Published in final edited form as:

J Pharm Biomed Anal. 2013 March 05; 75: 138–144. doi:10.1016/j.jpba.2012.11.022.

Determination of the glucuronide metabolite of ON 013100, a benzylstyrylsulfone antineoplastic drug, in colon cancer cells using LC/MS/MS

Sool Yeon Cho^a, Stephen C. Cosenza^b, Venkat Pallela^b, Gayatri Panda^b, M.V. Ramana Reddy^b, E. Premkumar Reddy^b, and John Roboz^{a,*}

^aThe Tisch Cancer Institute, Division of Hematology/Medical Oncology, Dept. of Medicine, Sciences, Mount Sinai School of Medicine, New York, NY 10029, USA

^bDept. of Oncological Sciences, Mount Sinai School of Medicine, New York, NY 10029, USA

Abstract

ON 013100, (E)-2,4,6-trimethoxystyryl-3-hydroxy-4-methoxybenzyl sulfone, is a potent kinase inhibitor whose phosphate form is in Phase I clinical trials in lymphoma and acute lymphoid leukemia. The objectives were to: (a) investigate the possible presence of the glucuronide metabolite of the drug in two representative colon cancer cell lines, a drug resistant (colo-205) and a drug sensitive (colo-320); (b) quantify the glucuronide metabolite and the unchanged drug in the cells after treatment with ON 013100. The glucuronide was synthesized and a selective LC/MS/MS method was developed and validated for the characterization and quantification of the metabolite. The glucuronide metabolite (570.6 Da) was found in the drug-resistant cells upon a 1 h incubation with ON 013100 (20 µg/ml). After treatment with the drug, the concentration of the metabolite gradually decreased from 0.84 µg/ml at 0 h through 0.21 µg/ml at 6 h to below detection limit of 8.0 ng/ml at 9 h. No glucuronide metabolite was detected in the drug-sensitive cells. The concentrations of intact ON 013100 in the drug-resistant cells gradually decreased from 0.41 µg/ml (0 h) to 0.06 µg/ml (9 h). The corresponding concentrations of the intact drug in the drug-sensitive cells were from 2.88 µg/ml to 0.94 µg/ml.

Keywords

Quantification; Glucuronidation; LC/MS/MS; Colon cancer cells; Resistant

1. Introduction

ON 013100 is a novel, synthetic benzylstyrylsulfone with a chemical composition (E)-2,4,6-trimethoxystyryl-3-hydroxy-4-methoxybenzyl sulfone, C₁₉H₂₂O₇S (394.4 Da) (Fig. 1A) [1]. While the drug has little or no effect on normal cell viability, it induces apoptosis, and has

*Corresponding author at: Division of Hematology/Medical Oncology, The Tisch Cancer Institute and Department of Medicine, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box #1247, New York, NY 10029, USA. Tel.: +1 212 241 7382; fax: +1 212 241 8774. sool.cho@mssm.edu (S.Y. Cho), Stephen.cosenza@mssm.edu (S.C. Cosenza), Venkat.pallela@mssm.edu (V. Pallela), Gayatri.panda@mssm.edu (G. Panda), r.reddy@mssm.edu (M.V.R. Reddy), ep.reddy@mssm.edu (E.P. Reddy), john.robosz@mssm.edu (J. Roboz).

activity against most human cancer cell lines in vitro (>100 tumor types tested, including drug resistant lines) as well against a broad spectrum of human xenografts in mice [2]. As a potent mitotic inhibitor the drug also inhibits cyclin D1 expression and induces selective G2/M arrest of tumor cells characterized by spindle abnormalities leading to apoptosis [3]. The phosphate salt of the drug is currently in phase I clinical trials for mantle cell lymphoma [4].

The objectives of the present work were to a) investigate the possible presence of the glucuronide metabolite of ON 013100 in two representative colon cancer cell lines, a drug resistant (colo-205) and a drug sensitive (colo-320); (b) quantify the glucuronide metabolite and the unchanged drug in the cells after treatment with ON 013100. The glucuronide (Fig. 1B) was synthesized and a selective LC/MS/MS method was developed and validated for the characterization and quantification of the metabolite.

2. Materials and methods

2.1. Drugs, chemicals, and reagents

ON 013100 and ON 01370 (378.5 Da), a structural analog (Fig. 1C) used as the internal standard (IS), were provided by Onconova Therapeutics, Inc. (Newtown, PA). The glucuronide metabolite of ON 013100 (570.6 Da) (Fig. 1B) was synthesized in house, as described below. All reagents for the synthesis were purchased from Sigma-Aldrich Co. (St. Louis, MO) and were used without further purification. Acetonitrile (ACN), formic acid, and water were of HPLC grade, and all other chemicals and reagents were of the highest available purity (Thermo Fisher Scientific, Waltham, MA).

2.2. Synthesis of the glucuronide of ON 013100

Fig. 2 shows the scheme for the synthesis of the glucuronide metabolite, (E)-3,4,5-Trihydroxy-6-{2-methoxy-5-[2-(2,4,6-trimethoxy-phenyl)ethenesulfonyl-methyl]-phenoxy}-tetrahydropyran-2-carboxylic acid (**4**) of ON 013100, the parent compound (**1**) via an intermediate compound, (E)-3,4,5-triacetoxy-6-{2-methoxy-5-[2-(2,4,6-trimethoxyphenyl)-ethenesulfonyl methyl]-phenoxy}-tetrahydropyran-2-carboxylic acid methyl ester (**3**).

To a solution of ON 013100 (**1** in Fig. 2) (250 mg or 0.63 mmol) and benzyltriethylammonium chloride (BTEAC) (72 mg or 0.315 mmol) in chloroform (5 ml) acetobromo- α -D-glucuronic acid methyl ester (**2** in Fig. 2) (675.6 mg or 1.7 mmol) and 5% aq. sodium hydroxide (1.9 ml) were added. The mixture was heated to reflux and stirred for 35 h, followed by dilution with chloroform and washing with sodium chloride solution. Next, the organic layer was dried over anhydrous sodium sulfate, filtered and the solvent evaporated under reduced pressure. The crude residue was purified using column chromatography with petroleum ether/ethyl acetate (1:1) to yield the desired intermediate compound (**3** in Fig. 2). Next, to a stirred solution of the intermediate compound (55 mg or 77.4 μ mol) in methanol (2 ml) a solution of NaOH (256 μ l, 5% in water) was added until the mixture became yellow. After reacting for 2 h, the solution was diluted with ethyl acetate and acidified with HCl (0.1 N) to a pH of approximately 3. Next, mixture was then washed with water and brine, the organic layer was dried over anhydrous sodium sulfate, filtered and

the solvent evaporated under reduced pressure. The crude residue was purified by column chromatography using ethyl acetate/methanol/water/acetic acid (60:30:9:1) to yield the desired metabolite of ON 013100 (**4** in Fig. 2).

The reactions were monitored by thin layer chromatography (TLC) on pre-coated silica gel F₂₅₄ plates (Sigma-Aldrich) with UV detection at 254 nm. Column chromatography was performed with Merck 70–230 mesh silica gel 60. For characterization using ¹H NMR, spectra were obtained with a Bruker Avance 600 MHz spectrometer.

2.3. Preparation of colon cancer cell lines Colo-205 and Colo-320.

Human colon cancer cells, purchased from ATCC (Manassas, VA), were cultured in RPMI 1640 medium (CellGro, Manassas, VA), containing 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 mg/ml glucose, 1.5 µg/ml sodium bicarbonate, 1 unit/ml penicillin-streptomycin (Gibco, Grand Island, NY), and 10% fetal bovine serum (Cell-Generation, Fort Collins, CO).

The cells were incubated in 5% CO₂, 95% air at 37 °C. Cells at 80–90% confluence were sub-cultured every 7 days. Cells were dissociated with 0.2 mg/ml EDTA (Life Technologies, Inc., Grand Island, NY) and incubated with ON 013100 (20 µg/ml) for 1 hour at 37 °C. Each sample plate had 3 million cells, was washed with PBS three times, further incubated for 0, 3, 6, or 9 h, and frozen to pellet. Pellet samples were extracted with 150 µl of acetonitrile and centrifuged for 30 min at 9000 × *g*. The supernatant were transferred to sample vials for LC/MS/MS analysis. Three million cells without being treated with ON 013100 were also prepared for the calibration curves used for quantification.

2.4. LC/MS for metabolite identification

Both identification/confirmation and quantification (see later) analyses were performed using a Model QuattroLC system (Waters, Co., Milford, MA) which consisted of a Model 1525 HPLC system (Waters, Co., Milford, MA) interfaced (integrated) with a triple quadrupole type mass spectrometer. The HPLC system consisted of an autosampler (Model 717 Plus, Waters, Co., Milford, MA) and a binary pump (Model 1525 µ, Waters, Co.). Components of the sample mixtures were resolved on a C18 type HPLC column (XSelect, 2.1 × 30 mm, 3.5 µm particle size, Waters, Co.), maintained at room temperature. Two mobile phases were used: water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Gradient conditions: linear from 20% B (0 min) to 100% B in 30 min, followed by 100% B for 10 min. The flow rate of the mobile phase was 0.3 ml/min. Total run time for single injection, including a 10-min period for pre-equilibration, was 50 min.

Electrospray ionization (ESI) mass spectra were obtained in the positive ion mode under the following operating conditions: capillary voltage 3 kV, cone voltage 15 V, source temperature 80 °C, desolvation temperature 250 °C, nitrogen nebulizer gas flow 80 l/h, desolvation gas flow 800 l/h. The mass scale was calibrated to *m/z* 2500 using sodium iodide. After injecting 10 µl sample aliquots, total ion chromatograms (TIC) were obtained in the “continuum” mode (not centroid mode) by monitoring masses in the *m/z* 100–1000

range (scan time 4 s). The operation of the mass spectrometer and data acquisition were controlled with a Masslynx software (version 4.0; Waters, Co.).

2.5. LC/MS/MS for quantification of the glucuronide metabolite and the unchanged parent compound

Cell pellet samples were extracted with 135 μ l of acetonitrile and 15 μ l of IS, centrifuged to for 30 min at $9000 \times g$ and the supernatant was transferred to a sample vial for analysis. For MS/MS analysis, collision energy was 15 eV for IS and ON 013100, 5 eV for ON 013100-glucuronide. Mass transitions were monitored from m/z 379.0 to 121.0 for IS, m/z 395.5 to 137.1 for ON 013100, and m/z 571.6 to 395.5 for the glucuronide of ON 013100.

2.6. Validation of the LC/MS/MS method for quantification

The method was validated with respect to analyte stability, selectivity, linearity, precision, accuracy, detection, and quantification limits, following the recommendations of the International Conference on Harmonisation (2005) guidelines [5]. The *stability* of the parent compound and the glucuronide metabolite was assessed using samples of standard solutions (5.0 μ g/ml) left at room temperature for an hour and then analyzed (in triplicate). Method *selectivity* was assessed with respect to possible interferences from the cell pellet by monitoring, both in samples and the synthetic standard, unique, selective reactions for each analyte; from m/z 395.5 to 136.7 for ON 013100, and from m/z 571.6 to 395.5 for the glucuronide; retention time were also monitored and compared.

The *linearity* of the calibration curves was assessed using the *internal standard* method with up to six different concentrations. Stock solutions of 0.5 mg/ml of ON 013100, the glucuronide, and the internal standard were prepared in acetonitrile and stored at -20°C . Standards of ON 013100 and its glucuronide with known concentrations (0.01–10 μ g/ml) were prepared by further diluting the stock solution with acetonitrile. The working solution of the IS was prepared by diluting the stock solution to a concentration of 10 μ g/ml. Each calibration sample was injected in triplicate, calibration curves were constructed from peak area ratios (analyte to IS) using linear regression equations from least-squares treatment of the data. Both *precision* and *accuracy* were determined at two concentration levels (0.5 and 5 μ g/ml) as prescribed by the ICH guidelines. *Instrumental repeatability* was assessed by analyzing, in triplicate, selected samples of known concentration. *Inter-day reproducibility* was checked, by preparing and analyzing two separate samples in triplicate on three different days. The *limit of detection* was determined by injecting solutions of low analyte concentrations (0.01 μ g/ml) using signal to noise ratio = 3. The *limit of quantification* was then calculated using signal to noise ratio of 10.

3. Results and discussion

3.1. Synthesis of the glucuronide of ON 013100 (4 in Fig. 2)

When the crude fraction containing the intermediate compound (3 in Fig. 2) was purified by column chromatography, the collected fraction containing the analyte yielded 67.2 mg, 15% (There was no need to optimize the yield of the intermediate product). The molecular mass

of the $[M + H]^+$ ion was determined by LC/MS as 711.4 Da compared to the expected 711.2 Da. This was considered adequate for identification of the intermediate product.

Additional characterization of the intermediate product was made by ^1H NMR. The solvent was DMSO- d_6 , the IS was tetramethylsilane (Me_4Si). Chemical shifts were obtained in parts per million (δ) downfield; spin multiplicities were obtained as s (singlet), d (doublet), br s (broad singlet), m (multiplet), and q (quartet). Coupling constants (J values) were measured in hertz (Hz). The relevant results of analysis were: δ 2.34 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 2.41 (s, 3H, OCH_3), 4.14 (s, 3H, OCH_3), 4.18 (s, 9H, 3 x OCH_3), 4.30–4.37 (m, 4H, CH_2), 4.48 (s, 2H, CH_2), 4.98–4.99 (d, 1H, CH), 5.31–5.39 (m, 4H, CH), 5.52–5.56 (m, 1H, CH), 6.50 (s, 2H, aromatic-H), 7.21–7.22 (d, 1H, aromatic-H), 7.32–7.34 (d, 1H, $J = 15.78$ Hz, CH = CH), 7.43–7.44 (d, 1H, aromatic-H), 8.05–8.08 (d, 1H, $J = 15.66$ Hz, CH = CH). These NMR results were considered adequate to confirm the expected identity of the intermediate compound.

When the crude fraction containing compound **4** was purified by column chromatography, the fraction containing the analyte yielded 82% (36.2 mg). Using LC/MS/MS analysis, the glucuronide (compound **4** in Fig. 2) was detected in the extracted ion chromatogram (XIC) at the expected mass, m/z 571 (Fig. 3). The mass spectrum of the LC peak revealed the expected $[M + H]^+$ ion of the glucuronide, at m/z 571, and also a fragment ion of m/z 395, which corresponds to the molecular mass $[M + H]^+$ of ON 013100 (Fig. 4). The product ion mode of MS/MS was selected to prove a direct relationship between the parent drug and the synthetic glucuronide metabolite to be used as standard. The product ion mass spectrum, obtained by MS/MS from m/z 571 from compound **4** showed a major peak at m/z 395.3, which corresponds to the molecular peak of ON 013100 (Fig. 5). It is clear from this MS/MS spectrum that the ion of m/z 571 has a product ion of m/z 395.3. This proves that the production originated from the parent compound, ON 013100, as a major fragment, after the loss of the glucuronide moiety. These results provide confirmation of the identity of the synthetic product as the expected glucuronide of ON 013100.

Additional characterization of the glucuronide was also made using NMR (as above for the intermediate compound). The relevant results of analysis by ^1H NMR (DMSO- d_6) were: δ 3.14 (br s, 1H, OH), 3.21 (br s, 1H, OH), 3.48 (br s, 1H, OH), 3.64 (br s, 1H, OH), 3.78 (s, 3H, OCH_3), 3.88 (s, 9H, 3 x OCH_3), 4.36 (s, 2H, CH_2), 4.43 (s, 1H, CH), 4.98 (s, 1H, CH), 5.07 (s, 1H, CH), 5.26 (s, 1H, CH), 6.31 (s, 2H, aromatic-H), 6.96–6.99 (m, 2H, aromatic-H), 7.12–7.14 (d, 1H, $J = 15.3$ Hz, CH = CH), 7.57–7.59 (d, 1H, $J = 15.3$ Hz, CH = CH).

3.2. Presence of the glucuronide metabolite in colon cancer cells

To look for the glucuronide metabolite of ON 013100, an extracted ion chromatogram (XIC) of m/z 571 peak was obtained from the extract of a pellet sample from a colon cancer cell line (Colo-205) known to be resistant to ON 013100, at 0 h after treatment with the drug (20 $\mu\text{g}/\text{ml}$), for 1 h at 37 °C (Fig. 6). The peak at retention time 11.2 min is the glucuronide metabolite of the study compound, ON 013100, at m/z 571. Based on this information, we established the detection window for glucuronide metabolite by monitoring m/z transition from 571 to 395. This peak represents the (E)-form of the glucuronide metabolites and this is now shown in the figure. The peak at 10.8 min was identified as the (Z)-form of the

glucuronide, and this is now also shown in the figure. It is a fact that in the synthesis of the standard (Fig. 1), the chemical structures of the compounds used were all of (E)-configuration—thus, in principle, there should be no (Z)-isomer in the synthetic product. However, a small amount was found – see Fig. 3. We speculate that a minor isomeric conversion has occurred during synthesis. The likely reason for the presence of (Z)-form in larger quantity relative to the (E)-form (Fig. 6) is that some conversion occurred during the process of incubation. At any rate, we consider that this is not important with respect to our objective of quantification because all calibration curves were obtained using the (E)-isomer. With respect to the identity of the peaks at 9.0 and 9.2 min, we have used MS/MS to ascertain if these peaks might be metabolites of ON 013100. Using the “parent ion” mode, these peaks did not produce m/z 395, suggesting that they have not originated from compound ON 013100. In the “product ion” mode, the mass of these peaks (m/z 571) did not reveal the presence of m/z 395, suggesting that these peaks have no relationship with the compound being investigated. We have concluded that these peaks are not metabolites of our study compound.

The mass spectra of the peaks of 10.8 min and 11.2 min both revealed an ion at m/z 571, suggesting that this mass represented the molecular mass of the glucuronide metabolite of ON 013100, i.e., 176 mass units larger than the molecular mass (m/z 395) of the parent compound (Fig. 7) [6]. The peak at 10.8 min was confirmed as the (Z)-isomer of glucuronide metabolite for ON 013100 by comparison to the retention time and mass spectrum of the synthetic glucuronide standard. The peak at 11.2 min was confirmed as the (E)-isomer of glucuronide metabolite for ON 013100. The other peaks were shown to be not relevant.

When the sensitive colon cell line (Colo-320) was treated with ON 013100 using the same experimental conditions described above, the XIC at m/z 571 showed no peak corresponding ON 013100 glucuronide (Fig. 8).

3.3. Validation of the LC/MC/MS technique for quantification

The method developed for quantification of both the parent compound and its glucuronide metabolite was validated with respect to analyte stability, selectivity, linearity, accuracy, precision, and limit of detection and quantification following the guidelines of the ICH (see Methods). Stability: No significant difference was observed between peak areas (measured in triplicate) between samples of the parent compound and the metabolite (of known concentrations) left standing at room temperature for 1 h. Selectivity: No interference was detected in any sample analyzed as described in Methods. Linearity: Calibration curves for both the parent compound and the glucuronide showed linearity ($r^2 = 0.999$) in the 0.01 to 10 $\mu\text{g/ml}$ range. Accuracy: Recovery was 105 and 101%, respectively, at concentrations of 0.5 and 5 $\mu\text{g/ml}$. Precision: The calculated percent relative standard deviations of intra-day repeatability and inter-day reproducibility were always 9% RSD or less. Limit of detection (signal to noise ratio = 3): 7.8 ng/ml for the parent drug and 8.0 ng/ml for the glucuronide. Calculated limit of quantification (signal to noise ratio = 10): 26.0 ng/ml for the parent drug and 26.7 ng/ml for the glucuronide.

3.4. Quantification of parent compound and the glucuronide metabolite in colon cancer cells

The concentration of the glucuronide metabolite in resistant colon cells (Colo-205 line) was 0.84 µg/ml at immediately (0 h) after treatment with ON 013100 for 1 h, followed by a gradual decrease to 0.45 µg/ml at 3 h, 0.21 µg/ml at 6 h, and not detectable at 9 h (Fig. 9A and Table 1). In contrast, the glucuronide metabolite was not detected at any time in the responsive colon cells (Colo-320 line); the limit of detection was 8.0 ng/ml.

A reverse situation was observed with respect to the parent drug, ON 013100, while in the sensitive colon cells (Colo-320 line), where the glucuronide metabolite was not detected, the initial drug concentration of 2.88 µg/ml decreased to 0.94 µg/ml during the 0–9 h period after treatment (Figure 9B and Table 1). However, in the resistant colon cells (Colo-205), even the initial concentration of the parent drug was 0.41 µg/ml, significantly lower than in the Colo-320 line, suggesting that the glucuronidation was a very rapid process. The further decrease of the drug concentration was very slow, 0.26 µg/ml at 3 h, 0.15 µg/ml at 6 h, and eventually 0.06 µg/ml at 9 h (Fig. 9B and Table 1).

3.5. Possible mechanism of glucuronidation

Glucuronidation, the addition of glucuronic acid to a substrate, is often involved in the xenobiotic metabolism of drugs. The process consists of the transfer of an intermediate, glucuronic acid component of UDP-glucuronic acid (glucuronic acid linked via a glycosidic bond to uridine diphosphate, formed in the liver) to a substrate catalyzed by one of several types of UDP-glucuronosyltransferase [7]. Glucuronidation was shown to be a mechanism of intrinsic drug resistance in colon cancer cells, contributing to the transport of drugs by proteins [8]. UGTs have also been shown to be a resistance mechanism utilized by hepatic and lung cancer cells against resistant anticancer drugs [9]. Combretastatin A4, a natural product with potent tubulin polymerization and cell growth inhibition properties, has been shown to be a substrate for UGT1A9 [10]. The present work establishes that the formation of the metabolite ON 13100 correlated with the drug losing activity by 1000-fold in resistant colon cancer cells (Colo-205 line), while drug sensitive colon cells (Colo-320 line) did not show the presence of the glucuronide metabolite.

4. Conclusions

The glucuronide metabolite of the parent drug, ON 013100, was synthesized and its composition confirmed by mass spectrometry and nuclear magnetic resonance. The presence of the glucuronide metabolite was detected and confirmed in cells of a drug resistant colon cell line. The metabolite could not be detected in the cells of a drug sensitive colon cell line. An LC/MS/MS technique was developed and validated for the quantification of both the parent drug and the glucuronide metabolite in colon cells. The intrinsic resistance due to the activity of drug-metabolizing uridine 5'- diphosphoglucuronosyl transferases (UGTs) in colon tumors should be considered as a clinically important factor when determining possible therapies with agents that may be UGT substrates.

Acknowledgements

This research work was supported in part by Onconova Therapeutic, Inc. and authors wish to thank for the generous help. Dr. E. Premkumar Reddy owns equity and receives financial compensation for providing consulting services and for serving on the Board of Directors of Onconova Therapeutics, Inc. In addition, Dr. E. Premkumar Reddy is a named inventor on pending and issued patents filed by Temple University and licensed to Onconova Therapeutics that are related to novel cancer treatments. The outcome of this research project could affect the value of these patents and of Onconova Therapeutics. Drs. S. Cosenza, V. Pallela, and M.V. R. Reddy own equity and receive financial compensation for providing consulting services to Onconova Therapeutics, Inc. Dr. J. Roboz receives financial compensation for providing consulting services to Onconova Therapeutic, Inc.

References

- [1]. Reddy MVR , Mallireddigari M , Cosenza S , Pallela V , Iqbal N , Robell K , Kang A , Reddy EP , Design synthesis, and biological evaluation of (E)-styrylbenzylsulfones as novel anticancer agents, *J. Med. Chem* 51 (2008) 86–100.18088089
- [2]. Park I , Reddy MVR , Reddy EP , Groopman JE , Evaluation of novel cell cycle inhibitors in mantle cell lymphoma, *Oncogene* 26 (2007) 5635–5642.17369860
- [3]. Prasad A , Park I , Allen H , Zhang X , Reddy MVR , Boominathan R , Reddy EP , Groopman JE , Styryl sulfonyl compounds inhibit translation of cyclin D1 in mantle cell lymphoma cells, *Oncogene* 28 (2009) 1518–1528.19198627
- [4]. Safety Study of ON 013105 in Lymphoma and Acute Lymphoid Leukemia, H. Lee Moffitt Cancer Center & Research Institute Tampa, FL 33612, United States, http://clinicaltrials.gov/ct2/show/NC_T01049113?term=ON+013105@rank=1
- [5]. International Conference on Harmonisation (2005) ICH-Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals of Human Use, Geneva, Switzerland.
- [6]. Maggs JL , Pirmhamed M , Kitteringham NR , Park BK , Characterization of the metabolites of carbamazepine in patient urine by liquid chromatography/mass spectrometry, *Drug Metab. Dispos* 25 (1997) 275–280.9172943
- [7]. Ritter JK , Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions, *Chem. Biol. Interact* 129 (2000) 171–193.11154740
- [8]. Cummings J , Zelcer N , Allen JD , Yao D , Boyd G , Maliepaard M , Friedberg TH , Smyth JF , Jodrell DI , Glucuronidation as a mechanism of intrinsic drug resistance in colon cancer cells: contribution of drug transport proteins, *Biochem. Pharmacol* 67 (2004) 31–39.14667926
- [9]. Cummings J , Ethell BT , Jardine L , Burchell B , Glucuronidation of SN-38 and NU/ICRF 505 in human colon cancer and adjacent normal colon, *Anticancer Res* 26 (2006) 2189–2196.16821585
- [10]. Aprile S , Del Grosso E , Grosa G , Identification of the human UDPglucuronosyltransferases involved in the glucuronidation of combretastatin A-4, *Drug Metab. Dispos* 38 (2010) 1141–1146.20375181

Fig. 1.

Formulas of A: ON 013100, (E)-2,4,6-trimethoxystyryl-3-hydroxy-4-methoxybenzyl sulfone, C₁₉H₂₂O₇S, 394.4 Da; B: Glucuronide of ON 013100, (E)-3,4,5-trihydroxy-6-{2-methoxy-5-[2-(2,4,6-trimethoxyphenyl)-ethenesulfonylmethyl]-phenoxy}-tetrahydropyran-2-carboxylic acid (570.5 Da); C: Structural analog of ON 013100, (E)-2,4,6-trimethoxystyryl-4-methoxybenzyl sulfone (378.5 Da), used as the internal standard (IS).

Fig. 2. Synthetic scheme for glucuronide metabolite of (E)-2, 4, 6-Trimethoxystyryl-3-hydroxy-4-methoxybenzylsulfone (**4**) from ON 013100 (**1**) via compound (**3**).

Fig. 3. Extracted ion chromatogram at m/z 571 of the synthetic metabolite (**4** in Fig. 2). The peak at retention 11.2 min is the (E)-isomer, the peak at retention 10.8 min it the (Z)-isomer.

Fig. 4.
Mass spectrum of the peak at 11.2 min retention time.

Fig. 5.
Product ion mass spectrum of m/z 571 from the synthetic standard (compound 4 in Fig. 2).

Fig. 6. Extracted ion chromatogram at m/z 571. Pellet sample from Colo-205 cells incubated with ON 013100 (20 $\mu\text{g}/\text{ml}$) for 1 h.

Fig. 7.
Mass spectrum of the peak at 11.2 min retention time.

Fig. 8. Extracted ion chromatogram at m/z 571. Pellet sample from Colo-320 cells incubated with ON 013100 (20 $\mu\text{g/ml}$) for 1 h.

Fig. 9. Concentration (µg/ml) of A: ON 013100 glucuronide and B: ON 013100 in cells after 1 h incubation with ON 013100. Data from Table 1.

Table 1

Quantity of ON 013100 and its glucuronide in Colo-205 and Colo-320 cells ($\mu\text{g/ml}$).

$\mu\text{g/ml}$ hour	ON 013100 glucuronide		ON 013100	
	Colo-205	Colo-320	Colo-205	Colo-320
0	0.84	Not detected	0.41	2.88
3	0.45	Not detected	0.26	2.32
6	0.21	Not detected	0.15	1.98
9	Not detected	Not detected	0.06	0.94