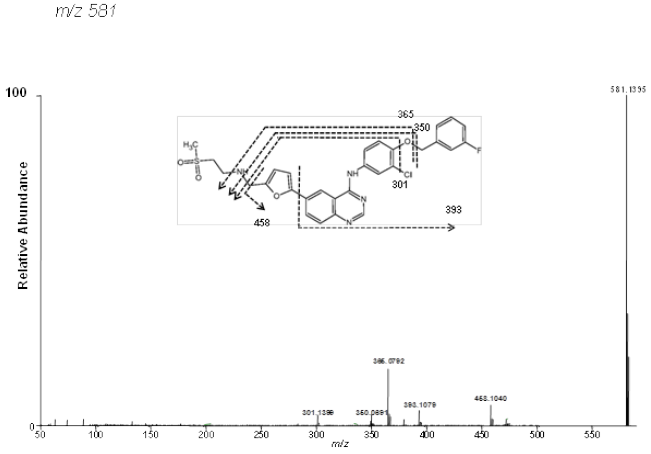

Figure 2: MS/MS spectrum of Lapatinib.

Potential precursors m/z	Intensity ratio (M+2/M)	Interpretation	
124.0431	0.06	contains S	
350.0692	0.30	contains Cl	Lapatinib fragments
368.0798	0.30	contains Cl	
458.1067	0.30	contains Cl	
473.1046	0.35	contains Cl and S	M1
489.0996	0.35	contains Cl and S	M2
536.2825	0.06	contains S	
581.1421	0.35	contains Cl and S	Lapatinib
597.1371	0.35	contains Cl and S	M4

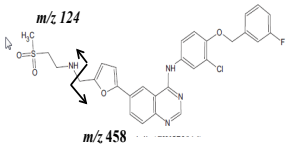

Figure 3: Lapatinib daughter ions structural elucidation.

n= 3 ou 4 (no time retention nor fold-change criteria). The data are then sorted out according to their ascending mass and a mass difference calculated between M, M+1 and M+2. Between M and M+1 a Δ of ca 1.0033 amu is expected while between M+2 and M+1 a Δ of ca 0.9925 amu is expected. Overall a total mass difference between M and M+2 of around 1.9958 amu should be expected for ions containing either chlorine or a sulfur atom or both. As stated before the exact masses difference only is not enough to differentiate between isotopic patterns and intensity ratio needed to be included to fully confirm the structural identity. In this way, the pairs of M/M+2 matching the masses difference criteria are gathered and the intensity ratio between them is calculated and structural interpretation could be done (Figure 3). The major metabolites of lapatinib could be then identified and their structure confirmed using this comprehensive scheme. Fragment ions at m/z 350 and 368 could have also their structure confirmed, the latter being most likely an adduct of 350 (+18). For the ion m/z 458 it possesses the same retention time as lapatinib which can be originated from in-source reaction. The ion at m/z 536 could not be identified as it only contains sulfur and most likely lost a chlorine ion. This comprehensive workflow enabled to go one step further in the structural elucidation and can be seen as an alternative tool to tedious classical deuterium-exchange experiment, chemical synthesis or radiolabelling.

Investigation of GSH-adduct formation

In the next set of experiments, lapatinib was studied in terms of its ability to form GSH-adducts. The formation of RM is suspected to play a major role in idiosyncratic hepatoxicity of a large proportion of drugs [5]. These RM are highly reactive and can interfere with cellular molecules *via* covalent interactions and thus affect critical cell function and promote cell deaths. Lapatinib has been shown to be extensively metabolized (Figure 4) and an electrophilic quinoneimine reactive intermediate can be generated from further oxidation of the O-dealkylated lapatinib [6]. To detect the generation of RMs, GSH-trapping was performed using HLM incubations. The O-dealkylated ion is detected at m/z 473.1045 (M1) in full scan mode, a subsequent dehydrogenation ($-H_2$) leading to the formation of the quinoneimine intermediate that is immediately trapped by the gluthatione. In theory the generated adduct should lead to an ion with m/z value of 778.1726. Extract chromatogram of this ion in full scan mode reveals a peak pattern around retention time of 3.3 min (Figure 5) but with a low signal to noise ratio that do not allow full confirmation (data not shown). Thereby, we modified several parameters in our approach, the injection volume was increased from 2 to 5 µL. In addition, and instead of using a HR full scan , a single ion monitoring (SIM) scan was used in a data dependent acquisition mode to generate the subsequent MS2 scan with a good quality (i.e. enhanced S/N) and provide substantial information for structural elucidation (Figure 5). Characteristic fragment ions of GSH adducts were thus identified as described in the literature [8]. This experimental set up enabled to confirm the presence of GSH-adduct and thereby the formation of a RM through a quinoneimine intermediate. Using Metworks add-on software we were enabled to predict in silico a M2 metabolite susceptible to form a quinonimine intermediate (Figure 4). It corresponds to the hydroxy O-dealkylated metabolite of lapatinib and therefore possesses the same chemical group that could lead to the formation of quinonimine intermediate.

In addition, the incubation with human recombinant CYP

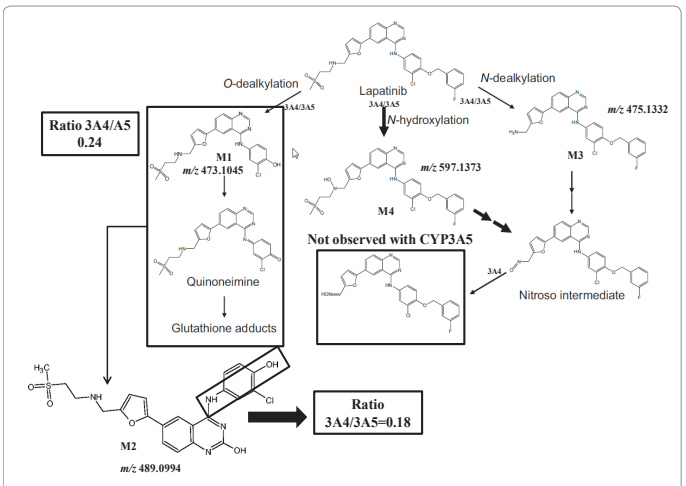

Figure 4: Lapatinib metabolic pathways. The structure and the reaction leading to the major known metabolites are described. The experimental ion abundancy ratio obtained following CYP isoforms incubation is presented for M1 and M2 which are susceptible to from quinonimine reactive species.

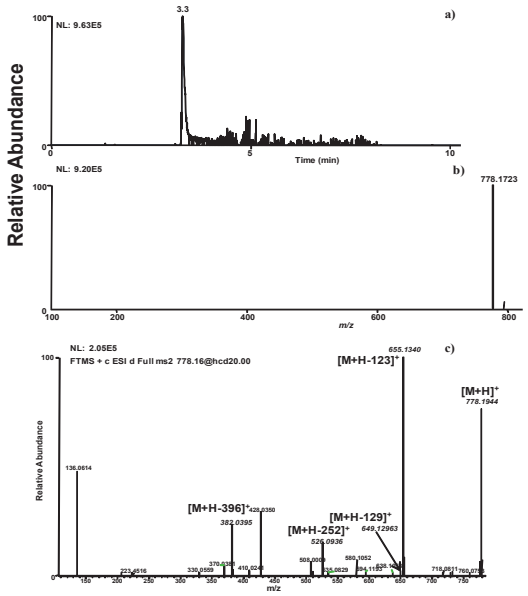

Figure 5: Identification of a reactive quinonimine intermediate: a) TIC chromatogram monitoring the masse value of 778.1726, b) Extract ion of *m/z* 581, 778 and 794 and c) data dependent spectra (MS^2) acquired upon the SIM acquisition.

isoenzymes revealed that the formation of both O-dealkylated and hydroxy O-dealkylated metabolites are regulated by both CYP3A4 and CYP3A5. The abundancies of these ions are higher with the CYP3A5 enzyme, several studies demonstrating that CYP3A4 is inactivated by another lapatinib metabolite that is generated most likely by a nitroso compound (Figure 4). This nitroso intermediate compound is not observed in the CYP3A5 incubation and it is known that frequency of polymorphic P450 3A5 expression differs among ethnic groups, e.g., P450 3A5 is found at high levels in 10 to 30% of Caucasian and 50% of African Americans [23,24]. Unlike CYP3A4, the level of CYP3A5 is dependent on the allele that determines its endogenous expression [25]. For instance, patients with the 3A5 *1/*1 allele have a CYP3A5 level that can represent up to 50% of the total CYP3A content. In other words, CYP3A5 is highly polymorphic compared with CYP3A4, making it a potentially significant contributor to pharmacokinetic variability for some substrates. For instance, drugs that are metabolized mainly by CYP3A5 would have altered hepatic clearance, depending on the expression levels of CYP3A5. This could be a problem if the affected drugs have narrow therapeutic indices or generate toxic reactive intermediates.

Incubations with human CYP isoenzymes 3A4 and 3A5 reveals different levels in terms of abundancies for M1 and M2 metabolites. For both ions higher abundancies are obtained with CYP 3A5 isoenzymes, corresponding ratios are reported in Figure 4.

For this reason, we decided to investigate if CYP3A5 isoenzyme could generate specifically other RMs from lapatinib and then explain partially the idiosyncratic toxicity of this drug.

Investigation of GSH-adduct formation from Human recombinant CYP3A5

A first attempt to identify GSH-adducts was performed using the neutral loss scan mode in a hybrid triple quadrupole linear ion trap instrument. The loss of pyroglutamic acid from glutathione conjugates

Compound	MRM1	MRM2	CE (eV)
Adduct-778	778->655	778->649	25
Adduit-794	794->671	794->665	25

Table 2: Selected reaction monitoring for the 2 quinonimine metabolites.

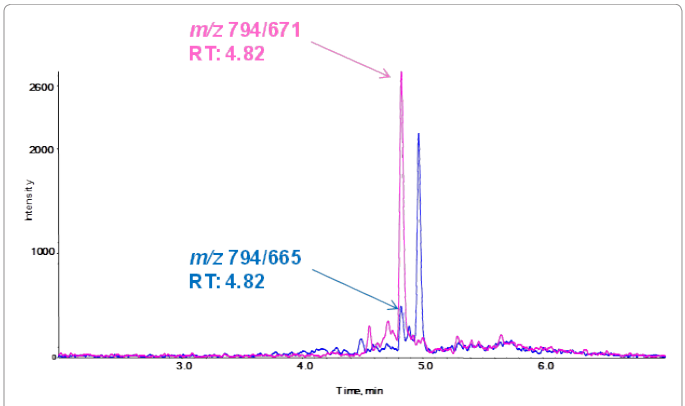

Figure 6: Predictive MRM transitions 794→ 671 and 794→ 665 observed in an enriched CYP3A5 human recombinant fraction.

results on a neutral mass of 129 Da. Neutral loss scanning of 129 Da using a triple-stage quadrupole mass spectrometer platform has been the method of choice for the characterization of glutathione conjugates [26]. A characteristic neutral mass loss (NL) of 129 corresponding to loss of pyroglutamic acid moiety of the GSH adduct was then monitored. Another neutral loss scan was monitored at m/z 123 to profile any additional GSH adduct that shares a common pattern with lapatinib (i.e., loss of the sulfonyl moiety). The incubation of human recombinant CYP3A5 isoenzyme was coupled to a GSH trapping experiment. The extracts were then injected (10 µL) and monitored in NL scan. Both NL scan (i.e., 129 and 123) revealed the presence of the already described GSH adduct at m/z=778, no other peak was identified in this experiment (data not shown). To gain in sensitivity, we decided to perform a MRM experiment following the transition corresponding to the NL of 123 and 129 for the GSH adduct at 778 but also to monitor the corresponding predictive transition for a GSH adduct generated by M2 (i.e., m/z 794). The transitions monitored along with their respective collision parameters are summarized in Table 2. This experiment enabled to detect a peak for the ion 794 at a retention time of 4.8 mins for the two transitions monitored (Figure 6). This finding coupled to the in-silico prediction suggests the presence of another quinonimine entity. Further experiment should be envisioned to fully confirm the identity of this compound, If this new quinonimine is fully confirmed this would be a step towards the understanding of idiosyncratic mechanism of Lapatinib.

Conclusion

Identification of reactive metabolites is considered a very challenging step in the drug metabolism workflow. In this work, we described a comprehensive approach based on HRMS and bioinformatics strategies to foster mapping of metabolism pathways and the detection of reactive metabolites. The use of *in vitro* metabolites formation *via* microsomal incubation enabled us to validate this workflow and further apply it to Lapatinib to understand the mechanism pathways leading to hepatoxicity in some patients. The use of high-resolution mass spectrometry enabled to identify lapatinib metabolites and among them a new potential quinonimine reactive metabolite. The strategy relies on UHPLC-HRMS and data treatment inspired from metabolomics data processing. The subsequent use of tandem mass

spectrometry was performed through data dependent and neutral loss scans for the structural elucidation of a new quinonimine reactive metabolite detected in LC-MS.

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