Postprint of: Potęga A., Żelaszczyk D., Mazerska Z., Electrochemical simulation of metabolism for antitumor-active imidazoacridinone C-1311 and in silico prediction of drug metabolic reactions, Journal of Pharmaceutical and Biomedical Analysis, Vol. 169 (2019), pp. 269-278, DOI: 10.1016/j.jpba.2019.03.017

Research highlights

- Phase I metabolism of C-1311 was successfully simulated in an electrochemical cell.
- The products of *N*-dealkylation and aliphatic hydroxylation reactions were detected.
- *In silico* analysis was used for the prediction of P450-mediated metabolites.
- We observed a good accordance between electrochemical and *in silico* results.
- Electrochemical and *in silico* methods are fast alternatives for enzymatic assays.

Title page

Title:

Electrochemical simulation of metabolism for antitumor-active imidazoacridinone C-1311 and *in silico* prediction of drug metabolic reactions.

Authors:

Agnieszka Potęga [a], Dorota Żelaszczyk [b], Zofia Mazerska [a]

Affiliations:

[a] Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, Gabriela Narutowicza St. 11/12, Gdańsk 80-233, Poland.
agnieszka.potega@pg.edu.pl (A.P.), zofia.mazerska@pg.edu.pl (Z.M.)

[b] Department of Organic Chemistry, Faculty of Pharmacy, Jagiellonian University, Medyczna St. 9, Kraków 30-688, Poland.
dorota.zelaszczyk@uj.edu.pl (D.Ż.)

Corresponding author:

Agnieszka Potęga
Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, Gabriela Narutowicza St. 11/12, Gdańsk 80-233, Poland.
e-mail: agnieszka.potega@pg.edu.pl, telephone: +48 58 347 15 93, fax: +48 58 347 11 44

Abstract

The metabolism of antitumor-active 5-diethylaminoethylamino-8-hydroxyimidazoacridinone (C-1311) has been investigated widely over the last decade but some aspects of molecular mechanisms of its metabolic transformation are still not explained. In the current work, we have reported a direct and rapid analytical tool for better prediction of C-1311 metabolism which is based on electrochemistry (EC) coupled on-line with electrospray ionization mass spectrometry (ESI-MS). Simulation of the oxidative phase I metabolism of the compound was achieved in a simple electrochemical thin-layer cell consisting of three electrodes (ROXY™, Antec Leyden, the Netherlands). We demonstrated that the formation of the products of *N*-dealkylation reactions can be easily simulated using purely instrumental approach. Newly reported products of oxidative transformations like hydroxylated or oxygenated derivatives become accessible. Structures of the electrochemically generated metabolites were elucidated on the basis of accurate mass ion data and tandem mass spectrometry experiments. *In silico* prediction of main sites of C-1311 metabolism was performed using MetaSite software. The compound was evaluated for cytochrome P450 1A2-, 3A4-, and 2D6-mediated reactions. The results obtained by EC were also compared and correlated with those of reported earlier for conventional *in vitro* enzymatic studies in the presence of liver microsomes and in the model peroxidase system. The *in vitro* experimental approach and the *in silico* metabolism findings showed a quite good agreement with the data from EC/ESI-MS analysis. Thus, we conclude here that the electrochemical technique provides the promising platform for the simple evaluation of drug metabolism and the reaction mechanism studies, giving first clues to the metabolic transformation of pharmaceuticals in the human body.

Keywords: Antitumor compound; On-line electrochemistry-mass spectrometry; Metabolite electrosynthesis; *In silico* site of metabolism prediction; Cytochrome P450; *In vitro* drug metabolism;

Abbreviations

C-1311, 5-diethylaminoethylamino-8-hydroxyimidazoacridinone; EC, electrochemistry, electrochemical; ESI, electrospray ionization; FA, formic acid; LC, liquid chromatography; *m/z*, mass-to-charge ratio; MS, mass spectrometry, mass spectrometer; MW, molecular weight; MS/MS, tandem mass spectrometry; P450, cytochrome P450; Q-TOF, quadrupole-time of flight

Parts of this work were presented at the 3rd International Summit on Toxicology & Applied Pharmacology (Chicago, IL, USA, 2014), at the 13th European ISSX Meeting (Glasgow, Scotland, 2015), and at the 20th North American ISSX Meeting (Orlando, FL, USA, 2015).

1. Introduction

Imidazoacridinones represent a group of the promising antitumor-active compounds developed in our laboratory [1]. 5-Diethylaminoethylamino-8-hydroxyimidazoacridinone (C-1311) (Fig. 1) has received significant attention due to its exceptionally high cytotoxic activity against a broad spectrum of human tumor cell lines in the National Cancer Institute *in vitro* screening system and of transplantable animal tumors [2,3]. This compound reached up the phase II clinical trials where exhibited activity against advanced solid tumors, and it was effective in women with metastatic breast cancer [4]. Furthermore, it turned out to be an agent with high-predicted activity in human bladder cancer [5].

The leading concept to find out how C-1311 causes high antitumor effect is the metabolic (oxidative and/or reductive) activation of the compound to species responsible for cell injury and death. It was demonstrated [6] that intercalation of C-1311 into DNA followed by its activation under enzymatic oxidative conditions gave rise to products capable of irreversible binding into DNA. Therefore, the knowledge about the route of metabolism of the drug candidate was desirable to determine its pharmacological and/or toxicological activity. Multiple studies on the molecular mechanism of the enzymatic activation of C-1311 with different liver drug-metabolizing enzymes were performed [7-10]. Various cytochrome P450 (P450) isoforms are among the many intracellular enzymes that catalyze activation reactions. Specifically, P450 enzymes are responsible for the biotransformation of about 70-80% of all drugs in clinical use [11]. However, no products of C-1311 metabolism were observed with any tested human recombinant P450s [8]. In contrast, C-1311 has been identified as a potent mechanism-based inactivator of P450 1A2 and 3A4 isoenzymes [9]. On the other hand, C-1311 was a good substrate for microsomal and the selected human recombinant flavin-containing monooxygenases and UDP-glucuronosyltransferases [8,10]. The knowledge on the biological background of C-1311 metabolism and its influence on human body is constantly increasing, but still molecular mechanisms have not been completely explained.

Drug metabolism studies are an integral part of the comprehensive characterization of a new chemical entity during various stages of the drug development process. In order to

address this challenging task, different methods for the simulation of metabolic reactions have been developed and applied. In practice, the studies on drug metabolism usually include *in vivo* experiments on the basis of animal models, or *in vitro* tests, where P450-containing matrices like hepatocytes, liver microsomes, and other cellular fractions derived from animal or human tissue are used [12] (Fig. 2A). Although the biological systems are still being improved, there are a number of drawbacks associated with these approaches (*e.g.*, the great expense of biological material and cofactors, the ethical aspect of using biological material of human or animal origin). From a practical aspect, both *in vitro* and *in vivo* systems inherently have a complex biological matrix that makes isolation and purification of metabolites time-consuming and laborious [13]. Therefore, analytical techniques which are able to mimic metabolic reactions taking place in the human body and which simultaneously allow to reduce the listed above limitations, gain strong attention.

In recent years a particular interest in the field of drug metabolism studies has been observed from electrochemistry. Complementary to the existing techniques, oxidation-reduction reactions catalyzed by liver P450 isoforms can be successfully simulated in an electrochemical (EC) cell coupled directly to a mass spectrometer (MS) [14,15]. This simple and pure instrumental technique allows for the generation of a number of potential oxidative metabolites, including reactive intermediates, and for the detection of the sites labile towards oxidation in a drug molecule [16,17]. The greatest advantage of the electrochemical studies on oxidative metabolic reactions over other *in vitro* methods is the absence of proteins in the reaction medium. Moreover, in EC system it is possible to control the reaction rate by the applications of electrode potentials specific for synthesis of the expected products [15].

Considering the mechanistic differences between EC and enzymatic oxidations, not all metabolic pathways can be mimicked by EC. Thus, EC cannot replace conventional metabolism studies in biological systems. However, this technique may serve as an attractive alternative tool for the initial investigation of drug metabolism in a single-step experiment. In this work, we explored the on-line combination of EC with electrospray ionization (ESI) MS for metabolism studies of antitumor drug candidate C-1311 (Fig. 2B). The main focus of the

present study was to put on the simulation of potential oxidation transformations of the compound with the prediction of metabolite structures by the identification of sites susceptible to oxidation. Further, the obtained results were supported by *in silico* computational method (Fig. 2C). It is usually a starting point of metabolic pathway studies, which may also assist in the process of drug/lead optimization [18]. MetaSite software, applied in this study, considered the enzyme-substrate recognition and the chemical transformations induced by the P450 enzymes on the most reactive sites. It attributed the more probable sites of metabolism, and therefore predicted the main metabolites that can be formed in various human tissues (liver, skin, brain, and lungs) [19]. Our choice of two major human P450s: 1A2 and 3A4 resulted from our earlier findings revealing that these P450 isoenzymes were irreversible inhibited by the studied imidazoacridinone, probably according to mechanism-based manner [9]. However, no reactive intermediate products, which would be responsible for autoinactivation of P450 enzymes, have been detected so far. In turn, P450 2D6 isoform was taken into account due to its specific involvement in aliphatic and aromatic hydroxylations [20]. In order to obtain insight into the potential of EC for the simulation of drug metabolism, the results of EC/MS analyses were also referred to the data of previous *in vitro* experiments on C-1311 in the presence of rat and human liver microsomes [7] and in the model activation system, horseradish peroxidase/hydrogen peroxide [21]. An overview of the compared approaches used in drug metabolism studies was shown in Fig. 2.

The comparison and correlation of the results obtained in various systems for drug metabolism studies are important for better understanding of C-1311 metabolic pathways. Conventional enzymatic approaches remain methods of choice for determination of phase I of drug metabolism. In turn, EC and *in silico* methods, that enable determinations of metabolism patterns, may be considered as fast alternatives to *in vitro* schemes, even though the results are not unrestrictedly transferable to the situation in the human liver. Further, structural characterization of metabolites allows the modification of drug molecule to decrease the metabolic clearance or to avoid unwanted metabolic transformations, what may contribute to the development of new derivatives with improved metabolic properties [14,22].

2. Materials and methods

2.1. Chemicals

Imidazoacridinone derivative, a 5-diethylaminoethylamino-8-hydroxyimidazoacridinone (C-1311) was synthesized and purified in our laboratory according to the method described earlier [1]. Formic acid (FA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium formate (NH_4HCO_2) was ordered from Fisher Scientific (Loughborough, UK). Methanol (MeOH) and acetonitrile (ACN) (both in gradient grade quality for liquid chromatography) were obtained from Merck KGaA (Darmstadt, Germany). All other commercially available chemicals and reagents were of the highest possible grade available. Ultrapure water (conductivity 18.2 MΩ cm), used in all the experiments, was passed through a Milli-Q water purification system from Merck KGaA.

2.2. General instrumentation

2.2.1. Electrochemistry (EC)

The EC system used for electrochemical experiments simulating P450-mediated oxidative reactions was set up as reported in previous investigations [23]. The ROXY™ potentiostat was equipped with a commercial electrochemical thin-layer ReactorCell™ and a SP2-ROXY™ Dual-Piston Syringe Pump (Antec Leyden, Zoeterwoude, The Netherlands). A three-electrode configuration was used in the present study, *i.e.*, a disc glassy carbon working electrode (ϕ = 8 mm; A = 0.502 cm^2), the HyREF™ palladium-hydrogen (Pd/H_2) reference electrode, and the carbon-loaded PTFE (polytetrafluoroethylene) auxiliary electrode. Representative results of at least three independent experiments were considered. The glassy carbon working electrode surface was wiping with a tissue wetted with methanol and/or was finished with a polishing disc and diamond slurry provided by the manufacturer (Antec Leyden) prior to each experiment.

2.2.2. Mass spectrometry (MS)

For the generation of two-dimensional (2-D) mass voltammograms, the outlet of the ROXY™ EC system was directly connected to the standard ESI source of an Agilent 6500 Series Accurate-Mass Quadrupole-Time of Flight (Q-TOF) mass spectrometer (Agilent Technologies,

Santa Clara, CA, USA) by means of 50 cm long PEEK tubing (130 µm I.D.). The system was controlled by Agilent MassHunter Workstation software (Agilent Technologies). The scan range was set from 50 to 600 mass-to-charge ratio (*m/z*) and mass spectrometric detection was carried out in the positive ionization mode (+). Tandem mass spectrometry (MS/MS) mode was used to provide additional structural information for the ions of interest. To ensure accurate mass during the experiment, the mass spectrometer was calibrated daily using calibration solution (ES-TOF reference mix, Agilent Technologies). The corresponding conditions for the ESI-Q-TOF-MS measurements and the optimized parameters for the ESI-ion trap MS/MS experiments can be viewed in Table 1.

The delay time between product formation within the EC cell and detection in the MS was taken into account. It was calculated on the basis of the dead volume between the reactor cell and the ion source of the MS (for the supplied tubing this volume was 12.7 µL) and the flow rate of the infusion syringe pump (30 µL min^{-1}). The transfer time to the capillary was estimated at about 0.7 min. Therefore, the oxidation behavior of C-1311 was observed in real time during application of a potential ramp to the EC cell.

2.2.3. Liquid chromatography (LC)

The mixture of C-1311 and its putative oxidation products leaving the EC cell was also collected and analysed by LC/MS. Liquid chromatographic separations were performed on a reversed-phase (RP) 5-µm Suplex pKb-100 analytical column (4.6 mm x 250 mm, C18) (Supelco Inc., Bellefonte, PA, USA) with Agilent 6500 Series Accurate-Mass Q-TOF LC/MS system (Agilent Technologies) controlled by Agilent MassHunter Workstation software. The mobile phase consisted of 0.05 M aqueous NH_4HCO_2 buffer (pH 3.4 adjusted with FA; solvent A) and MeOH (solvent B). The LC analyses were carried out at a flow rate of 1 mL min^{-1} with the following mobile phase system: a linear gradient from 15% to 80% B in A for 25 min, followed by a linear gradient from 80% to 100% B in A for 3 min, and 2 min isocratic 100% B. During all LC separations, the column was operated at a room temperature. The eluates were monitored at 380 nm. The MS conditions were identical to those described above.

2.3. Electrochemical simulation of the oxidative metabolism of C-1311

A stock solution of C-1311 was prepared in an electrolyte (composition described below) at a concentration of 1 mg mL^{-1}. It was used to prepare fresh working solutions of C-1311 (0.01 mg mL^{-1}) shortly before each experiment. Two electrolytes of different pH values were used for optimization of the electrochemical conditions: (1) H_2O-MeOH (1:1, *v/v*) with 0.1% FA, pH 3.3; (2) 20 mM NH_4HCO_2-ACN (1:1, *v/v*), pH 7.4. Simulation of the oxidative phase I metabolism of C-1311 in the EC cell was accomplished at a constant flow rate of 30 µL min^{-1}. The potential of the working electrode was ramped linearly from 0 V up to 2.5 V at 10 mV s^{-1} and was controlled by the Dialogue software (Antec Leyden). The relevant EC conditions are listed in Table 1. The outlet of the reactor cell was connected on-line to the ESI-MS source.

2.4. *In silico* prediction of cytochrome P450-mediated sites of C-1311 metabolism

The MetaSite software tool (version 5.1.1; Molecular Discovery Ltd., Hertfordshire, UK) is a computational algorithm used to identify most likely metabolic soft-spots of xenobiotics. The software considers two factors: chemical reactivity of the substrate and structural complementarity between the active site of the P450 enzyme and the ligand and comes up with the most optimal orientation [24]. A 2-D structure of the C-1311 was imported into the interface of MetaSite to predict phase I sites of metabolism and structures of metabolites in liver related to P450 1A2-, 3A4-, and 2D6-mediated reactions. Only metabolites with a molecular mass higher than 150 Da and with a likelihood ranking >50% were considered.

3. Results and discussion

The main focus of this study was directed to the combination and comparison of *in vitro* and *in silico* approaches for the determination of C-1311 metabolism. First, the oxidation of C-1311 in an electrochemical thin-layer cell was employed for fast and easy generation of the possible phase I metabolites. Then, *in silico* prediction of the sites of C-1311 biotransformation mediated by cytochromes P450 using the MetaSite software tool was carried out to support the experimental data found via EC. The results deriving from both the EC and *in silico* studies were also compared with those of the most common *in vitro* investigations with rat and human liver microsomes and in the model peroxidase system, which were discussed in detail elsewhere [7,21].

3.1. Electrochemical simulation of C-1311 oxidative metabolism

The determination of the metabolic fate of drugs is an essential and important part of the drug development process. Progress in this research area depends critically on the improvement of methods involved in the generation and analysis of various types of drug metabolites. In the current study, the on-line coupling of EC with ESI-MS (Fig. 2B) is an effective analytical tool for the studies on the oxidation products of antitumor-active compounds developed in our laboratory, since experiments are done under controlled conditions using pure solvents and reagents. Additionally, the absence of the components of biological matrices in the electrochemical reaction medium, prevents the further laborious tasks related to isolation and identification of metabolic products formed in conventional enzymatic systems. Consequently, data analysis process is accelerated [25].

3.1.1. Optimization of the electrochemical conditions

The optimization of the electrochemical conditions is of great importance for a successful comparison with conventional *in vitro* enzymatic approaches. The conversion efficiency in the EC cell may be influenced by the properties of the sample solvent. Therefore, the electrochemical simulation of the oxidative metabolism of C-1311 has been performed in two electrolyte solutions with different composition and pH value (see Materials and methods section). The flow rate of the sample solution through the reactor cell and the potential applied

at a working electrode may also affect the EC efficiency. All these parameters were optimized for the best conditions for oxidation and identification processes. In result, the most effective conversion of C-1311 into its expected metabolites was attained with use of the H_2O-MeOH (1:1, *v/v*) with 0.1% FA electrolyte (pH 3.3) and glassy carbon as a working electrode material for voltage range between 0 and 2.5 V. Additionally, we have observed that methanol was better than acetonitrile in diminishing the adsorption of the electrochemical products on the surface of the working electrode what resulted in greater signal intensity of the selected mass ions. Moreover, H_2O-MeOH electrolyte solution produced the lowest mass background noise in positive total ion chromatograph (data not shown). Details regarding the optimized EC conditions used for measurements can be found in Table 1.

3.1.2. On-line EC/MS experiments

Initially, a solution of C-1311 was directly introduced from the syringe pump into the EC cell without applying a voltage to the working electrode (cell off). Due to the presence of the nitrogen atoms in the side chain, the C-1311 molecule can easily be protonated and detected with high intensities as $[M+H]^+$ ion (*m/z* 351.1820) (Fig. 3A) in the positive ionization mode of the ESI-Q-TOF mass spectrometer. In turn, applying a voltage to the EC cell (cell on) led to C-1311 oxidation and resulted in a reduction in ion signal corresponding to the substrate. Simultaneously, oxidation products of C-1311 were formed and new signals of the $[M+H]^+$ ions corresponding to putative oxidation products were observed (Fig. 3B). To provide a concise overview of the oxidation products, 2-D mass voltammograms for the ions of interest were generated (Fig. 4) by plotting the intensities of the extracted ions against time of electrochemical analysis. A total of eight putative products of C-1311 were generated by EC. The electrosynthesis of various products required operation at different potential settings. The vast majority of products were generated at potentials between 1 and 2 V, and to form the products at *m/z* 365 and *m/z* 367 the higher values of potentials were required.

3.1.3. Characterization of the products

Combining EC system on-line with MS allowed the initial characterization of the formed products by an increasing signal intensity of the corresponding *m/z*. Their molecular structures have been derived based on calculations with accurate mass data (mass ≤ 2 ppm). The fragmentation (MS/MS) spectra were also recorded. In turn, the separation of oxidation products by reversed-phase LC can overcome ion suppression effects that may be encountered during on-line EC/MS thus providing additional information about the range of oxidation products and, consequently, about the reaction mechanism [26]. However, unstable products may escape analysis. Therefore, both approaches (EC/MS and LC/MS) were employed for a comprehensive analysis. LC method was established based on the preliminary results from 2-D mass voltammograms. Extracted ion chromatograms of C-1311 and its products that were generated electrochemically are presented in Fig. 5.

A comprehensive summary of the products detected following oxidative transformation of C-1311 in the EC cell was shown in Table 2. *N*-Dealkylation is a characteristic type of *in vitro* and *in vivo* metabolic transformation catalyzed by cytochrome P450 enzymes and it is a major metabolism pathway for drugs containing secondary and tertiary amines [27]. This process can also be easily induced electrochemically [14,15,28]. A signal at *m/z* 252 (P1) involves a reduction in mass by 99 Da what can be explained by a loss of diethylaminoethyl moiety as a result of complete *N*-dealkylation in the side chain of C-1311 molecule. In turn, the P3 product (*m/z* 323; - 28 Da) may arise from an *N*-deethylation reaction. Postulated mechanism of the electrochemical *N*-dealkylation of *N*-alkylamines proceeds through one-electron transfer to generate an imine intermediate that, after hydrolysis and intramolecular rearrangement, gives the product of *N*-dealkylation [27]. For further confirmation of the *N*-dealkylated derivatives, the fragmentation patterns, generated by in-source fragmentation in the ESI interface, have been studied. The Q-TOF-MS/MS spectrum obtained for *m/z* 323 ion (Fig. 6A) showed fragment ions at *m/z* 252 (- 99 Da) and *m/z* 278 (- 73 Da). While the former can be assigned to the *N*-dealkylation process, the latter can indicate an intermediate product of a side chain degradation (P2) that was also detected in the EC system. In the elution profile (according to gradient profile used for LC analysis) the P1 peak was eluted at 20.47 min (Fig. 5) so that it

appeared after the peak corresponding to the mass ion at *m/z* 351 (C-1311; t_R 14.33 min). Hence, it would indicate its less polar nature than that of the substrate. P3 product was found in trace amounts in LC analysis.

Dealkylation is usually considered as an element of the detoxification pathway of xenobiotics [27]. In this sense, the metabolic transformation leading to the *N*-dealkylated products should give probably the less toxic, inactive metabolites. Both *N*-dealkylated products, P1 and P3, were also observed in horseradish peroxidase/hydrogen peroxide system [21], while only the product P3 was detected in similar amounts after incubation with rat and human microsomes [7]. Consequently, the presented electrochemical reactor seems to be better suited for simulating the P450-catalyzed *N*-dealkylation reactions than liver microsomes. This may be related to the fact that, although liver microsomes contain the physiologically relevant combinations of drug-metabolizing enzymes, expression of different P450 isoforms may be very diverse in the organism. On the other hand, primary amines are the most common quasi-irreversible P450 inactivators [29]. The resulting primary amine may be further oxidized to nitroso derivative (via the intermediate hydroxylamine metabolite), which can form a metabolic intermediate complex with the ferrous form of the heme iron atom. Therefore, secondary and tertiary amines can serve as suitable precursors for enzyme inactivation provided they are *N*-dealkylated to primary amines. This is in agreement with the previous knowledge on the metabolic activation of C-1311 [9].

The dehydrogenation reaction is a special feature of P450 in which it actually behaves as an oxidase-dehydrogenase, and converts molecular oxygen to water [27]. In addition to *N*-dealkylation, dehydrogenation can also be imitated by direct electrochemical oxidation where it is proposed to undergo through two successive electron/proton transfer reactions [14,15]. The obtained mass ion at *m/z* 349 correlates with the loss of two hydrogen atoms (- 2 Da) what indicates the generation of the dehydrogenated product (P4). To elucidate the nature of this compound, the LC/MS analysis was performed. The peak at *m/z* 349 (Fig. 5) was eluted ahead of C-1311 at 11.88 min what suggests rather higher hydrophilicity of the respective compound than that of the substrate. It is assumed that a carbon-carbon double bond is formed in the

side chain what is energetically advantageous because provides an extra stability due to the possibility of resonance with the electrons of the aromatic system. Electrochemical dehydrogenation of a carbon-carbon bond has been observed for a number of drug compounds [14,28,30].

Electrochemical conversion of C-1311 mainly led to the formation of the products P5 and P6, resulting in the signals at *m/z* 365 and *m/z* 367, respectively (Fig.4B). In the case of the first one, an increase in mass by 14 Da, compared with *m/z* 351 (C-1311), can be explained by a two-step process, consisting of oxidation (+ O), followed by the dehydrogenation (- 2H). This may indicate a route leading to an aldehyde or a ketone. The second mass ion suggests the introduction of one oxygen atom into the C-1311 molecule, shown by a mass increase of 16 Da. The insertion of an oxygen atom may take place under the formation of a hydroxyl group (aromatic or aliphatic hydroxylation) or by *N*-oxidation. Carbon hydroxylation is a very common P450-catalyzed reaction [27] and the utility of electrochemistry for simulation of this process for pharmaceutical drugs and xenobiotics is well documented [14,16,17]. The LC separation of the electrochemically generated products revealed that two different isomers (peaks P6a and P6b in Fig. 5) of a species with *m/z* 367, showing similar intensities, could be detected. Their retention times of 14.79 min and 16.22 min, respectively, were higher than C-1311 what confirmed the assumption that the hydroxylated products might have been formed. Moreover, these peaks may be attributable to hydroxylation on different sites of the C-1311 molecule.

MS/MS spectrum of *m/z* 367 showed the fragment ions at *m/z* 350 and the most abundant *m/z* 336 (Fig. 6C). The existence of fragment ion at *m/z* 350 may indicate the loss of one hydroxyl group (- 17 Da), what can hardly be in agreement with an *N*-oxide structure, while the latter may arise from the heterolytic beta cleavage of carbon-carbon bound that released a CH_2OH molecule (- 31 Da). Hence, one of the isomers of the P6 product may be associated with a singly hydroxylated species bearing the additional oxygen atom at the terminal carbon atom (ω position) of the ethyl group of the nitrogen atom. In this study, working at higher potentials enabled aliphatic carbon to be hydroxylated. According to previous studies on C-

1311 metabolism [7,8,21], the likely hydroxylation site in the C-1311 molecule may also be the imidazoacridinone moiety. However, no evidence was found for the formation of an aromatic alcohol and our findings strongly show that hydroxylation on the side chain of the compound appears to be more preferential in electrochemistry. On the other hand, *N*-oxidation could not be excluded, since the product at *m/z* 367 was obtained in both rat as well as human liver microsomes [7] and in metabolism of C-1311 with FMO [8], and have been proposed to be *N*-oxide derivative on the *ω*-nitrogen atom of C-1311. The peak of the 6a isomer, with a slightly higher retention time than C-1311, may just point out the presence such a product. Interestingly, the identical fragment ion at *m/z* 336 was also seen in MS/MS spectrum of *m/z* 365 (Fig. 6B). This time it is implied that the loss of COH molecule took place what confirms the further oxidation of the P6 to the corresponding aldehyde.

The next oxidation products P7 and P8 exhibited strong signals at *m/z* 379 and *m/z* 381, respectively. Despite showing quite good intensities in the EC/MS system, both products were not found in LC/MS analysis and no MS/MS data were accessible for them. This may be due to the limited stability of these reaction products. Thus, the assigned structures of P7 and P8 are to be regarded as tentative. The determined exact mass of *m/z* 379.1764 indicates the formation of a derivative with an additional formyl group (-CHO; calculated: *m/z* 379.1764, mass error 0.0 ppm) rather than a product of double oxidation (+ 2O, - 4H; calculated: *m/z* 379.1401, mass error 95.8 ppm). It presumably occurred through the reaction of C-1311 with formic acid present in the electrolyte solution. In turn, the P8 product is proposed to be the P7 corresponding alcohol. A general overview of the proposed pathways of C-1311 electrochemical oxidation is shown in Fig. 7.

3.2. *In silico* prediction of cytochrome P450-mediated sites of C-1311 metabolism

No stable final products of C-1311 metabolism with P450 isoenzymes have been observed so far [8], but the results of cytochrome P450 1A2 and 3A4-inhibitory activities of the compound [9] strongly suggest the possibility of any metabolic activation pathway. C-1311, as a prodrug, could be also activated by the action of P450 2D6 that is an isoenzyme specifically involved in aliphatic and aromatic hydroxylations [20]. Our former studies and these presented above

pointed that the most reactive site in the C-1311 molecule was expected to be diethylamino functional group. In this study, for the demonstration of the suitability of the electrochemical system in drug metabolism studies, prediction of C-1311 metabolism was performed with the use of MetaSite 5.1.1 software tool (Molecular Discovery Ltd.). The results of the proposed the most probable metabolites with their molecular weight and calculated cLogP (the logarithm of the partition coefficient between *n*-octanol and water) are presented in Table 3.

Five and six top ranked phase I metabolite structures were predicted in the case of P450 1A2 and 3A4 isoforms, respectively. The dialkylaminoalkylamino moiety was proved to be the most probable site of P450 attack in the C-1311 molecule. In both cases, the *N*-deethylation products were obtained (MW 322.14; 50% and 100% of likelihood for P450 1A2 and 3A4, respectively), wherein complete *N*-dealkylation (MW 251.07; 100% of likelihood) is likely only with the participation of P450 1A2 isoenzyme. MetaSite also indicated the possibility of hydroxylation (MW 366.17; 100% of likelihood for P450 1A2) and oxidation (MW 364.15) on various positions of the side chain but with the indication of the ω - 1 carbon atom of the terminal ethylamino group as preferential (50% and 100% of likelihood for P450 1A2 and 3A4, respectively). The most likely metabolite for P450 1A2 activity was also formed by oxidative deamination and the following oxidation on the imidazoacridinone ring (MW 266.03; 100% of likelihood). This electrophilic *ortho*-quinone derivative may potentially be implicated in the covalent apoprotein modification of P450 isoenzymes. Hence, its existence may be associated with the observed stronger inactivation of P450 1A2 than 3A4 by C-1311 [9]. Probably due to its high chemical reactivity, this product was not detected in the EC/MS system. Oxidative deamination product (MW 293.08) and its corresponding carboxylic acid (MW 309.07), both with an equal likelihood of 50%, were predicted in the case of P450 3A4. Another main metabolite of this P450 isoenzyme was that derived by dehydrogenation in the ethylamino chain (MW 348.16; 50% of likelihood). Considering P450 2D6-mediated sites of C-1311 metabolism, MetaSite predicted the *ortho* position to the hydroxyl group in the acridinone moiety as the most probable site of C-1311 hydroxylation (MW 366.17; 100% of likelihood). The resulting diol derivative may contribute to overall cytotoxic activity of the compound

because it is a preffered structure for the substitution by nucleophiles existing in a living organism (*i.e.*, glutathione, thiols of proteins or purine and pyrimidine bases of DNA). However, the predicted hydroxylation would take place also in the aliphatic chain giving a compound with the identical MW of 366.17 but with 50% of likelihood. These structures are the result of hydroxylation reactions typical for P450 2D6 catalytic activity. Two other products provided by MetaSite, with the same 50% of likelihood, were identical with those indicated for P450 1A2 (*N*-deethylated product) and 3A4 (dehydrogenated product), whereas the structure of the oxygenated product (MW 364.15; 50% of likelihood) was different.

In case of all predicted metabolites the calculated cLogP values were in the range of -1.56 to 2.67 (for P450 1A2), 1.56 to 2.52 (for P450 3A4), and 1.21 to 2.62 (for P450 2D6). It means that they are rather more hydrophilic substances compared to the parent compound (cLogP 2.78). This is with accordance with general rule that metabolism of chemicals generates polar water-soluble metabolites what causes their poorer absorption or permeation in biological systems but improves excretion from the body.

Summing up, MetaSite-based *in silico* predictions for sites of C-1311 metabolism supported electrochemical findings. MetaSite correctly predicted the identical *N*-dealkylation and dehydrogenation products generated in the EC system. A number of hydroxylation and oxidation products were also predicted by *in silico* analysis. However, we found that there are some discrepancies in the sites of hydroxylation or oxidation between *in silico* and electrochemical predictions. Firstly, the formation of different isomers cannot be observed from direct on-line EC/MS(/MS), as they often show similar fragmentation patterns in tandem MS. To obtain the stereochemistry induced by different P450 isoenzymes, it would be necessary to modify the electrode surfaces. Secondly, EC yields products resulting from the most labile sites in a molecule sensitive to oxidation or reduction. *In silico* system is based on the prediction of enzyme-substrate interactions (specific to individual P450 isoform), where, in contrast to the EC method, the regioselectivity of drug oxidation is often governed by the topology of the active site of P450's [14,16,17]. Table 4 represents a quick overview of all

438 oxidative products of C-1311 detected by application of various methods for metabolism
439 studies.

4. Conclusions

The present work is a clear demonstration of the usefulness of electrochemistry connected on-line to mass spectrometry for the drug metabolism studies. Electrochemical conversion of the studied antitumor imidazoacridinone C-1311 resulted in the products of *N*-dealkylation, dehydrogenation, hydroxylation and oxidation reactions what confirmed that the cytochrome P450-mediated metabolism of pharmaceuticals can be efficiently simulated in an electrochemical cell. The foregoing experiments revealed that some of the products generated electrochemically were in a good agreement with those predicted by *in silico* analysis and those previously reported from *in vitro* enzymatic incubations. Furthermore, electrochemical method was also found to provide unique features not identified with other approaches. The observed discrepancies between EC- and P450-generated metabolites may be due to the mechanistic differences between EC and enzymatic oxidations or may result from the efficiency of a given method. Nonetheless, electrochemically-mediated transformations supported with *in silico* methods can be considered as a convenient and simple alternative to *in vitro* enzymatic assays. The simplicity of the EC system, and the ease and speed with applying to a large number of compounds, make it a useful tool for the initial investigation of drug metabolism.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors thank Professor Agata Kot-Wasik (Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, Poland) for cooperation in the field of MS(/MS) analysis.

Funding

This work was supported by the National Science Center, Poland [the grant number 2012/07/D/NZ7/03395].

References

[1] W. M. Cholody, S. Martelli, J. Paradziej-Lukowicz, J. Konopa, 5-[(Aminoalkyl)amino]imidazo[4,5,1-de]acridin-6-ones as a novel class of antineoplastic agents. Synthesis and biological activity, J. Med. Chem. 33 (1990a) 49-52.

[2] B. Berger, H. Marquardt, J. Westendorf, Pharmacological and toxicological aspects of new imidazoacridinone antitumor agents, Cancer Res. 56 (1996) 2094-2104.

[3] A. M. Burger, J. A. Double, J. Konopa, M. C. Bibby, Preclinical evaluation of novel imidazoacridinone derivatives with potent activity against experimental colorectal cancer, Br. J. Cancer 74 (1996) 1369-1374.

[4] N. Isambert, M. Campone, E. Bourbouloux, M. Drouin, A. Major, W. Yin, P. Loadman, R. Capizzi, C. Grieshaber, P. Fumoleau, Evaluation of the safety of C-1311 (SYMADEX) administered in a phase I dose-escalation trial as a weekly infusion for 3 consecutive weeks in patients with advanced solid tumors, Eur. J. Cancer 46 (2010) 729-734.

[5] S. C. Smith, D. M. Havaleshko, K. Moon, A. S. Baras, J. Lee, S. Bekiranov, D. J. Burke, D. Theodorescu, Use of yeast chemigenomics and COXEN informatics in preclinical evaluation of anticancer agents, Neoplasia 13 (2011) 72-80.

[6] Z. Mazerska, J. Dziegielewski, J. Konopa, Enzymatic activation of a new antitumour drug, 5-diethylaminoethylamino-8-hydroxyimidazoacridinone, C-1311, observed after its intercalation into DNA, Biochem. Pharmacol. 61 (2001) 685-694.

[7] A. Wiśniewska, A. Chrapkowska, A. Kot-Wasik, J. Konopa, Z. Mazerska, Metabolic transformations of antitumor imidazoacridinone, C-1311, with microsomal fractions of rat and human liver, Acta Biochim. Pol. 54 (2007) 831-838.

[8] A. Potega, E. Dabrowska, M.Niemira, A. Kot-Wasik, S. Ronseaux, J. C. Henderson, C. R. Wolf, Z. Mazerska, The imidazoacridinone antitumor drug, C-1311, is metabolized by FMOs but not cytochrome P450s, Drug Metab. Dispos. 39 (2011) 1423-1432.

[9] A. Potęga, B. Fedejko-Kap, Z. Mazerska, Imidazoacridinone antitumor agent C-1311 as a selective mechanism-based inactivator of human cytochrome P450 1A2 and 3A4 isoenzymes, Pharmacol. Rep. 68 (2016) 663-670.

[10] B. Fedejko-Kap, S. M. Bratton, M. Finel, A. Radominska-Pandya, Z. Mazerska, Role of human UDP-glucuronosyltransferases in the biotransformation of the triazoloacridinone and imidazoacridinone antitumor agents C-1305 and C-1311: highly selective substrates for UGT1A10, Drug Metab. Dispos. 40 (2012) 1736-1743.

[11] U. M. Zanger, M. Schwab, Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation, Pharmacol. Ther. 138 (2013) 103-141.

[12] S. Asha, M. Vidyavathi, Role of human liver microsomes in in vitro metabolism of drugs - a review, Appl. Biochem. Biotechnol. 160 (2010) 1699-1722.

[13] E. F. Brandon, C. D. Raap, I. Meijerman, J. H. Beijnen, J. H. Schellens, An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons, Toxicol. Appl. Pharmacol. 189 (2003) 233-246.

[14] U. Jurva, H. V. Wikstrom, L. Weidolf, A. P. Bruins, Comparison between electrochemistry/mass spectrometry and cytochrome P450 catalyzed oxidation reactions, Rapid Commun. Mass Spectrom. 17 (2003) 800-810.

[15] E. Nouri-Nigjeh, R. Bischoff, A. P. Bruins, H. P. Permentier, Electrochemistry in the mimicry of oxidative drug metabolism by cytochrome P450s, Curr. Drug Metab. 12 (2011) 359-371.

[16] U. Bussy, M. Boujtita, Advances in the electrochemical simulation of oxidation reactions mediated by cytochrome P450, Chem. Res. Toxicol. 27 (2014) 1652-1668.

[17] A. Baumann, U. Karst, Online electrochemistry/mass spectrometry in drug metabolism studies: principles and applications, Expert Opin. Drug Metab. Toxicol. 6 (2010) 715-731.

[18] M. Zheng, X. Luo, Q. Shen, Y. Wang, Y. Du, W. Zhu, H. Jiang, Site of metabolism prediction for six biotransformations mediated by cytochromes P450, Bioinformatics 25 (2009) 1251-1258.

[19] A. Tarcsay, G. M. Keseru, In silico site of metabolism prediction of cytochrome P450-mediated biotransformations, Expert Opin. Drug Metab. Toxicol. 7 (2011) 299-312.

[20] P. R. Ortiz De Montellano, J. J. De Voss, Substrate oxidation by cytochrome P450 enzymes, in: P. R. Ortiz De Montellano (Ed.), Cytochrome P450: Structure, Mechanism, and Biochemistry, Kluwer Academic/Plenum Publishers, New York, 2005, pp. 183-245.

[21] Z. Mazerska, P. Sowiński, J. Konopa, Molecular mechanism of the enzymatic oxidation investigated for imidazoacridinone antitumor drug, C-1311, Biochem. Pharmacol. 66 (2003) 1727-1736.

[22] T. A. Baillie, M. N. Cayen, H. Fouda, R. J. Gerson, J. D. Green, S. J. Grossman, L. J. Klunk, B. LeBlanc, D. G. Perkins, L. A. Shipley, Drug metabolites in safety testing, Toxicol. Appl. Pharmacol. 182 (2002) 188-196.

[23] A. Potęga, D. Garwolińska, A. M. Nowicka, M. Fau, A. Kot-Wasik, Z. Mazerska, Phase I and phase II metabolism simulation of antitumor-active 2-hydroxyacridinone with electrochemistry coupled on-line with mass spectrometry, Xenobiotica (2018) DOI: 10.1080/00498254.2018.1524946.A.

[24] M. Trunzer, B. Faller, A. Zimmerlin, Metabolic soft spot identification and compound optimization in early discovery phases using MetaSite and LC-MS/MS validation, J. Med. Chem. 52 (2009) 329-335.

[25] H. Orhan, N. P. E. Vermeulen, Conventional and novel approaches in generating and characterization of reactive intermediates from drugs/drug candidates, Curr. Drug Metab. 12 (2011) 383-394.

[26] J. J. Pitt, Principles and applications of liquid chromatography-mass spectrometry in clinical bioanalysis, Clin. Biochem. Rev. 30 (2009)19-34.

[27] F. P. Guengerich, Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity, Chem. Res. Toxicol. 14 (2001) 611-650.

[28] W. Lohmann, U. Karst, Generation and identification of reactive metabolites by electrochemistry and immobilized enzymes coupled on-line to liquid chromatography/mass spectrometry, Anal. Chem. 79 (2007) 6831-6839.

[29] S. T. M. Orr, S. L. Ripp, T. E. Ballard, J. L. Henderson, D. O. Scott, R. S. Obach, H. Sun, A. S. Kalgutkar, Mechanism-based inactivation (MBI) of cytochrome P450 enzymes:

549 structure−activity relationships and discovery strategies to mitigate drug−drug interaction
550 risks, J. Med. Chem. 55 (2012) 4896-4933.
551 [30] W. Lohmann, U. Karst, Simulation of the detoxification of paracetamol using on-line
552 electrochemistry/liquid chromatography/mass spectrometry, Anal. Bioanal. Chem. 386
553 (2006) 1701-1708.

Figure captions

Figure 1

Molecular structure and atomic numbering of antitumor-active imidazoacridinone C-1311.

Figure 2

Schematic diagram of the approaches used to study drug or drug candidate metabolic reactions, and/or generation of metabolites. (A) *In vivo* / *in vitro* models; (B) An instrumental set-up used for the electrochemical simulation of phase I oxidative metabolism; (C) *In silico* prediction of sites of metabolism. See the text for a detailed description.

Figure 3

Representative ESI mass spectra of C-1311 phase I oxidative transformation in the EC reactor (A) cell off and (B) cell on (positive ionization mode).

Figure 4

Representative two-dimensional (2-D) mass voltammograms for the selected ions, resulting from the electrochemical oxidation of C-1311 at a glassy carbon working electrode (extracted ion intensity versus the progress of the electrochemical oxidation; positive ionization mode). The *m/z* ratios shown correspond to the protonated $[M+H]^+$ C-1311 (*m/z* 351) and its putative products (see legend), and have been rounded to the nearest integer. The signal is dependent on the voltage used in the EC reactor (two and a half EC cycles are shown – see graph A) Experimental conditions: potential range 0 – 2.5 V; scan rate 10 mV s^{-1}, continuous; T = 21 ^{o}C; ϕ glassy carbon working electrode 8 mm.

Figure 5

Extracted ion chromatograms of the selected mass ions, found in the mixture of C-1311 and its putative oxidation products leaving the electrochemical cell (extracted ion intensity versus the retention time; positive ionization mode). The *m/z* ratios shown have been rounded to the nearest integer. Peak names correspond to compounds presented in Table 2.

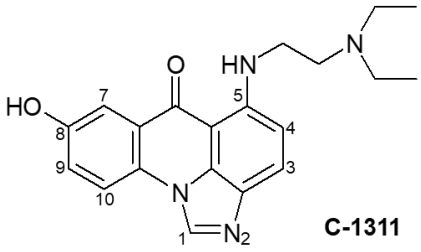
Figure 6

580 Representative Q-TOF-MS/MS spectra of the selected products of C-1311 electrochemical
581 oxidation with proposed fragmentation assignments (insets). Q-TOF-MS/MS, quadrupole-time
582 of flight-tandem mass spectrometry.

583 **Figure 7**

584 General overview of the proposed pathways of C-1311 electrochemical oxidation. Product
585 structures were derived on the basis of measured masses and MS/MS fragmentation patterns.
586 Product names correspond to compounds presented in Table 2.

Figure 1



C-1311

Figure 2

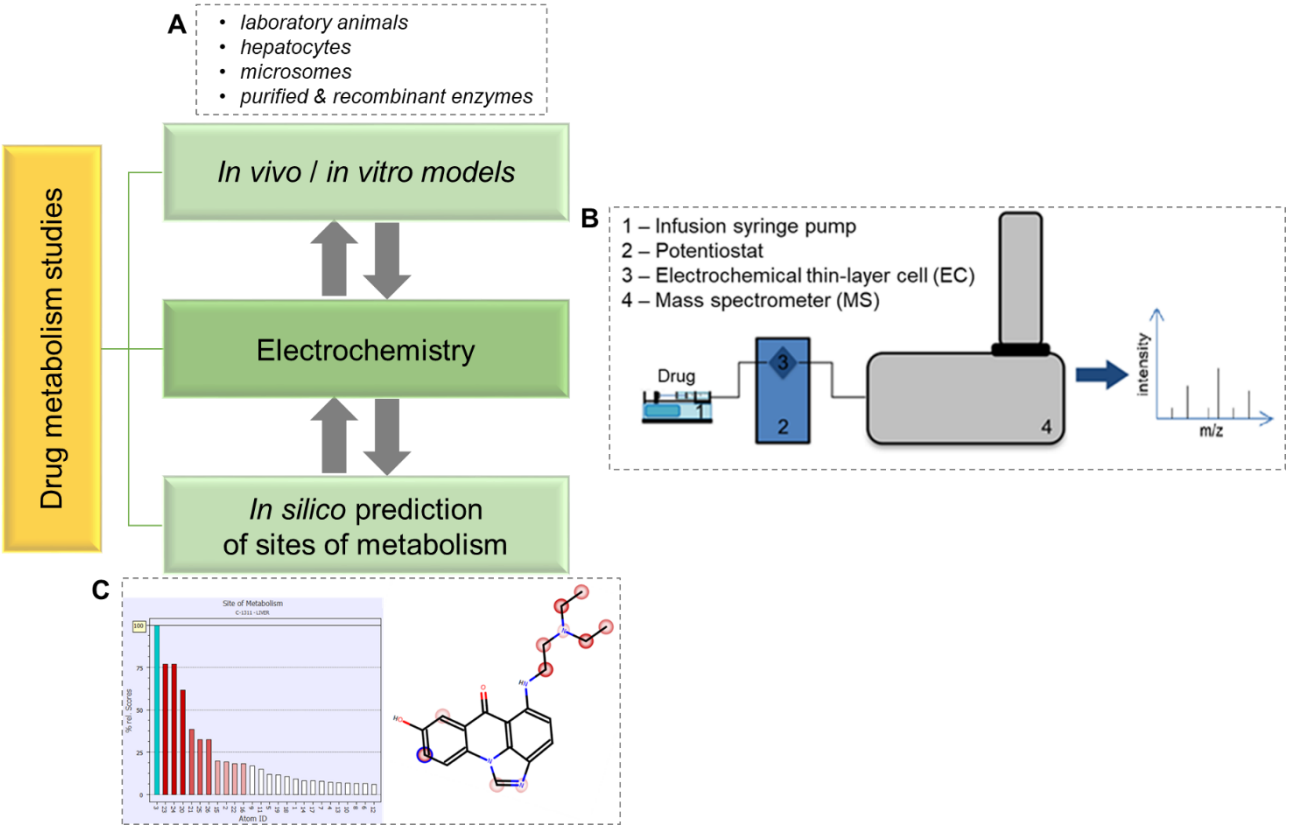


Figure 3

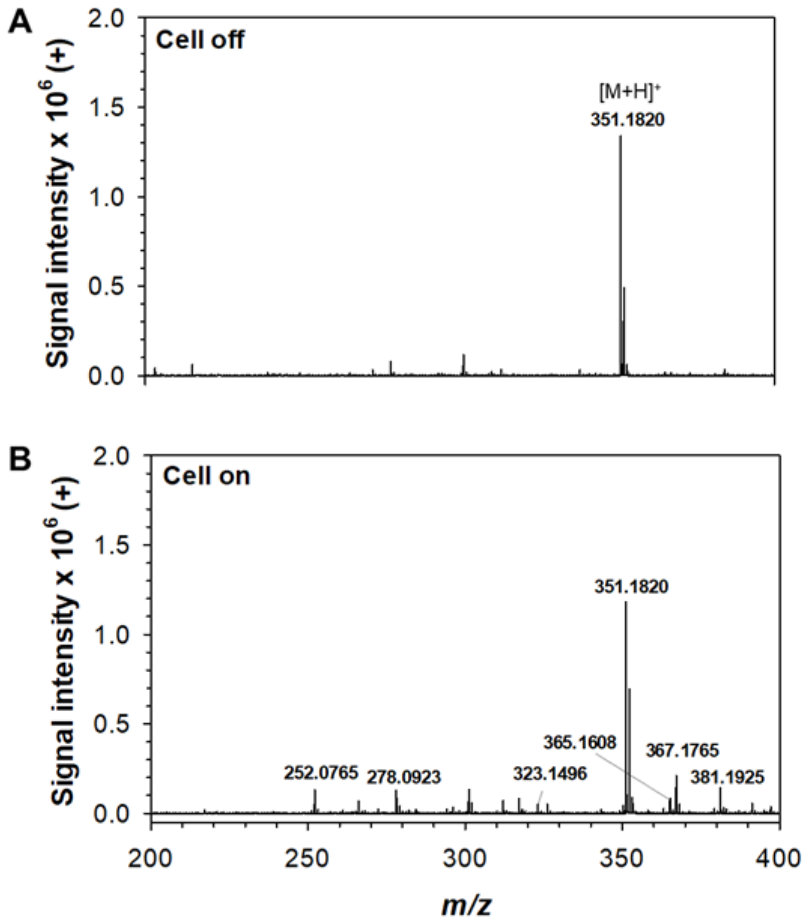


Figure 4

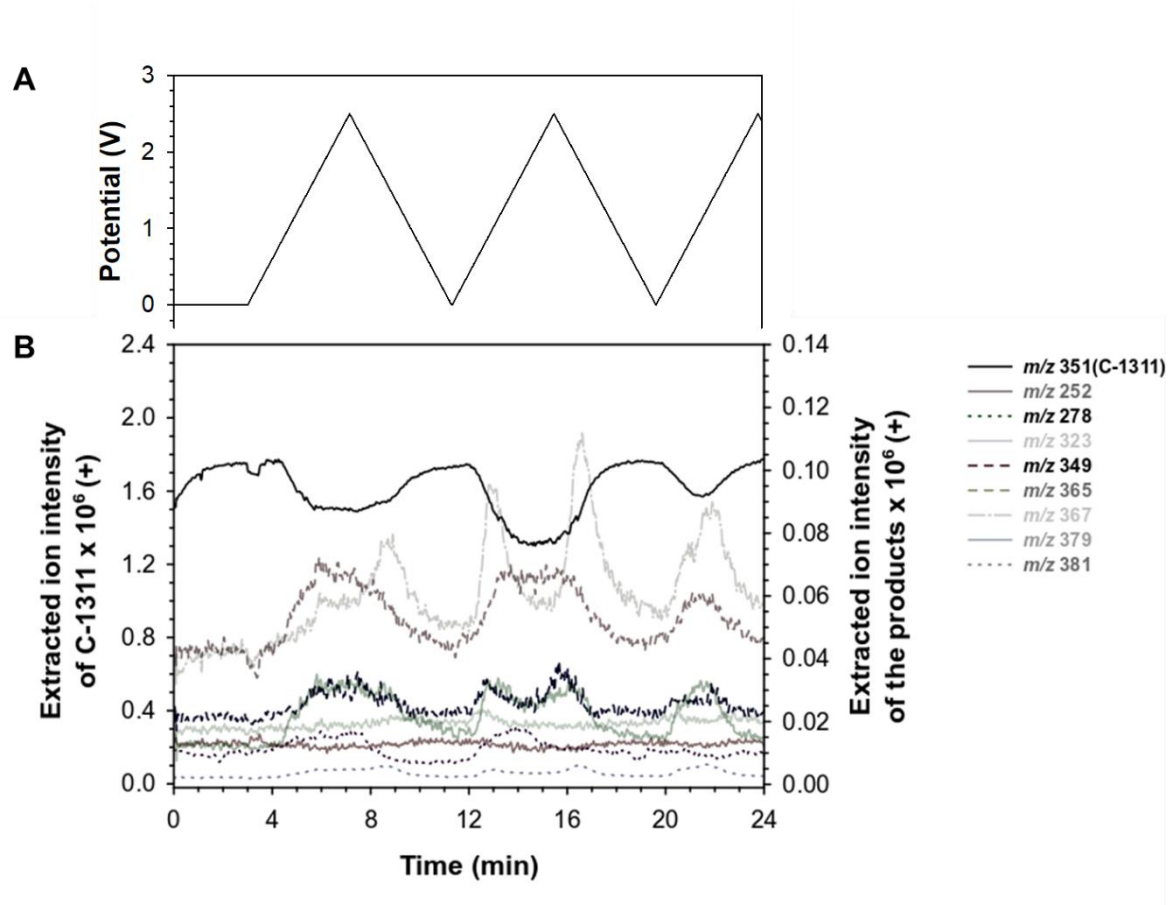


Figure 5

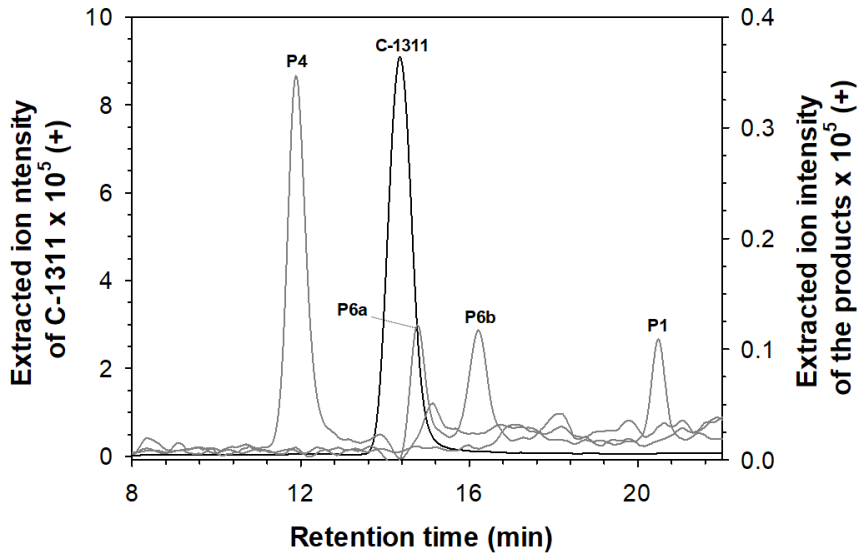


Figure 6

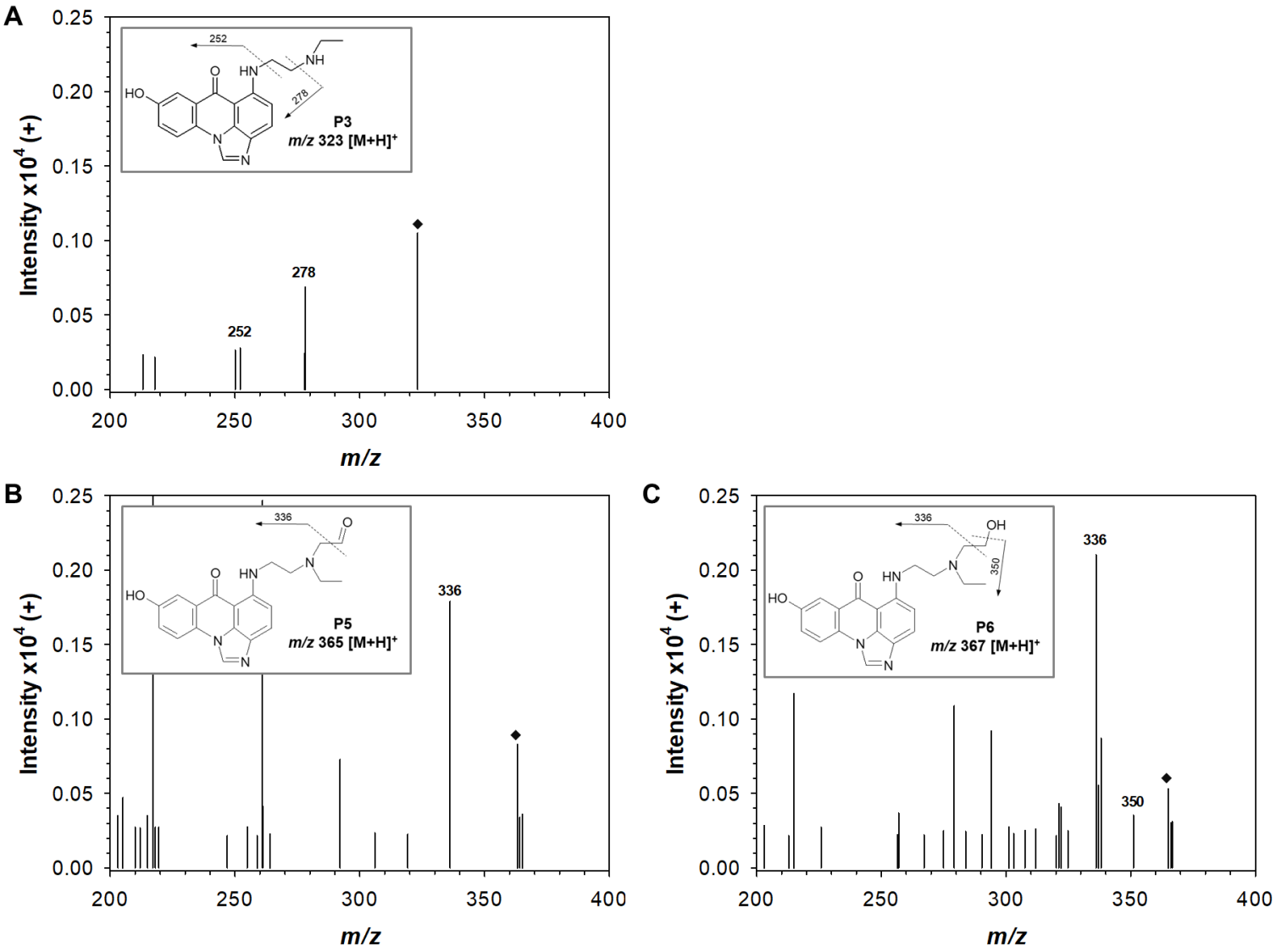


Figure 7

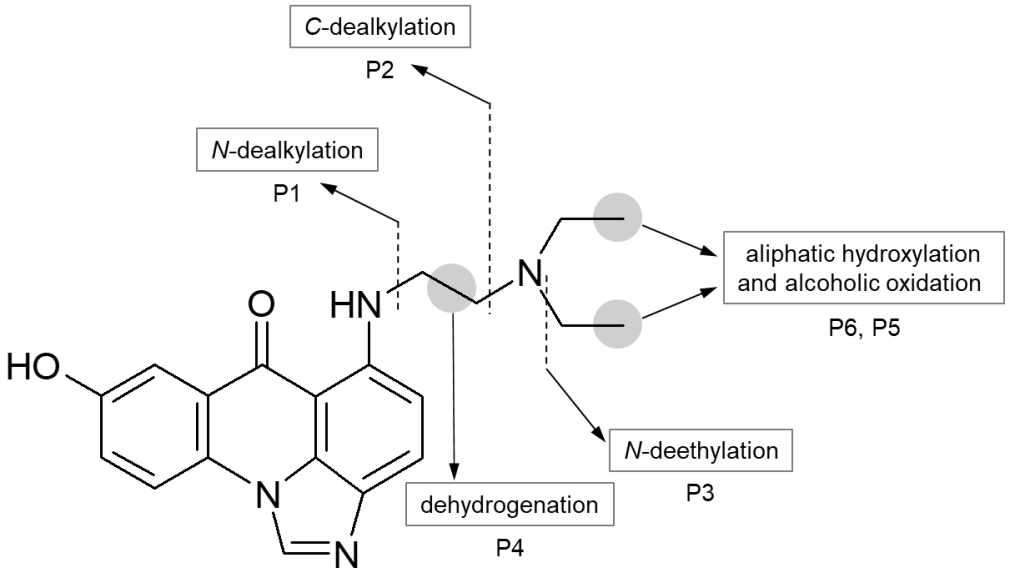


Table 1

EC, ESI-Q-TOF-MS, and ESI-ion trap MS/MS parameters as applied in direct EC/MS(/MS) experiments for determination of accurate masses of product ions and getting ion fragmentation.

	Parameter	Value or setting
EC settings	Flow rate	30 µL min^{-1}
	Potential	0 – 2.5 V (10 mV steps)
	EC operating mode	Scan
	Cycle	Continuous
MS(/MS) settings	Ion source	Dual electrospray
	MS operating mode	Scan
	Ion polarity	Positive
	The range of *m/z*	50 – 600
	Capillary voltage	3500 V
	Nebulizer gas (N_2) pressure	35 psig
	Drying gas (N_2) flow	10 L min^{-1}
	Drying gas temperature	325 °C
	Fragmentor	175 V
	Skimmer	45 V
	OCT 1 RF Vpp	750 V
	Rate	1,5 spectra s^{-1}
	MS/MS method	targeted
	- Slope	- 4 *m/z*
	- Offset	- 5 V

Table 2

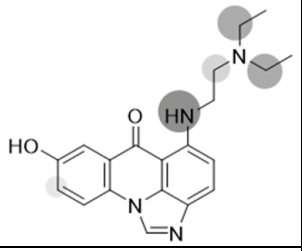
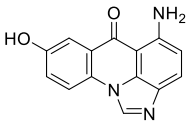
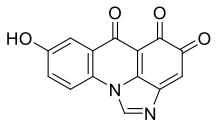
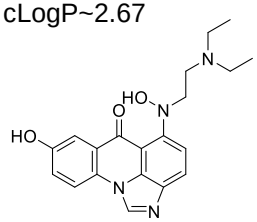
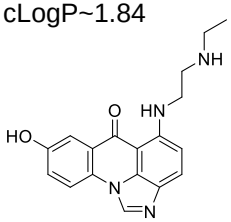
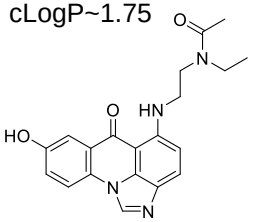
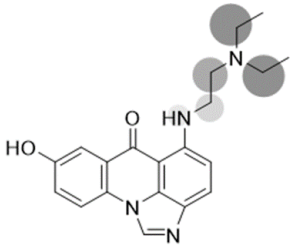
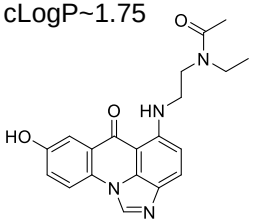
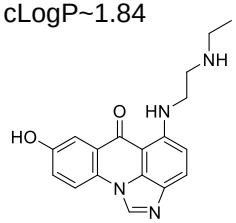
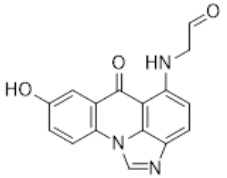
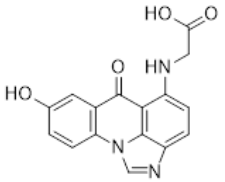
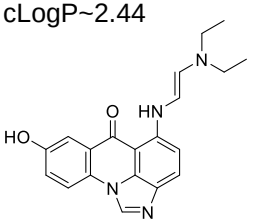
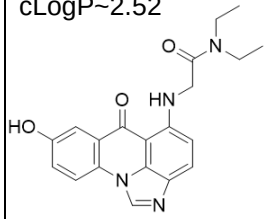
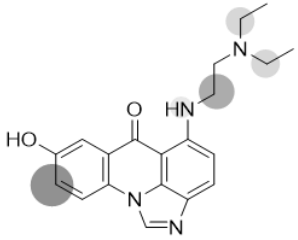
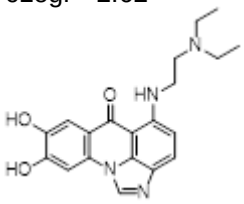
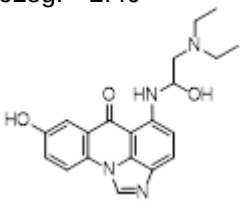
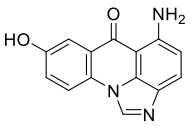
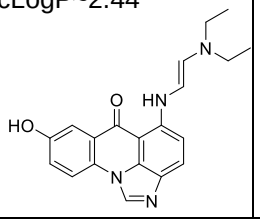
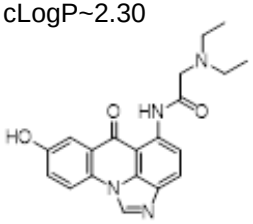
Accurate mass ion data and elemental composition changes associated with C-1311 products detected following electrochemical oxidation of the compound at a glassy carbon working electrode in a potential range of 0 – 2.5 V versus Pd/H_2.

Name	Retention time (min)	Representative *m/z* $[M+H]^+$	Mass error [a] (ppm)	Elemental composition	Elemental composition change	Transformation of C-1311
C-1311	14.33	351.1820	1.3	$C_{20}H_{22}N_4O_2$	-	-
P1	20.47	252.0765	-1.0	$C_{14}H_9N_3O_2$	- $C_6H_{13}N$	*N*-dealkylation (complete)
P2	ND [b]	278.0923	0.0	$C_{16}H_{11}N_3O_2$	- $C_4H_{10}N$	*C*-dealkylation
P3	Trace [c]	323.1496	-2.0	$C_{18}H_{18}N_4O_2$	- C_2H_4	*N*-deethylation
P4	11.88	349.1665	1.7	$C_{20}H_{20}N_4O_2$	- 2H	dehydrogenation
P5	ND [b]	365.1608	0.0	$C_{20}H_{20}N_4O_3$	+ O - 2H	oxidation
P6a, b	14.79; 16.22	367.1765	0.1	$C_{20}H_{22}N_4O_3$	+ O	hydroxylation/*N*-oxidation
P7	ND [b]	379.1764	0.0	$C_{21}H_{22}N_4O_3$	+ O - 2H + CH_2	oxidation + methylation
P8	Trace [c]	381.1925	1.0	$C_{21}H_{24}N_4O_3$	+ O + CH_2	hydroxylation + methylation

[a] Exact masses were calculated using Molecular Mass Calculator freeware version v2.02.
[b] ND, not detected.
[c] Trace, trace amounts observed.

Table 3

Most probable sites of metabolism (SoMs) and top ranked P450 1A2-, and 3A4- and 2D6-mediated metabolites predicted for C-1311 using MetaSite software.

	SoMs for C-1311 (MW 350.17, cLogP 2.78)	Most probable metabolites predicted (n = 5 for P450 1A2 and 2D6, n = 6 for P450 3A4) and respective likelihood ranking					
P450 1A2		100% MW 251.07 cLogP~1.21 	100% MW 266.03 cLogP~-1.56 	100% MW 366.17 cLogP~2.67 	50% MW 322.14 cLogP~1.84 	50% MW 364.15 cLogP~1.75 	
P450 3A4		100% MW 364.15 cLogP~1.75 	100% MW 322.14 cLogP~1.84 	50% MW 293.08 cLogP~2.08 	50% MW 309.07 cLogP~1.56 	50% MW 348.16 cLogP~2.44 	50% MW 364.15 cLogP~2.52 
P450 2D6		100% MW 366.17 cLogP~2.62 	50% MW 366.17 cLogP~2.40 	50% MW 251.07 cLogP~1.21 	50% MW 348.16 cLogP~2.44 	50% MW 364.15 cLogP~2.30 	

The functional groups that most likely will be metabolized are marked: the darker the color and the greater the circle of marked functional group – the higher the probability of metabolism to occur. MW, molecular weight; cLogP, the logarithm of partition coefficient.

Table 4

Representation of putative oxidative products of C-1311 and their detection in various *in vitro* metabolism generation platforms or predicted with the use of MetaSite. The *m/z* ratios shown have been rounded to the nearest integer.

	Occurrence or predicted by					
m/z of putative metabolite	EC	*In vitro*		*In silico* (MetaSite)		
		RLMs or HLMs [7]	HRP/H_2O_2 [21]	P50 1A2	P450 3A4	P450 2D6
252	+	-	+	+	-	+
278	+	-	-	-	-	-
323	+	+	+	+	+	-
349	+	-	-	-	+	+
365	+	-	-	+	+	+
367	+	+	-	+	-	+
379	+	-	-	-	-	-
381	+	-	-	-	-	-

EC, electrochemistry reaction in ROXY™ system; RLMs, rat liver microsomal incubations; HLMs, human liver microsomes; HRP/H_2O_2, horseradish peroxidase/hydrogen peroxide.