Vol. 9, No. 1, 2018
ISSN 2233-4203/ e-ISSN 2093-8950
INVITED ARTICLE www.msletters.org | Mass Spectrometry Letters

DC23, a Triazolothione Resorcinol Analogue, Is Extensively Metabolized to Glucuronide Conjugates in Human Liver Microsomes

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Received February 08, 2018; Accepted February 21, 2018
First published on the web March 31, 2018; DOI: 10.5478/MSL.2017.9.1.24

Abstract : DC23, a triazolothione resorcinol analogue, is known to inhibit heat shock protein 90 and pyruvate dehydrogenase kinase which are up-regulated in cancer and diabetes, respectively. This study was performed to elucidate the metabolism of DC23 in human liver microsomes (HLMs). HLMs incubated with DC23 in the presence of uridine 5'-diphosphoglucuronic acid (UDPGA) and/or β-nicotinamide adenine dinucleotide phosphate (NADPH) resulted in the formation of four metabolites, M1-M4. M1 was identified as DC23-*N*-Oxide, on the basis of LC-MS/MS analysis. DC23 was further metabolized to its glucuronide conjugates (M2, M3, and M4). In vitro metabolic stability studies conducted with DC23 in HLMs revealed significant glucuronide conjugation with a $t_{1/2}$ value of 1.3 min. The inhibitory potency of DC23 on five human cytochrome P450s was also investigated in HLMs. In these experiments, DC23 inhibited CYP2C9-mediated tolbutamide hydroxylase activity with an IC_{50} value of 8.7 μM, which could have implications for drug interactions.

Keywords : DC23, microsomes, oxidation, glucuronidation, drug interaction

Introduction

Resorcinol is a common scaffold that is found in a class of chemotherapeutic agents.[1,2] Compounds having resorcinol scaffold showed high affinity toward heat shock protein 90 (Hsp90), which is a drug target for anticancer treatments.[3-8] Onalespib,[9] ganetespib,[10] luminespib,[11] VER-50589,[12] and BX-2819[13] are the representative Hsp90 inhibitors having resorcinol scaffold. Among them, onalespib and luminespib are in clinical trials as anticancer agents. Recently, triazolothione resorcinols including DC23 and PS10 are also known to inhibit pyruvate dehydrogenase kinase (PDK) isoforms which are up-regulated in obesity, diabetes, and cancer.[14] Therefore, resorcinylic compounds have been evaluated as good candidates for the treatment of cancer, obesity, and diabetes.[12-14]

Although some resorcinylic compounds have strong pharmacological activities, to date, there has been no report on their druggability such as drug-drug interaction and metabolic stability. In this study, we investigated metabolism of DC23,[13,14] one of triazolothione resorcinol compounds, in human liver microsomes (HLMs). We also examined the inhibitory effects of DC23 on the metabolism of five P450 isoform selective substrates to predict the probability of drug interactions.

Experimental

Chemicals

DC23 (5-(2,4-dihydroxyphenyl)-4-(1-naphthyl)-2,4-dihydro-3H-1,2,4,-triazole-3-thion, purity > 95%) was purchased from ChemBridge Corporation (San Diego, CA, USA). Alamethicin, uridine 5'-diphosphoglucuronic acid (UDPGA), and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents were LC-MS grade (Merck, Darnstadt, Germany). Pooled HLMs (HLM 150, mixed gender, #452117) were purchased from Corning (Woburn, MA, USA).

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In vitro phase I and II metabolism of DC23 in HLMs

To evaluate microsomal metabolism of DC23, pooled HLMs (1.0 mg/mL, Corning), alamethicin (25 μg/mg microsomal protein), and phosphate buffer (100 mM, pH 7.4) were mixed and placed on ice for 15 min. After pre-incubation for 5 min with DC23 (100 μM), the reaction of DC23 was initiated by the NADPH (1 mM) and UDPGA (5 mM) system for 2 h with agitation. The reaction was terminated by the adding 250 μL of ice-cold acetonitrile. After termination of reaction, the mixture was vortexed and centrifuged at 18,000 × *g* for 5 min. Finally, the supernatant was injected into LC-MS/MS analysis.

Metabolic stability of DC23 in HLMs

To evaluate phase I metabolic stability of DC23, pooled HLMs (1.0 mg/mL), potassium phosphate buffer, and DC23 (1 μM) were pre-incubated (37°C, 5 min). To initiate the reaction, NADPH generating system with DC23 (1 μM) was added to the samples and incubated for specific time points (0, 10, 20, 40, and 60 min). Then, sample aliquots were taken and terminated with acetonitrile.

To evaluate phase II metabolic stability of DC23, alamethicin was initially added to the HLMs (1.0 mg/mL) with potassium phosphate buffer and placed on ice for 15 min to allow pore formation. To start the reaction, DC23 (1 μM) and UDPGA were added to the samples and incubated for specific time points (0, 10, 20, 40, and 60 min) with agitation. Then, sample aliquots were taken and terminated with acetonitrile. After centrifugation, supernatants were analyzed using LC-MS/MS to determine the concentration of DC23 in sample.

LC-MS/MS analysis of DC23 and its metabolites

Separation and identification of all metabolites were carried out using a Thermo Accela HPLC system with Thermo Vantage Triple quadrupole mass spectrometer (ThermoFischer Scientific, San Jose, CA, USA). Compound separation was performed on a Phenomenex Kinetex XB-C18 column (100 × 2.1 mm, 2.6 μm). The mobile phase consisted (A) of water with 0.1% formic acid, and (B) of acetonitrile with 0.1% formic acid. To achieve chromatographic separation, a gradient elution program was optimized as follows: 0-0.5 min, 30% B; 0.5-1.5 min, 30-60% B; 1.5-2 min, 60-85% B, which was kept for 2 min until re-equilibration. The flow rate was 0.2 mL/min and the injection volume was 1 μL. Electrospray ionization was used for positive ion modes, the mass transition used for quantitation of DC23 in metabolic stability study was 336→136 (collision energy 35 eV).

Inhibitory potency of DC23 on five cytochrome P450 activities in HLMs

The inhibitory potential on CYP 1A2, 2C9, 2C19, 2D6, and 3A enzymes were determined using cytochrome P450 assays by combining cocktail incubation and tandem mass spectrometry, as described previously with some modifications.[15,16] Brief, the assay mixtures containing substrates cocktail (Table 1), pooled HLMs (0.25 mg/mL), and DC23 (0-50 μM), were pre-prewarmed at 37°C for 5 min. The enzymatic reaction was initiated by the addition of the NADPH and incubated for 10 min at 37°C with

Table 1. Inhibitory potency of DC23 on five major cytochrome P450 activities in human liver microsomes.

Enzyme activity	P450	IC_{50} (μM)
Phenacetin *O*-deethylation	1A2	>50
Tolbutamide 4-methylhydroxylation	2C9	8.7
S-Mephenytoin 4-hydroxylation	2C19	30.8
Dextromethorphan *O*-demethylation	2D6	>50
Midazolam 1'-hydroxylation	3A	20.4

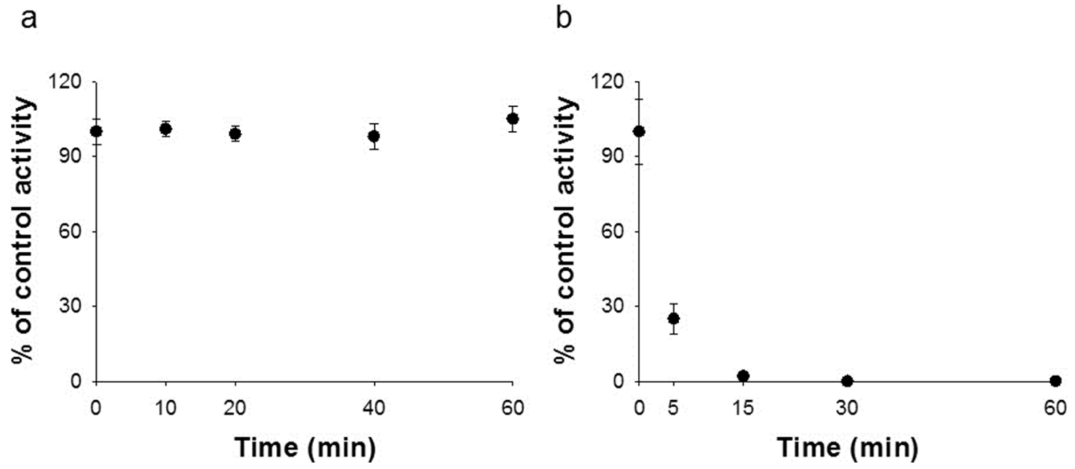

Figure 1. Phase I and phase II metabolic stability of DC23 in human liver microsomes in the presence of NADPH (A) and UDPGA (B), respectively. Data are the means of triplicate experiments.

agitation. The reaction was stopped by addition of ice-cold acetonitrile containing 5 ng/mL terfenadine as the internal standard. All incubations were carried out in triplicate for each concentration.

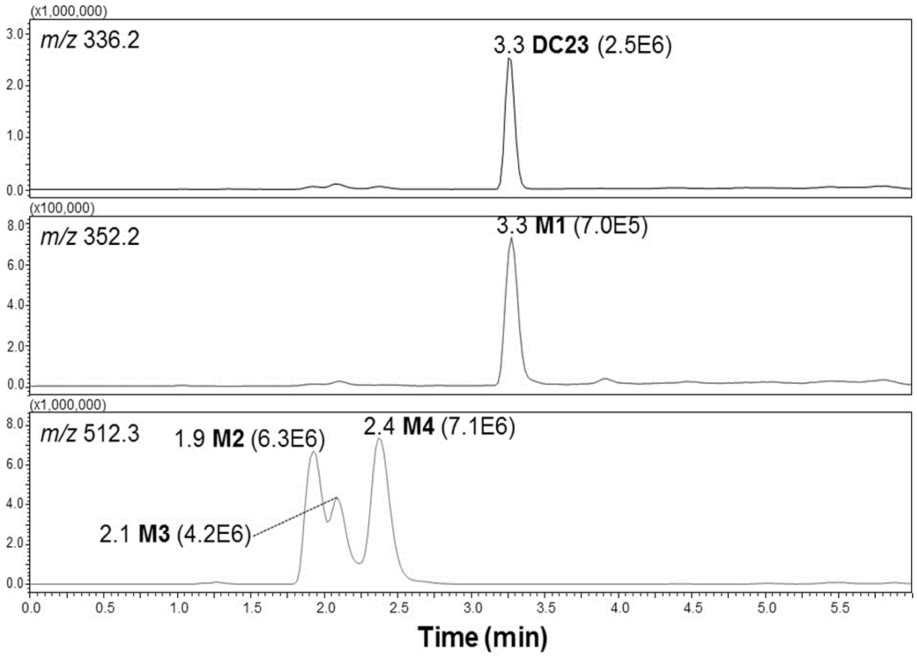

Figure 2. Extracted ion chromatograms of DC23 metabolites on microsomal incubation with NADPH and UDPGA.

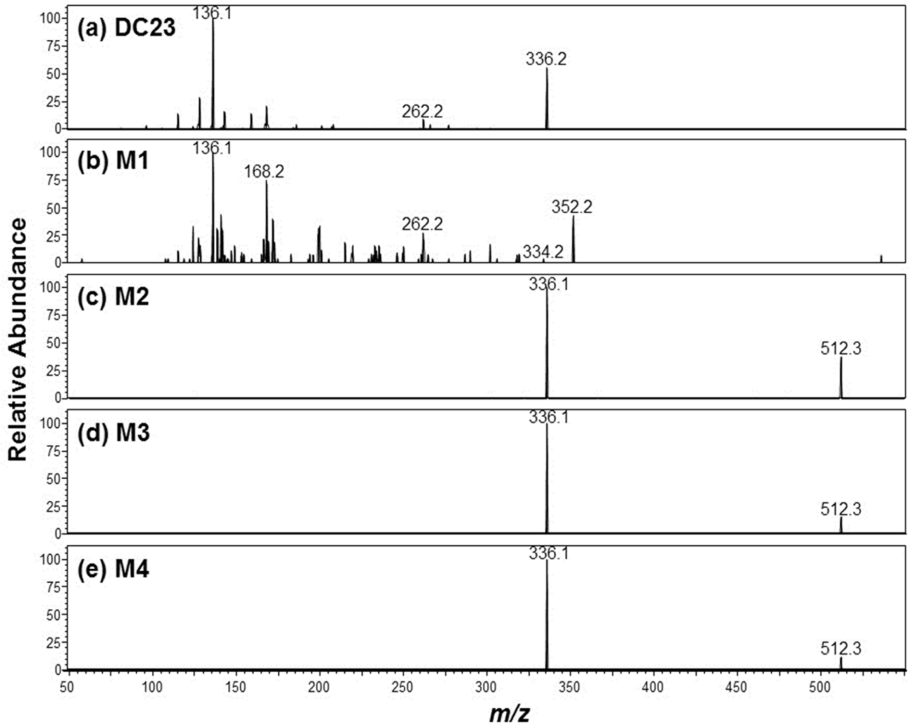

Figure 3. Product ion scan mass spectra of DC23, its one phase I metabolite (M1), and three phase II metabolites (M2, M3, and M4) obtained by LC-MS/MS analysis following incubation of human liver microsomes with DC23 in the presence of a NADPH and UDPGA.

Results and Discussion

Metabolic stability of DC23 in HLMs

Metabolic stability of DC23 in HLMs is presented in Figure 1. When incubated with HLMs in the presence of NADPH and UDPGA, *in vitro* $t_{1/2}$ values for DC23 were > 60 and 1.3 min, respectively. *In vitro* metabolic stability studies with DC23 in HLMs revealed that DC23 was significantly metabolized to glucuronide conjugates, whereas oxidative degradation was negligible.

DC23 metabolites identification in HLMs

Following the incubation of DC23 with HLMs in the presence of an UDPGA and NADPH, the parent compound DC23 and one minor phase I metabolites (M1) and three major phase II metabolites (M2, M3, and M4) were profiled and tentatively identified by LC-MS/MS (Figure 2). The ions of retention time at 1.9, 2.1, and 2.4 min in *m/z* 336 channel were in-source dissociation[17] product of M2, M3, and M4 by loss of a glucuronic acid moiety (Figure 2). Further analysis of DC23 and its metabolites using LC-MS/MS produced a product ion for structural elucidation (Figure 3). The MS/MS spectrum of DC23, which has a protonated molecular ion $[M+H]^+$ at *m/z* 336, showed fragment ions at *m/z* 262 and 136 due to cleavage of triazolothione ring (Figure 4A).

Metabolites M1 was tentatively identified as DC23-*N*-Oxide (Figure 3 and 4B). The mass spectra of M1 contained a protonated molecular ion peak $[M+H]^+$ at *m/z* 352, suggesting one oxygen atom was added in the DC23 molecule ($[M+H]^+$, *m/z* 336). The MS/MS spectra of oxygenated metabolite also showed fragmentation patterns similar to DC23 (Figure 4A and 4B). The MS/MS spectrum of M1 also showed fragment ions at *m/z* 262, 168, and 136. This suggests that the oxidation site in the M1 metabolite is the *N*-naphthyl triazolothion group (Figure 4B). However, the exact hydroxylation site in the triazolothion group of M1 compound could not be determined. M1 gave a fragment ion at *m/z* 334 due to the loss of a water molecule. The other metabolites, M2, M3 and M4 have deprotonated molecular ion peaks $[M+H]^+$ at *m/z* 512 (Figure 3). These three metabolites were confirmed as DC23 glucuronide by the diagnostic loss of the glucuronosyl moiety (176 amu) to form the aglycone product ion at *m/z* 336 (Figure 4C).[17] The exact site of glucuronidation of DC23 glucuronide could not be determined.

In vitro metabolism of DC23

An *in vitro* metabolism study found DC23 to be a substrate for P450 and UGT enzymes. As shown in Figure 5, DC23 was metabolized by oxidation (M1) and by glucuronidation (M2, M3 and M4). The peak height of M2, M3, and M4 were higher than that of M1 (Figure 2). When 100 μM DC23 was incubated in HLMs in the presence of UDPGA and NADPH, ca. 6.5% of the DC23 remained after 60 min of incubation. The percentages of M1, M2, M3, and M4 were about 2.5%, 30%, 21%, and 40%, respectively. This suggests that glucuronidation might be a

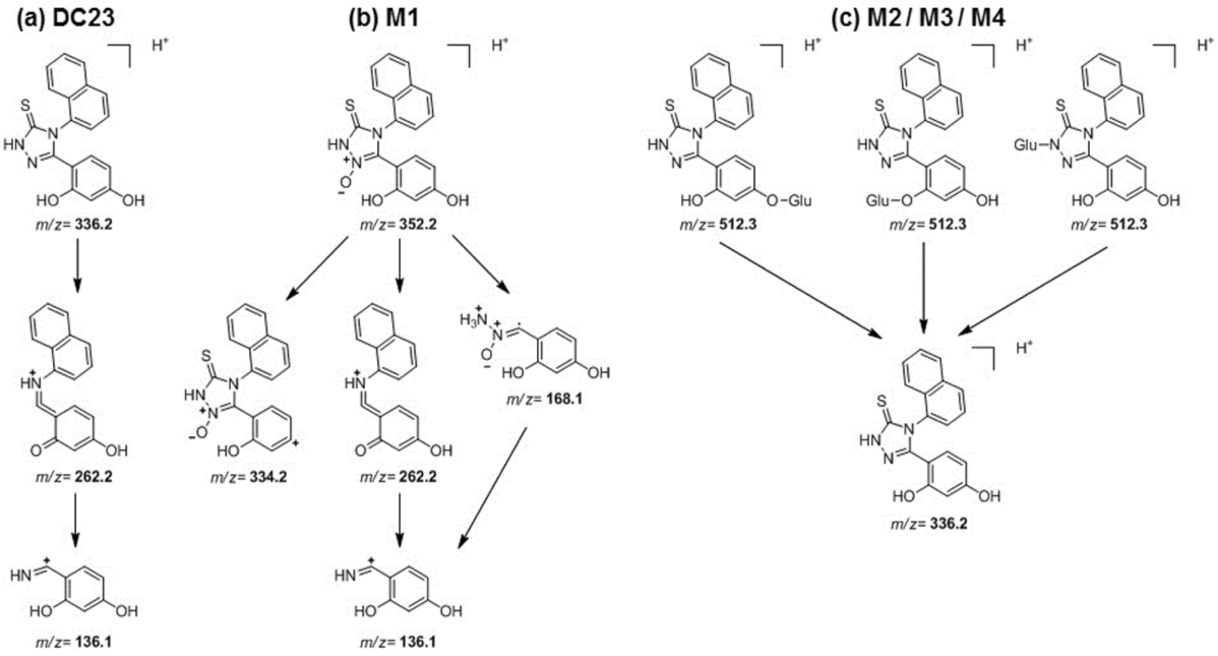

Figure 4. MS/MS fragmentation schemes for DC23 (A), one phase I metabolite M1 (B), and three phase II metabolites M2-M4 (C). The oxygen is depicted attached to the 1 position of the triazolothione moiety, for convenience. Our results do not allow determination of the exact oxidation position.

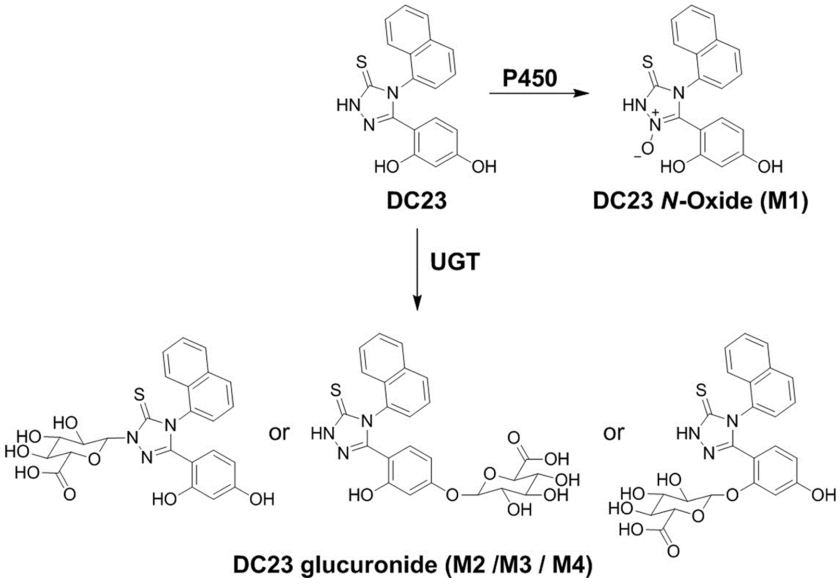

Figure 5. Proposed metabolic pathway of DC23 in human liver microsomes.

major metabolism of DC23 in HLMs. A metabolic pathway for DC23 in HLMs is suggested in Figure 5.

***In vitro* inhibition of P450 enzymes by DC23**

Inhibition of P450 activity of DC23 (up to 50 μM) was evaluated to investigate the effect on P450-mediated drug interactions in HLMs (Table 1). DC23 was found to be an inhibitor of CYP2C9-mediated tolbutamide hydroxylase activity *in vitro* with an IC_{50} values of 8.7 μM, which is higher than IC_{50} values (0.3~1.5 μM) of the well-known potent CYP2C9 inhibitor sulfaphenazole.[16,18] DC23 also acted as an inhibitor of omeprazole hydroxylase activity (CYP2C19) and midazolam 1'-hydroxylase activity (CYP3A) with IC_{50} values of 30.8 and 20.4 μM, respectively. DC23 at a concentration of 50 μM did not affect the activities of CYP1A2 and CYP2D6 isoforms. These findings suggest that clinical interactions between DC23 and P450s such as CYP1A2 and CYP2D6 would not be expected.

Conclusions

We conducted in vitro metabolism study that determined DC23 to be a substrate of UGT-mediated metabolism (Figure 5). DC23 is metabolized to M1 by oxidation, and to M2, M3, and M4 by glucuronidation in HLMs. Glucuronidation was the predominant DC23 biotransformation pathway in HLMs. DC23 was also found to inhibit the metabolism of a CYP2C9 substrate in vitro.

Acknowledgments

Research reported in this publication was supported by a grant of the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111345).

Reference

1. Sidera, K.; Patsavoudi, E. *Recent Pat Anticancer Drug Discov.* **2014**, 9, 1.
2. Biamonte, M. A.; R. Van de Water; Arndt, J. W.; Scannevin, R. H.; Perret, D.; Lee, W. C. *J. Med. Chem.* **2010**, 53, 3.
3. Taddei, M.; Ferrini, S.; Giannotti, L.; Corsi, M.; Manetti, F.; Giannini, G.; Vesci, L.; Milazzo, F. M.; Alloatti, D.; Guglielmi, M. B.; Castorina, M.; Cervoni, M. L.; Barbarino, M.; Fodera, R.; Carollo, V.; Pisano, C.; Armaroli, S.; Cabri, W. *J. Med. Chem.* **2014**, 57, 2258.
4. Zhang, C.; Wang, X.; Liu, H.; Zhang, M.; Geng, M.; Sun, L.; Shen, A.; Zhang, A. *Eur. J. Med. Chem.* **2017**, 125, 315.
5. Sharp, S. Y.; Roe, S. M.; Kazlauskas, E.; Cikotiene, I.; Workman, P.; Matulis, D.; Prodromou, C. *PLoS One*, **2012**, 7, e44642.
6. Jeong, J. H.; Oh, Y. J.; Lho, Y.; Park, S. Y.; Liu, K. H.; Ha, E.; Seo, Y. H. *Eur. J. Med. Chem*. **2016**, 124, 1069.
7. Fuhrmann-Stroissnigg, H.; Ling, Y. Y.; Zhao, J.; McGowan, S. J.; Zhu, Y.; Brooks, R. W.; Grassi, D.; Gregg, S. Q.; Stripay, J. L.; Dorronsoro, A.; Corbo, L.; Tang, P.; Bukata, C.; Ring, N.; Giacca, M.; Li, X.; Tchkonia, T.; Kirkland, J. L.; Niedernhofer, L. J.; Robbins, P. D. *Nat. Commun.* **2017**, 8, 422.
8. Jeong, J. H.; Oh, Y. J.; Kwon, T. K.; Seo, Y. H. *Arch. Pharm. Res.* **2017**, 40, 96.
9. Woodhead, A. J.; Angove, H.; Carr, M. G.; Chessari, G.; Congreve, M.; Coyle, J. E.; Cosme, J.; Graham, B.; Day,

P. J.; Downham, R.; Fazal, L.; Feltell, R.; Figueroa, E.; Frederickson, M.; Lewis, J.; McMenamin, R.; Murray, C. W.; O'Brien, M. A.; Parra, L.; Patel, S.; Phillips, T.; Rees, D. C.; Rich, S.; Smith, D. M.; Trewartha, G.; Vinkovic, M.; Williams, B.; Woolford, A. J. *J. Med. Chem.* **2010**, 53, 5956.
10. London, C. A.; Bear, M. D.; McCleese, J.; Foley, K. P.; Paalangara, R.; Inoue, T.; Ying, W.; Barsoum, J.; *PLoS One* **2011**, 6, e27018.
11. Eccles, S. A.; Massey, A.; Raynaud, F. I.; Sharp, S. Y.; Box, G.; Valenti, M.; Patterson, L.; de Haven Brandon, A.; Gowan, S.; Boxall, F.; Aherne, W.; Rowlands, M.; Hayes, A.; Martins, V.; Urban, F.; Boxall, K.; Prodromou, C.; Pearl, L.; James, K.; Matthews, T. P.; Cheung, K. M.; Kalusa, A.; Jones, K.; McDonald, E.; Barril, X.; Brough, P. A.; Cansfield, J. E.; Dymock, B.; Drysdale, M. J.; Finch, H.; Howes, R.; Hubbard, R. E.; Surgenor, A.; Webb, P.; Wood, M.; Wright, L.; Workman, P.; *Cancer Res.* **2008**, 68, 2850.
12. Sharp, S. Y.; Prodromou, C.; Boxall, K.; Powers, M. V.; Holmes, J. L.; Box, G.; Matthews, T. P.; Cheung, K. M.; Kalusa, A.; James, K.; Hayes, A.; Hardcastle, A.; Dymock, B.; Brough, P. A.; Barril, X.; Cansfield, J. E.; Wright, L.; Surgenor, A.; Foloppe, N.; Hubbard, R. E.; Aherne, W.; Pearl, L.; Jones, K.; McDonald, E.; Raynaud, F.; Eccles, S.; Drysdale, M.; Workman, P. *Mol. Cancer Ther.* **2007**, 6, 1198.
13. Feldman, R. I.; Mintzer, B.; Zhu, D.; Wu, J. M.; Biroc, S. L.; Yuan, S.; Emayan, K.; Chang, Z.; Chen, D.; Arnaiz, D. O.; Bryant, J.; Ge, X. S.; Whitlow, M.; Adler, M.; Polokoff, M. A.; Li, W. W.; Ferrer, M.; Sato, T.; Gu, J. M.; Shen, J.; Tseng, J. L.; Dinter, H.; Buckman, B.; *Chem. Biol. Drug Des.* **2009**, 74, 43.
14. Tso, S. C.; Qi, X.; Gui, W. J.; Wu, C. Y.; Chuang, J. L.; Wernstedt-Asterholm, I.; Morlock, L. K.; Owens, K. R.; Scherer, P. E.; Williams, N. S.; Tambar, U. K.; Wynn, R. M.; Chuang, D. T.; *J. Biol. Chem.* **2014**, 289, 4432.
15. Lee, B.; Ji, H. K.; Lee, T.; Liu, K. H.; *Drug Metab. Dispos.* **2015**, 43, 1137.
16. Kim, M. J.; Kim, H.; Cha, I. J.; Park, J. S.; Shon, J. H.; Liu, K. H.; Shin, J. G.; *Rapid Commun. Mass Spectrom.* **2005**, 19, 2651.
17. Joo, J.; Lee, D.; Wu, Z.; Shin, J. H.; Lee, H. S.; Kwon, B. M.; Huh, T. L.; Kim, Y. W.; Lee, S. J.; Kim, T. W.; Lee, T.; Liu, K. H.; *Biopharm. Drug Dispos*. **2013**, 34, 195.
18. Walsky, R. L.; Obach, R. S.; *Drug Metab. Dispos.* **2003**, 31, 343.