Research highlights

- Phase I metabolism of C-1311 was successfully simulated in an electrochemical cell.

- Th*e* 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.

1 **Title page**

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3 **Title:**

4 Electrochemical simulation of metabolism for antitumor-active imidazoacridinone C-1311 and

5 *in silico* prediction of drug metabolic reactions.

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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.

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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

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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

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87   address this challenging task, different methods for the simulation of metabolic reactions have

88   been developed and applied. In practice, the studies on drug metabolism usually include *in*

89   *vivo* experiments on the basis of animal models, or *in vitro* tests, where P450-containing

90   matrices like hepatocytes, liver microsomes, and other cellular fractions derived from animal

91   or human tissue are used [12] (Fig. 2A). Although the biological systems are still being

92   improved, there are a number of drawbacks associated with these approaches (*e.g.*, the great

93   expense of biological material and cofactors, the ethical aspect of using biological material of

94   human or animal origin). From a practical aspect, both *in vitro* and *in vivo* systems inherently

95   have a complex biological matrix that makes isolation and purification of metabolites time-

96   consuming and laborious [13]. Therefore, analytical techniques which are able to mimic

97   metabolic reactions taking place in the human body and which simultaneously allow to reduce

98   the listed above limitations, gain strong attention.

99   In recent years a particular interest in the field of drug metabolism studies has been

100 observed from electrochemistry. Complementary to the existing techniques, oxidation-

101 reduction reactions catalyzed by liver P450 isoforms can be successfully simulated in an

102 electrochemical (EC) cell coupled directly to a mass spectrometer (MS) [14,15]. This simple

103 and pure instrumental technique allows for the generation of a number of potential oxidative

104 metabolites, including reactive intermediates, and for the detection of the sites labile towards

105 oxidation in a drug molecule [16,17]. The greatest advantage of the electrochemical studies on

106 oxidative metabolic reactions over other *in vitro* methods is the absence of proteins in the

107 reaction medium. Moreover, in EC system it is possible to control the reaction rate by the

108 applications of electrode potentials specific for synthesis of the expected products [15].

109 Considering the mechanistic differences between EC and enzymatic oxidations, not all

110 metabolic pathways can be mimicked by EC. Thus, EC cannot replace conventional

111 metabolism studies in biological systems. However, this technique may serve as an attractive

112 alternative tool for the initial investigation of drug metabolism in a single-step experiment. In

113 this work, we explored the on-line combination of EC with electrospray ionization (ESI) MS for

114 metabolism studies of antitumor drug candidate C-1311 (Fig. 2B). The main focus of the

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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].

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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^{TM} potentiostat was equipped with a commercial electrochemical thin-layer ReactorCell^{TM} and a SP2-ROXY^{TM} 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^{TM} 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^{TM} 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,

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171   Santa Clara, CA, USA) by means of 50 cm long PEEK tubing (130 µm I.D.). The system was

172   controlled by Agilent MassHunter Workstation software (Agilent Technologies). The scan

173   range was set from 50 to 600 mass-to-charge ratio (*m/z*) and mass spectrometric detection

174   was carried out in the positive ionization mode (+). Tandem mass spectrometry (MS/MS) mode

175   was used to provide additional structural information for the ions of interest. To ensure accurate

176   mass during the experiment, the mass spectrometer was calibrated daily using calibration

177   solution (ES-TOF reference mix, Agilent Technologies). The corresponding conditions for the

178   ESI-Q-TOF-MS measurements and the optimized parameters for the ESI-ion trap MS/MS

179   experiments can be viewed in Table 1.

180   The delay time between product formation within the EC cell and detection in the MS was

181   taken into account. It was calculated on the basis of the dead volume between the reactor cell

182   and the ion source of the MS (for the supplied tubing this volume was 12.7 µL) and the flow

183   rate of the infusion syringe pump (30 µL min^{-1}). The transfer time to the capillary was estimated

184   at about 0.7 min. Therefore, the oxidation behavior of C-1311 was observed in real time during

185   application of a potential ramp to the EC cell.

186   **2.2.3. Liquid chromatography (LC)**

187   The mixture of C-1311 and its putative oxidation products leaving the EC cell was also

188   collected and analysed by LC/MS. Liquid chromatographic separations were performed on a

189   reversed-phase (RP) 5-µm Suplex pKb-100 analytical column (4.6 mm x 250 mm, C18)

190   (Supelco Inc., Bellefonte, PA, USA) with Agilent 6500 Series Accurate-Mass Q-TOF LC/MS

191   system (Agilent Technologies) controlled by Agilent MassHunter Workstation software. The

192   mobile phase consisted of 0.05 M aqueous NH_4HCO_2 buffer (pH 3.4 adjusted with FA; solvent

193   A) and MeOH (solvent B). The LC analyses were carried out at a flow rate of 1 mL min^{-1} with

194   the following mobile phase system: a linear gradient from 15% to 80% B in A for 25 min,

195   followed by a linear gradient from 80% to 100% B in A for 3 min, and 2 min isocratic 100% B.

196   During all LC separations, the column was operated at a room temperature. The eluates were

197   monitored at 380 nm. The MS conditions were identical to those described above.

198   **2.3. Electrochemical simulation of the oxidative metabolism of C-1311**

199   A stock solution of C-1311 was prepared in an electrolyte (composition described below) at a

200   concentration of 1 mg mL^{-1}. It was used to prepare fresh working solutions of C-1311 (0.01 mg

201   mL^{-1}) shortly before each experiment. Two electrolytes of different pH values were used for

202   optimization of the electrochemical conditions: (1) H_2O-MeOH (1:1, *v/v*) with 0.1% FA, pH 3.3;

203   (2) 20 mM NH_4HCO_2-ACN (1:1, *v/v*), pH 7.4. Simulation of the oxidative phase I metabolism of

204   C-1311 in the EC cell was accomplished at a constant flow rate of 30 μL min^{-1}. The potential

205   of the working electrode was ramped linearly from 0 V up to 2.5 V at 10 mV s^{-1} and was

206   controlled by the Dialogue software (Antec Leyden). The relevant EC conditions are listed in

207   Table 1. The outlet of the reactor cell was connected on-line to the ESI-MS source.

208   **2.4. *In silico* prediction of cytochrome P450-mediated sites of C-1311 metabolism**

209   The MetaSite software tool (version 5.1.1; Molecular Discovery Ltd., Hertfordshire, UK) is a

210   computational algorithm used to identify most likely metabolic soft-spots of xenobiotics. The

211   software considers two factors: chemical reactivity of the substrate and structural

212   complementarity between the active site of the P450 enzyme and the ligand and comes up

213   with the most optimal orientation [24]. A 2-D structure of the C-1311 was imported into the

214   interface of MetaSite to predict phase I sites of metabolism and structures of metabolites in

215   liver related to P450 1A2-, 3A4-, and 2D6-mediated reactions. Only metabolites with a

216   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

245   at a working electrode may also affect the EC efficiency. All these parameters were optimized

246   for the best conditions for oxidation and identification processes. In result, the most effective

247   conversion of C-1311 into its expected metabolites was attained with use of the H_2O-MeOH

248   (1:1, *v/v*) with 0.1% FA electrolyte (pH 3.3) and glassy carbon as a working electrode material

249   for voltage range between 0 and 2.5 V. Additionally, we have observed that methanol was

250   better than acetonitrile in diminishing the adsorption of the electrochemical products on the

251   surface of the working electrode what resulted in greater signal intensity of the selected mass

252   ions. Moreover, H_2O-MeOH electrolyte solution produced the lowest mass background noise

253   in positive total ion chromatograph (data not shown). Details regarding the optimized EC

254   conditions used for measurements can be found in Table 1.

255   **3.1.2. On-line EC/MS experiments**

256   Initially, a solution of C-1311 was directly introduced from the syringe pump into the EC cell

257   without applying a voltage to the working electrode (cell off). Due to the presence of the

258   nitrogen atoms in the side chain, the C-1311 molecule can easily be protonated and detected

259   with high intensities as $[M+H]^+$ ion (*m/z* 351.1820) (Fig. 3A) in the positive ionization mode of

260   the ESI-Q-TOF mass spectrometer. In turn, applying a voltage to the EC cell (cell on) led to C-

261   1311 oxidation and resulted in a reduction in ion signal corresponding to the substrate.

262   Simultaneously, oxidation products of C-1311 were formed and new signals of the $[M+H]^+$ ions

263   corresponding to putative oxidation products were observed (Fig. 3B). To provide a concise

264   overview of the oxidation products, 2-D mass voltammograms for the ions of interest were

265   generated (Fig. 4) by plotting the intensities of the extracted ions against time of

266   electrochemical analysis. A total of eight putative products of C-1311 were generated by EC.

267   The electrosynthesis of various products required operation at different potential settings. The

268   vast majority of products were generated at potentials between 1 and 2 V, and to form the

269   products at *m/z* 365 and *m/z* 367 the higher values of potentials were required.

270   **3.1.3. Characterization of the products**

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271 Combining EC system on-line with MS allowed the initial characterization of the formed

272 products by an increasing signal intensity of the corresponding *m/z*. Their molecular structures

273 have been derived based on calculations with accurate mass data (mass

274 ≤ 2 ppm). The fragmentation (MS/MS) spectra were also recorded. In turn, the separation of

275 oxidation products by reversed-phase LC can overcome ion suppression effects that may be

276 encountered during on-line EC/MS thus providing additional information about the range of

277 oxidation products and, consequently, about the reaction mechanism [26]. However, unstable

278 products may escape analysis. Therefore, both approaches (EC/MS and LC/MS) were

279 employed for a comprehensive analysis. LC method was established based on the preliminary

280 results from 2-D mass voltammograms. Extracted ion chromatograms of C-1311 and its

281 products that were generated electrochemically are presented in Fig. 5.

282   A comprehensive summary of the products detected following oxidative transformation of

283 C-1311 in the EC cell was shown in Table 2. *N*-Dealkylation is a characteristic type of *in vitro*

284 and *in vivo* metabolic transformation catalyzed by cytochrome P450 enzymes and it is a major

285 metabolism pathway for drugs containing secondary and tertiary amines [27]. This process

286 can also be easily induced electrochemically [14,15,28]. A signal at *m/z* 252 (P1) involves a

287 reduction in mass by 99 Da what can be explained by a loss of diethylaminoethyl moiety as a

288 result of complete *N*-dealkylation in the side chain of C-1311 molecule. In turn, the P3 product

289 (*m/z* 323; - 28 Da) may arise from an *N*-deethylation reaction. Postulated mechanism of the

290 electrochemical *N*-dealkylation of *N*-alkylamines proceeds through one-electron transfer to

291 generate an imine intermediate that, after hydrolysis and intramolecular rearrangement, gives

292 the product of *N*-dealkylation [27]. For further confirmation of the *N*-dealkylated derivatives, the

293 fragmentation patterns, generated by in-source fragmentation in the ESI interface, have been

294 studied. The Q-TOF-MS/MS spectrum obtained for *m/z* 323 ion (Fig. 6A) showed fragment

295 ions at *m/z* 252 (- 99 Da) and *m/z* 278 (- 73 Da). While the former can be assigned to the *N*-

296 dealkylation process, the latter can indicate an intermediate product of a side chain

297 degradation (P2) that was also detected in the EC system. In the elution profile (according to

298 gradient profile used for LC analysis) the P1 peak was eluted at 20.47 min (Fig. 5) so that it

299   appeared after the peak corresponding to the mass ion at m/z 351 (C-1311; t_R 14.33 min).

300   Hence, it would indicate its less polar nature than that of the substrate. P3 product was found

301   in trace amounts in LC analysis.

302   Dealkylation is usually considered as an element of the detoxification pathway of

303   xenobiotics [27]. In this sense, the metabolic transformation leading to the N-dealkylated

304   products should give probably the less toxic, inactive metabolites. Both N-dealkylated

305   products, P1 and P3, were also observed in horseradish peroxidase/hydrogen peroxide

306   system [21], while only the product P3 was detected in similar amounts after incubation with

307   rat and human microsomes [7]. Consequently, the presented electrochemical reactor seems

308   to be better suited for simulating the P450-catalyzed N-dealkylation reactions than liver

309   microsomes. This may be related to the fact that, although liver microsomes contain the

310   physiologically relevant combinations of drug-metabolizing enzymes, expression of different

311   P450 isoforms may be very diverse in the organism. On the other hand, primary amines are

312   the most common quasi-irreversible P450 inactivators [29]. The resulting primary amine may

313   be further oxidized to nitroso derivative (via the intermediate hydroxylamine metabolite), which

314   can form a metabolic intermediate complex with the ferrous form of the heme iron atom.

315   Therefore, secondary and tertiary amines can serve as suitable precursors for enzyme

316   inactivation provided they are N-dealkylated to primary amines. This is in agreement with the

317   previous knowledge on the metabolic activation of C-1311 [9].

318   The dehydrogenation reaction is a special feature of P450 in which it actually behaves as

319   an oxidase-dehydrogenase, and converts molecular oxygen to water [27]. In addition to N-

320   dealkylation, dehydrogenation can also be imitated by direct electrochemical oxidation where

321   it is proposed to undergo through two successive electron/proton transfer reactions [14,15].

322   The obtained mass ion at m/z 349 correlates with the loss of two hydrogen atoms (- 2 Da) what

323   indicates the generation of the dehydrogenated product (P4). To elucidate the nature of this

324   compound, the LC/MS analysis was performed. The peak at m/z 349 (Fig. 5) was eluted ahead

325   of C-1311 at 11.88 min what suggests rather higher hydrophilicity of the respective compound

326   than that of the substrate. It is assumed that a carbon-carbon double bond is formed in the

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327   side chain what is energetically advantageous because provides an extra stability due to the

328   possibility of resonance with the electrons of the aromatic system. Electrochemical

329   dehydrogenation of a carbon-carbon bond has been observed for a number of drug

330   compounds [14,28,30].

331   Electrochemical conversion of C-1311 mainly led to the formation of the products P5 and

332   P6, resulting in the signals at *m/z* 365 and *m/z* 367, respectively (Fig.4B). In the case of the

333   first one, an increase in mass by 14 Da, compared with *m/z* 351 (C-1311), can be explained

334   by a two-step process, consisting of oxidation (+ O), followed by the dehydrogenation (- 2H).

335   This may indicate a route leading to an aldehyde or a ketone. The second mass ion suggests

336   the introduction of one oxygen atom into the C-1311 molecule, shown by a mass increase of

337   16 Da. The insertion of an oxygen atom may take place under the formation of a hydroxyl

338   group (aromatic or aliphatic hydroxylation) or by *N*-oxidation. Carbon hydroxylation is a very

339   common P450-catalyzed reaction [27] and the utility of electrochemistry for simulation of this

340   process for pharmaceutical drugs and xenobiotics is well documented [14,16,17]. The LC

341   separation of the electrochemically generated products revealed that two different isomers

342   (peaks P6a and P6b in Fig. 5) of a species with *m/z* 367, showing similar intensities, could be

343   detected. Their retention times of 14.79 min and 16.22 min, respectively, were higher than C-

344   1311 what confirmed the assumption that the hydroxylated products might have been formed.

345   Moreover, these peaks may be attributable to hydroxylation on different sites of the C-1311

346   molecule.

347   MS/MS spectrum of *m/z* 367 showed the fragment ions at *m/z* 350 and the most abundant

348   *m/z* 336 (Fig. 6C). The existence of fragment ion at *m/z* 350 may indicate the loss of one

349   hydroxyl group (- 17 Da), what can hardly be in agreement with an *N*-oxide structure, while the

350   latter may arise from the heterolytic beta cleavage of carbon-carbon bound that released a

351   CH_2OH molecule (- 31 Da). Hence, one of the isomers of the P6 product may be associated

352   with a singly hydroxylated species bearing the additional oxygen atom at the terminal carbon

353   atom (*ω* position) of the ethyl group of the nitrogen atom. In this study, working at higher

354   potentials enabled aliphatic carbon to be hydroxylated. According to previous studies on C-

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355   1311 metabolism [7,8,21], the likely hydroxylation site in the C-1311 molecule may also be the

356   imidazoacridinone moiety. However, no evidence was found for the formation of an aromatic

357   alcohol and our findings strongly show that hydroxylation on the side chain of the compound

358   appears to be more preferential in electrochemistry. On the other hand, *N*-oxidation could not

359   be excluded, since the product at *m/z* 367 was obtained in both rat as well as human liver

360   microsomes [7] and in metabolism of C-1311 with FMO [8], and have been proposed to be *N*-

361   oxide derivative on the *ω*-nitrogen atom of C-1311. The peak of the 6a isomer, with a slightly

362   higher retention time than C-1311, may just point out the presence such a product.

363   Interestingly, the identical fragment ion at *m/z* 336 was also seen in MS/MS spectrum of *m/z*

364   365 (Fig. 6B). This time it is implied that the loss of COH molecule took place what confirms

365   the further oxidation of the P6 to the corresponding aldehyde.

366   The next oxidation products P7 and P8 exhibited strong signals at *m/z* 379 and *m/z* 381,

367   respectively. Despite showing quite good intensities in the EC/MS system, both products were

368   not found in LC/MS analysis and no MS/MS data were accessible for them. This may be due

369   to the limited stability of these reaction products. Thus, the assigned structures of P7 and P8

370   are to be regarded as tentative. The determined exact mass of *m/z* 379.1764 indicates the

371   formation of a derivative with an additional formyl group (-CHO; calculated: *m/z* 379.1764,

372   mass error 0.0 ppm) rather than a product of double oxidation (+ 2O, - 4H; calculated: *m/z*

373   379.1401, mass error 95.8 ppm). It presumably occurred through the reaction of C-1311 with

374   formic acid present in the electrolyte solution. In turn, the P8 product is proposed to be the P7

375   corresponding alcohol. A general overview of the proposed pathways of C-1311

376   electrochemical oxidation is shown in Fig. 7.

377   **3.2. *In silico* prediction of cytochrome P450-mediated sites of C-1311 metabolism**

378   No stable final products of C-1311 metabolism with P450 isoenzymes have been observed

379   so far [8], but the results of cytochrome P450 1A2 and 3A4-inhibitory activities of the compound

380   [9] strongly suggest the possibility of any metabolic activation pathway. C-1311, as a prodrug,

381   could be also activated by the action of P450 2D6 that is an isoenzyme specifically involved in

382   aliphatic and aromatic hydroxylations [20]. Our former studies and these presented above

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383 pointed that the most reactive site in the C-1311 molecule was expected to be diethylamino

384 functional group. In this study, for the demonstration of the suitability of the electrochemical

385 system in drug metabolism studies, prediction of C-1311 metabolism was performed with the

386 use of MetaSite 5.1.1 software tool (Molecular Discovery Ltd.). The results of the proposed the

387 most probable metabolites with their molecular weight and calculated cLogP (the logarithm of

388 the partition coefficient between *n*-octanol and water) are presented in Table 3.

389 Five and six top ranked phase I metabolite structures were predicted in the case of

390 P450 1A2 and 3A4 isoforms, respectively. The dialkylaminoalkylamino moiety was proved to

391 be the most probable site of P450 attack in the C-1311 molecule. In both cases, the *N*-

392 deethylation products were obtained (MW 322.14; 50% and 100% of likelihood for P450 1A2

393 and 3A4, respectively), wherein complete *N*-dealkylation (MW 251.07; 100% of likelihood) is

394 likely only with the participation of P450 1A2 isoenzyme. MetaSite also indicated the possibility

395 of hydroxylation (MW 366.17; 100% of likelihood for P450 1A2) and oxidation (MW 364.15) on

396 various positions of the side chain but with the indication of the ω - 1 carbon atom of the

397 terminal ethylamino group as preferential (50% and 100% of likelihood for P450 1A2 and 3A4,

398 respectively). The most likely metabolite for P450 1A2 activity was also formed by oxidative

399 deamination and the following oxidation on the imidazoacridinone ring (MW 266.03; 100% of

400 likelihood). This electrophilic *ortho*-quinone derivative may potentially be implicated in the

401 covalent apoprotein modification of P450 isoenzymes. Hence, its existence may be associated

402 with the observed stronger inactivation of P450 1A2 than 3A4 by C-1311 [9]. Probably due to

403 its high chemical reactivity, this product was not detected in the EC/MS system. Oxidative

404 deamination product (MW 293.08) and its corresponding carboxylic acid (MW 309.07), both

405 with an equal likelihood of 50%, were predicted in the case of P450 3A4. Another main

406 metabolite of this P450 isoenzyme was that derived by dehydrogenation in the ethylamino

407 chain (MW 348.16; 50% of likelihood). Considering P450 2D6-mediated sites of C-1311

408 metabolism, MetaSite predicted the *ortho* position to the hydroxyl group in the acridinone

409 moiety as the most probable site of C-1311 hydroxylation (MW 366.17; 100% of likelihood).

410 The resulting diol derivative may contribute to overall cytotoxic activity of the compound

16

411   because it is a preffered structure for the substitution by nucleophiles existing in a living

412   organism (*i.e.*, glutathione, thiols of proteins or purine and pyrimidine bases of DNA). However,

413   the predicted hydroxylation would take place also in the aliphatic chain giving a compound with

414   the identical MW of 366.17 but with 50% of likelihood. These structures are the result of

415   hydroxylation reactions typical for P450 2D6 catalytic activity. Two other products provided by

416   MetaSite, with the same 50% of likelihood, were identical with those indicated for P450 1A2

417   (*N*-deethylated product) and 3A4 (dehydrogenated product), whereas the structure of the

418   oxygenated product (MW 364.15; 50% of likelihood) was different.

419   In case of all predicted metabolites the calculated cLogP values were in the range of -1.56

420   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

421   that they are rather more hydrophilic substances compared to the parent compound (cLogP

422   2.78). This is with accordance with general rule that metabolism of chemicals generates polar

423   water-soluble metabolites what causes their poorer absorption or permeation in biological

424   systems but improves excretion from the body.

425   Summing up, MetaSite-based *in silico* predictions for sites of C-1311 metabolism

426   supported electrochemical findings. MetaSite correctly predicted the identical *N*-dealkylation

427   and dehydrogenation products generated in the EC system. A number of hydroxylation and

428   oxidation products were also predicted by *in silico* analysis. However, we found that there are

429   some discrepancies in the sites of hydroxylation or oxidation between *in silico* and

430   electrochemical predictions. Firstly, the formation of different isomers cannot be observed from

431   direct on-line EC/MS(/MS), as they often show similar fragmentation patterns in tandem MS.

432   To obtain the stereochemistry induced by different P450 isoenzymes, it would be necessary

433   to modify the electrode surfaces. Secondly, EC yields products resulting from the most labile

434   sites in a molecule sensitive to oxidation or reduction. *In silico* system is based on the

435   prediction of enzyme-substrate interactions (specific to individual P450 isoform), where, in

436   contrast to the EC method, the regioselectivity of drug oxidation is often governed by the

437   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.

19

Conflicts of interest

There are no conflicts to declare.

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554   **Figure captions**

555   **Figure 1**

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

557   **Figure 2**

558   Schematic diagram of the approaches used to study drug or drug candidate metabolic

559   reactions, and/or generation of metabolites. (A) *In vivo* / *in vitro* models; (B) An instrumental

560   set-up used for the electrochemical simulation of phase I oxidative metabolism; (C) *In silico*

561   prediction of sites of metabolism. See the text for a detailed description.

562   **Figure 3**

563   Representative ESI mass spectra of C-1311 phase I oxidative transformation in the EC reactor

564   (A) cell off and (B) cell on (positive ionization mode).

565   **Figure 4**

566   Representative two-dimensional (2-D) mass voltammograms for the selected ions, resulting

567   from the electrochemical oxidation of C-1311 at a glassy carbon working electrode (extracted

568   ion intensity versus the progress of the electrochemical oxidation; positive ionization mode).

569   The *m/z* ratios shown correspond to the protonated [M+H]$^+$ C-1311 (*m/z* 351) and its putative

570   products (see legend), and have been rounded to the nearest integer. The signal is dependent

571   on the voltage used in the EC reactor (two and a half EC cycles are shown – see graph A)

572   Experimental conditions: potential range 0 – 2.5 V; scan rate 10 mV s^{-1}, continuous; T = 21 ^{o}C;

573   ϕ glassy carbon working electrode 8 mm.

574   **Figure 5**

575   Extracted ion chromatograms of the selected mass ions, found in the mixture of C-1311 and

576   its putative oxidation products leaving the electrochemical cell (extracted ion intensity versus

577   the retention time; positive ionization mode). The *m/z* ratios shown have been rounded to the

578   nearest integer. Peak names correspond to compounds presented in Table 2.

579   **Figure 6**

24

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.
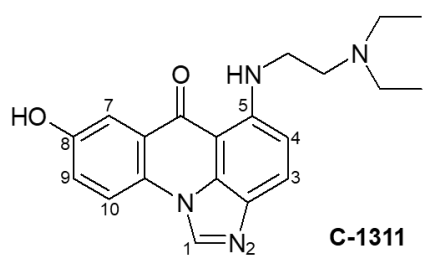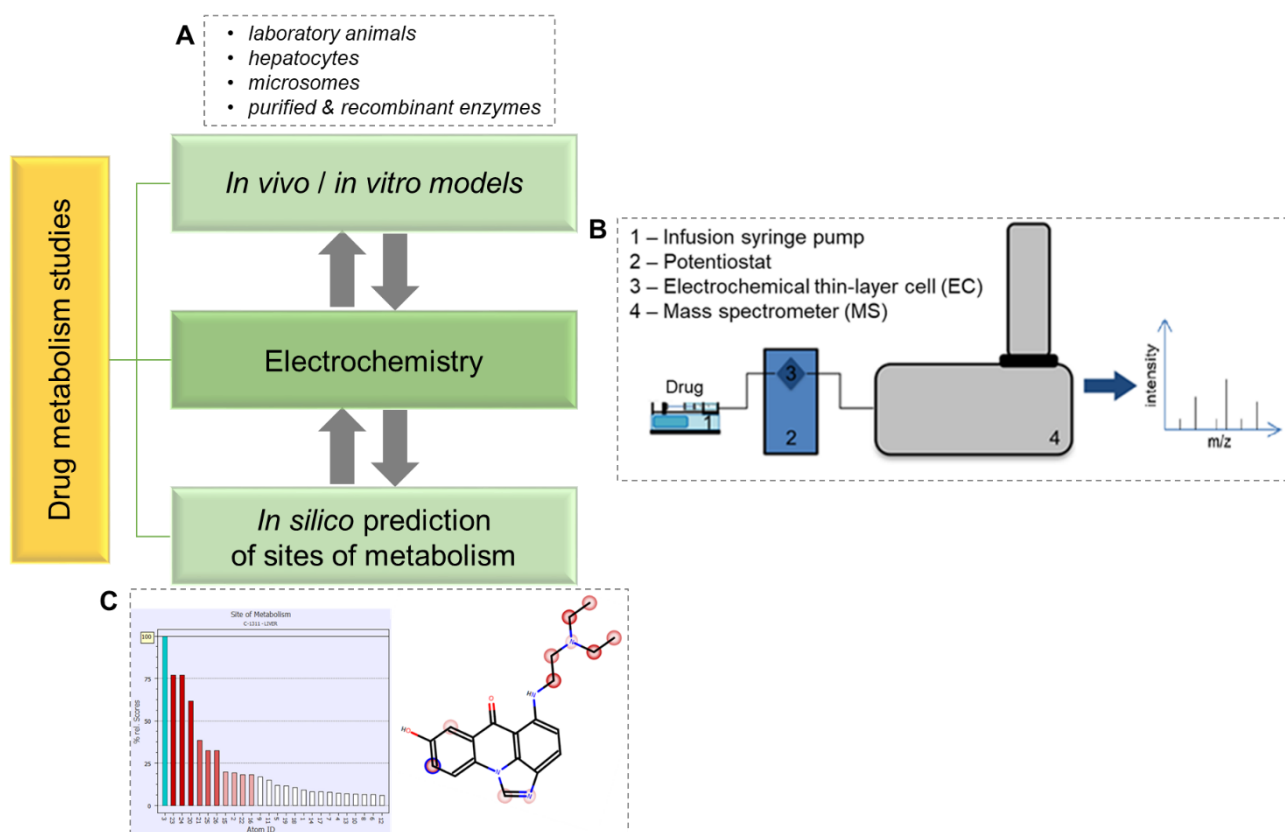
586 Product names correspond to compounds presented in Table 2.

Figure 1

C-1311
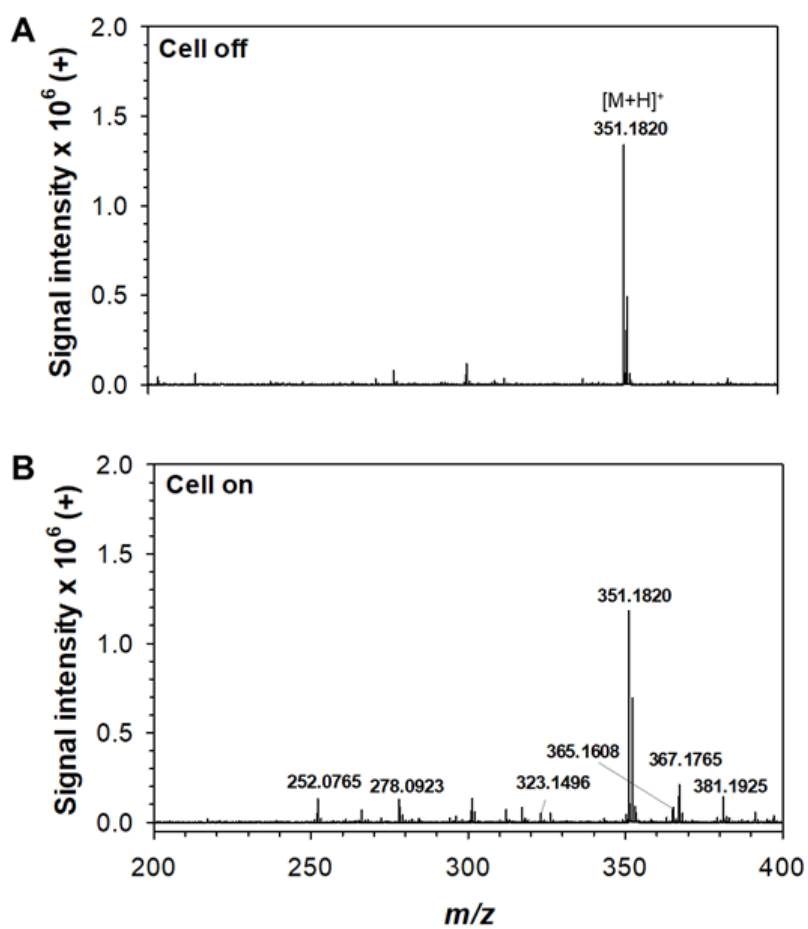
Figure 2
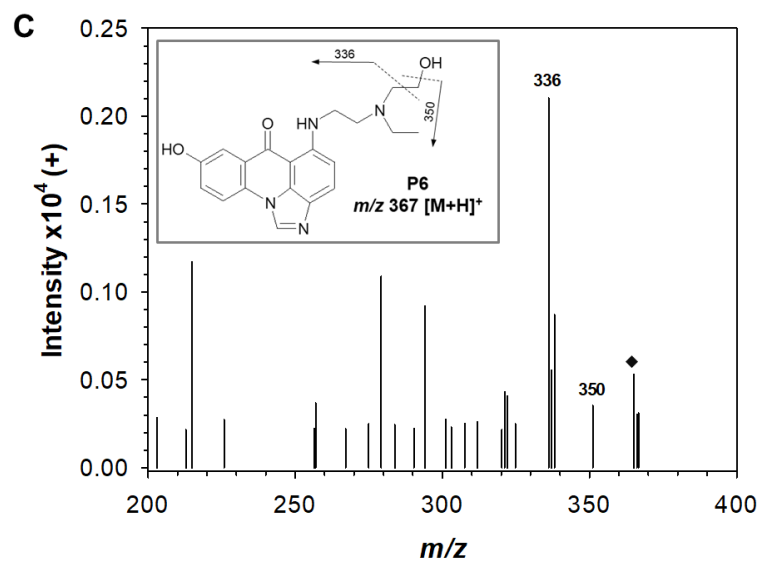
Figure 3

Figure 4
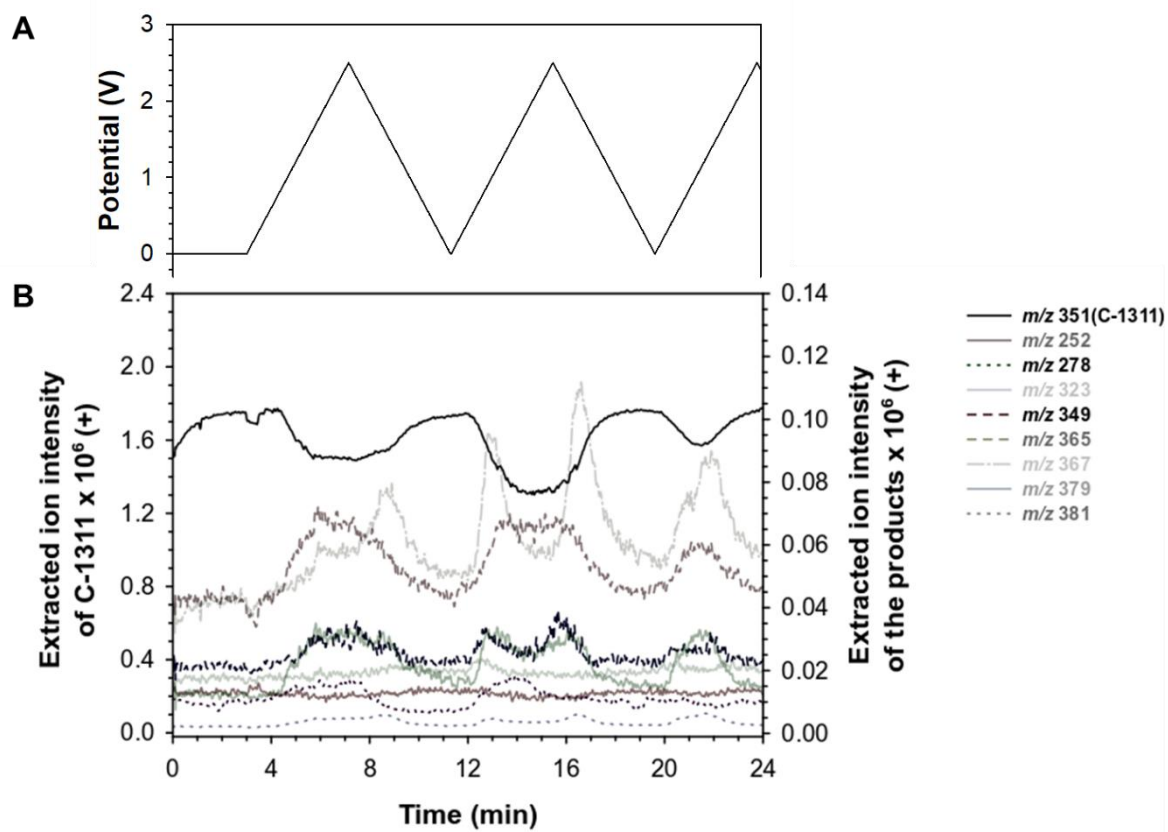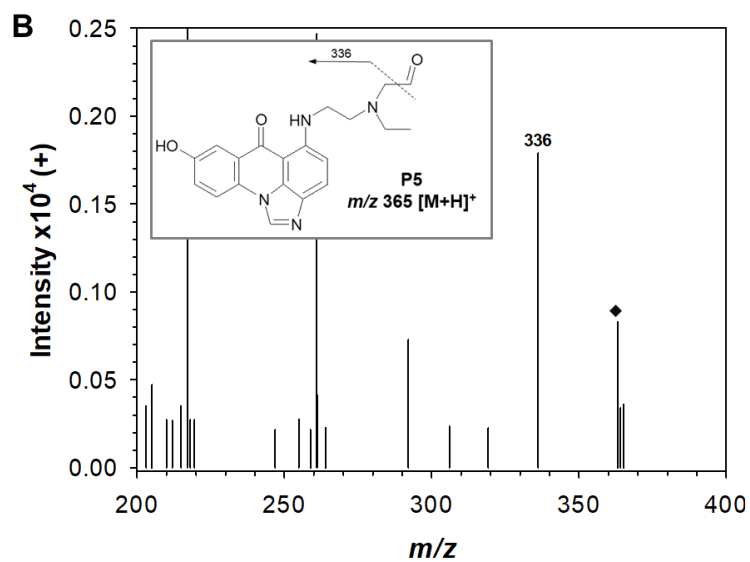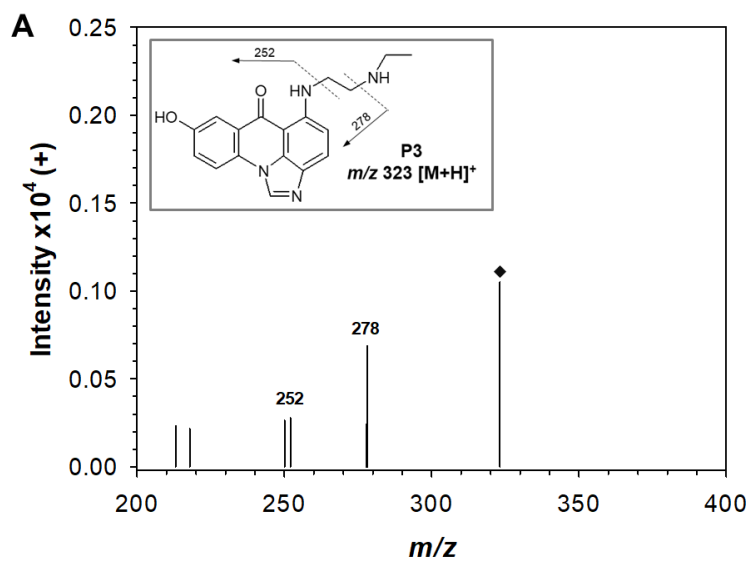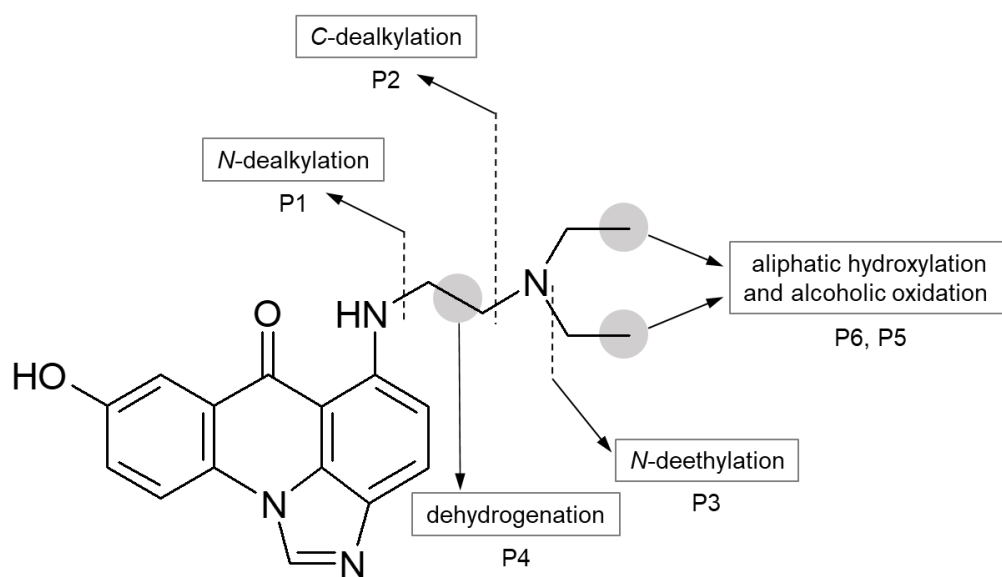
Figure 5
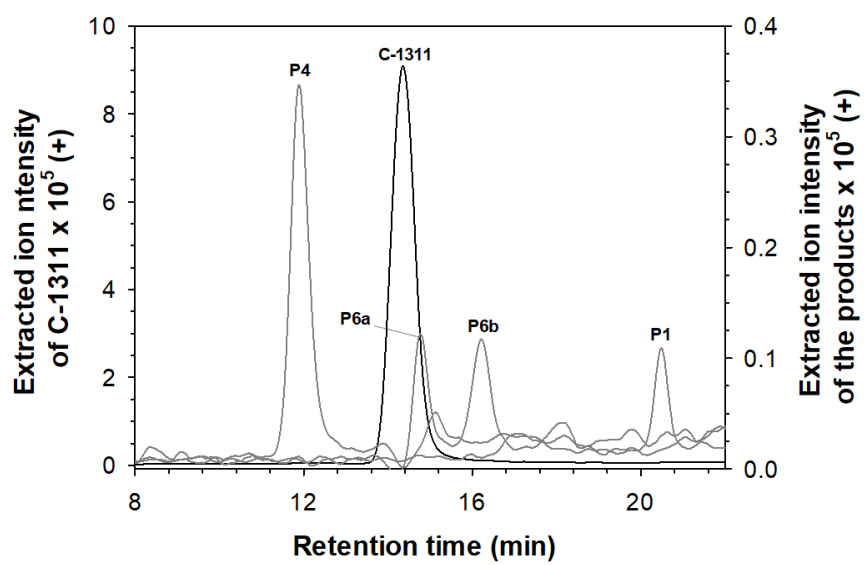
Figure 6

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$_4$O$_2$	-	-
P1	20.47	252.0765	-1.0	C$_{14}$H$_9$N$_3$O$_2$	- C$_6$H$_{13}$N	*N*-dealkylation (complete)
P2	ND ^{b}	278.0923	0.0	C$_{16}$H$_{11}$N$_3$O$_2$	- C$_4$H$_{10}$N	*C*-dealkylation
P3	Trace ^{c}	323.1496	-2.0	C$_{18}$H$_{18}$N$_4$O$_2$	- C$_2$H$_4$	*N*-deethylation
P4	11.88	349.1665	1.7	C$_{20}$H$_{20}$N$_4$O$_2$	- 2H	dehydrogenation
P5	ND ^{b}	365.1608	0.0	C$_{20}$H$_{20}$N$_4$O$_3$	+ O - 2H	oxidation
P6a, b	14.79; 16.22	367.1765	0.1	C$_{20}$H$_{22}$N$_4$O$_3$	+ O	hydroxylation/*N*-oxidation
P7	ND ^{b}	379.1764	0.0	C$_{21}$H$_{22}$N$_4$O$_3$	+ O - 2H + CH$_2$	oxidation + methylation
P8	Trace ^{c}	381.1925	1.0	C$_{21}$H$_{24}$N$_4$O$_3$	+ 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.

m/z of putative metabolite	Occurrence or predicted by					
	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.