Research Article

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Fragmentation pattern of certain isatin–indole antiproliferative conjugates with application to identify their in vitro metabolic profiles in rat liver microsomes by liquid chromatography tandem mass spectrometry

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Abstract: The fragmentation pattern of certain isatin-based compounds was carried out using collision-induced dissociation inside the triple quadrupole mass analyzer. These data were used as a clue for the identification of metabolites of the recently reported isatin-based antiproliferative agent, namely, \( N'\)-[5-bromo-1-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene]-5-methoxy-1H-indole-2-carbohydrazide (1) in rat liver microsomes (RLMs) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Prediction of the vulnerable sites for metabolic pathways in compound 1 was performed by WhichLP450 module of StarDrop software. In vitro metabolites for compound 1 were identified with the aid of rat liver microsomes. The in silico data were utilized as a guide for the practical work. Compound 1 was metabolized into three (hydroxylated, reduced and O-demethylated) metabolites in RLMs in the presence of NADPH. The chemical structures of those metabolites were elucidated, and the metabolic pathways were proposed by comparing the fragmentation pattern of the isatin–indole conjugates 1–7. The data presented in this paper provided useful information on the effect of different substituents on the ionization/fragmentation processes and can be used in the characterization of isatin derivatives.

In silico toxicity assessments for the title compounds 1–7 and for the metabolites of compound 1 were conducted utilizing the deductive estimation of risk from existing knowledge (DEREK) module of StarDrop software.

Keywords: Antiproliferative, Fragmentation pattern, Isatin derivatives, Metabolic profiling, Tandem mass spectrometry

1 Introduction

Cancer is a serious public health burden worldwide, and there is an increase in the mortality rate particularly in less economically developed countries [1]. Despite the availability of a large number of chemotherapeutic drugs, satisfactory management of cancer is still unmet due to serious side effects, nonselectivity and developments of resistance to the clinically available anticancer agents [2]. Therefore, there is an imperative need to get new anticancer candidates with better pharmacodynamic and pharmacokinetic profiles than the existing drugs.

Isatin and indole are privileged scaffolds because they are incorporated in myriad compounds endowed with various bioactivities including anticancer agents [3–5]. In view of these premises and as a part of our interest to develop new potent anticancer candidates, we were inspired to synthesize the isatin-based compounds 1–7 as potent antiproliferative compounds. Compounds 1–7 exhibited the antiproliferative activity, in which compounds \( N'\)-[5-bromo-1-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene]-5-methoxy-1H-indole-2-carbohydrazide (1) and \( N'\)-[1-benzyl-5-methoxy-2-oxo-1,2-dihydro-3H-indol-3-ylidene]-5-methoxy-1H-indole-2-carbohydrazide (7) are the most potent congeners with average IC\(_{50}\) values of 5.60 and 1.69 µM in vitro, respectively, against the tested human cancer cell lines [6].

For compounds under development for clinical use, certain types of studies are usually performed including fragmentation pattern and drug metabolism [7]. Drug metabolism research has become a fundamental part of
the drug discovery process, and it has evolved from being complementary to be an essential part [8]. In the metabolic profile studies, the compound is investigated to determine its identity and the number of its metabolites. Because of the importance of isatin–indole molecular hybrids, fragmentation patterns were conducted on the title isatin–indole conjugates 1–7. These data were applied for the identification of in vitro metabolites of compound 1 after incubation with rat liver microsomes (RLMs).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become a mainstay in the drug metabolism research [9,10]. The use of product ions for the identification of fragmentation pattern is another major advantage in LC-MS/MS techniques that have greatly facilitated the identification of related compounds including drug metabolites [11].

Our research group is extremely involved in synthesizing, evaluating biological activities and metabolic profiling of potential known drugs as well as newly developed potential future drug candidates [12–17]. Herein, we report the fragmentation pattern of the recently reported isatin-based antiproliferative agents 1–7 with the application to metabolic profiling of compound 1 in RLMs (Figure 1) [18,19].

In addition, an in silico study was conducted for prediction of the vulnerable metabolic sites in compound 1. The structural alerts in the chemical structures of compounds 1–7 and in the metabolites of compound 1 were obtained using deductive estimation of risk from the existing knowledge (DEREK) module of StarDrop software [20,21]. These alerts could be used as a new strategy for reducing the side effects of the newly developed bioactive compounds without affecting their pharmacological activity by incorporating targeted modifications to improve their safety while retaining their efficacy. In addition, the in silico toxicity assessments of compounds 1–7 were also performed using DEREK software.

2 Experimental

2.1 In silico prediction of compound 1 metabolites using WhichP450™ module of StarDrop software

The vulnerable metabolic key sites in compound 1 were identified utilizing the site lability in WhichP450™ module of the StarDrop software that was revealed by

Figure 1: Chemical structures of compounds 1–7.
the composite site lability (CSL). The outcomes were presented by the pie chart that was used for indication of the most likely CYP450 isoforms that have a principal role in the metabolism of compound 1.

2.2 General considerations

Liquid chromatography-mass spectrometry (LC-MS/MS) was used in the metabolic analysis using LC: Agilent 1200 series; MS: Agilent 6410 triple quadrupole (LC-MS/MS). The column used was Agilent eclipse plus C18 (Dim 150 × 2.1 mm), and the particle size was 3.5 µm. MS parameters are as follows: drying gas: N₂; gas temperature: 350°C; gas flow: 12.0 L/min; nebulizer pressure: 60 psi; ion source: ESI; capillary voltage: 4,000 V; mode: positive.

2.3 Fragmentation pattern studies

Fragmentation patterns of compound 1 and its related derivatives 2–7 were performed by the flow injection analysis through multiple injections of 2 µg/mL solution of each compound to tandem mass spectrometer (Agilent 6410 triple quadrupole).

Table 1: List of compounds with their fragment ions

| Compound ID | m/z | Substituted groups | Fragment ions |
|-------------|-----|--------------------|---------------|
| 1           | 427 | Br, CH₃            | 174           |
| 2           | 459 | H                  | 174           |
| 3           | 477 | Cl                 | 174           |
| 4           | 459 | Cl, Benzyl         | 174           |
| 5           | 335 | H, H               | 174           |
| 6           | 521 | Br, 4-Fluorobenzyl | 174           |
| 7           | 455 | OCH₃, Benzyl       | 174           |

Fragmentation of isatin-indole conjugates 1–7 yielded the same fragmentation pattern as it gave one fragment ion at m/z = 174 that represented the breakage of the carbohydrazide bond.

Figure 2: Proposed metabolic sites for compound 1 by the StarDrop WhichP450™ module. (Metabolic landscape indicates the lability of C24 (in the methoxy group), C17 and C19 (in the indole moiety), and C1 (in the N-methyl group) in compound 1 is proposed to be labile for metabolism. CYP3A4 was proposed to have the main effect in compound 1 metabolism.)
2.4 RLMs incubations

Metabolism of compound 1 (1 µL of 1 mM stock solution) was determined with 40 µL (1 mg/mL) of rat liver microsomes (RLMs) in 0.08 M potassium phosphate buffer (0.08 M KH₂PO₄/NaH₂PO₄, pH 7.4), at 37°C for 30 min, in a final incubation volume of 1 mL with freshly prepared ice-cold MgCl₂ solution (20.33 mg/mL). Test tubes containing the incubation mixtures were transferred to a shaking water bath and allowed to stand at a temperature of 37°C for 5 min. The reactions were initiated by the addition of a nicotinamide adenine dinucleotide phosphate (NADPH)–generating system containing 0.8 mM NADPH into the reaction mixture (8.33 mg/mL). The incubation was allowed to run for 2 h. The reaction was terminated by the addition of 2 mL of ice-cold acetonitrile. The mixture was centrifuged for 15 min at 14 000 rpm, and the supernatant was transferred to a fresh container, and the solvent was evaporated under a stream of nitrogen. The residue was reconstituted in 1 mL with the mobile phase and transferred to HPLC vials for analysis. All metabolic incubation experiments were repeated three times using controls (without RLMs or NADPH).

2.5 Metabolites characterization using LC-MS/MS

The chromatographic analysis was carried out using LC-MS/MS with an electrospray ionization (ESI) source. A reversed-phase column (Eclipse plus C₁₈: Dim 150 × 2.1 mm with particle size 3.5 µm) was utilized for the separation. Isocratic mobile phases consisted of A: HPLC water containing 1% formic acid and B: acetonitrile. A gradient program was used for the HPLC separation at a flow rate of 0.2 mL/min for 90 min to allow the separation of very closely related metabolites. The gradients steps involved solvent B at 5% (0–5 min), 5–60% (5–60 min), 60–90% (60–70 min) and 90–5% (70–90 min), with a post run time of 10 min. Nitrogen was used as the sheath gas at a flow rate of 11 L/min, and the capillary temperature was set at 350°C. Nebulizer pressure was 55 psi with a collision energy of 18 eV, the capillary voltage was 4,000 V, and ESI was operated in the positive ion mode. Compound 1 metabolites were confirmed by the absence of their chromatographic peaks in the control incubations.

2.6 In silico toxicological studies of compounds 1–7 and compound 1 metabolites M1–M3 using DEREK software

DEREK software was used for screening of the structural alerts and for predicting the toxicological side effects of compounds 1–7 and compound 1 metabolites M1–M3. The outcomes can be used to establish more safer compounds in the drug design process [22,23].

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

The synthesis and the characterization of the test compounds 1–7 have been previously reported [19,24]. Compound 1 manifested an in vitro antiproliferative activity with an average IC₅₀ value of 5.60 µM against HT-29, ZR-75 and A-549 human cancer cell lines, while the reference drug, sunitinib, showed an average IC₅₀ value of 8.11 µM against
Figure 3: continued
Figure 3: Product ion mass spectra of compounds 2 (a), 3 (b), 4 (c), 5 (d), 6 (e) and 7 (f). (PI fragmentation of compound 1–7 gave one fragment ion at m/z 174 that represented the breakage of the carbohydrazide bond. Compound 5 (without any substitutions) showed an additional m/z at 146 that represented 3-iminoindolin-2-one fragment.)
the same human cancer cell lines (6). Consequently, compound 1 was chosen for further metabolic profiling for the development of future potential drug candidates before entering into clinical trials. Since mass fragmentation (MS/MS) behavior studies are very important for elucidating the structures of the possible identified metabolites in the field of metabolic profiling, we therefore studied the mass fragmentation (MS/MS) behavior of those potential drug-like candidates 1–7.

3.1 *In silico* prediction of compound 1 metabolites

Metabolic landscape indicates the lability of each site in compound 1 with respect to metabolism by CYP3A4 in absolute terms to guide the prediction of compound 1 metabolites and also the optimization of its chemical structure for improving its metabolic stability. The current study indicated that C24 (in the methoxy group), C17 and C19 (in the indole moiety), and C1 (in the
**Table 2:** Compound 1 and its related possible metabolites

| Parent m/z | ID   | Fragments | Retention time | Metabolic reaction |
|------------|------|-----------|----------------|-------------------|
| 427        | 1    | 174       | 52.7           |                   |
| 431        | M1   | 176       | 49.1           | Reduction         |
| 443        | M2   | 425, 190, 174 | 44.3        | Hydroxylation     |
| 413        | M3   | 160       | 41.9           | O-demethylation   |

Fragmentation of compound 1 metabolites yielded the same fragmentation behavior.

\[\text{\textit{N-methyl group}}\text{ in compound } \text{1} \text{ are proposed to be labile for metabolism. The CSL is shown in the top-right of the metabolic landscape of compound 1 (Figure 2).}\]

\[\textbf{Scheme 4: MS/MS fragments of M1.}\]

The results from WhichP450™ module, represented by the pie chart, were utilized for indicating the most likely CYP450 isoforms that have a major role in compound 1 metabolism (Figure 2). CYP3A4 was proposed to have the main effect in compound 1 metabolism.

\[\textbf{Figure 5: PI chromatogram of M1 showing a chromatographic peak at 49.1 min (a) and PI mass spectrum of M1 (b). (PI fragmentation of M1 at } m/z \text{ 431 gave one fragment ion at } m/z \text{ 176 that showed 2 } m/z \text{ more units, which revealed the reduction metabolic reaction.)}\]
Figure 6: PI chromatogram of M2 showing a chromatographic peak at 44.3 min (a) and PI mass spectrum of M2 (b). (PI fragmentation of M2 at $m/z$ 443 gave two fragment ions at $m/z$ 425 and $m/z$ 190 that showed 16 $m/z$ more units, which revealed the hydroxylation metabolic reaction).

Scheme 5: MS/MS fragments of M2.
3.2 Fragmentation pattern of compounds 1–7

Compounds 1–7 were dissolved in DMSO at the concentration of 2 µg/mL and were directly injected through a connector into the LC-MS/MS system. The flow injection analysis was used for the optimization of mass spectrometric parameters for the studied compounds. Fragmentation of isatin–indole conjugates 1–7 yielded the same fragmentation pattern (Table 1) as explained in Scheme 1. Product ion (PI) fragmentation of compounds 1–7 gave one fragment ion at $m/z$ 160 that showed 14 $m/z$ less units, which revealed an O-demethylation metabolic reaction.

\[ m/z: 413 \rightarrow 160 \]

Scheme 6: MS/MS fragments of M3.

3.3 Metabolic profiling of compound 1

Mass spectrum of compound 1 manifested molecular ion peak at $m/z = 427 [M + H]^+$ at retention time (RT) = 52.7 min.
PI fragmentation of $m/z = 427$ gave one fragment ion at $m/z = 174$ that represented the breakage of the carbohydrazide bond (Figure 4b and Scheme 3). Compound 1 metabolites with their fragmentation pattern are listed in Table 2. M1, M2 and M3 chromatographic peaks were not found in the control incubations (either by omitting NADPH or RLMs).

### 3.3.1 Fragmentation pattern for M1 metabolite of compound 1

Mass spectrum of M1 manifested a molecular ion peak at $m/z = 431$ [M + H]$^+$ at retention time (RT) = 49.1 min (Figure 5a). PI fragmentation of $m/z = 431$ gave one fragment ion at $m/z = 176$ that showed 2 $m/z$ more units as compared with compound 1 fragments, which revealed a reduction metabolic reaction (Figure 5b and Scheme 4).

### 3.3.2 Fragmentation pattern for M2 metabolite of compound 1

Mass spectrum of M2 manifested a molecular ion peak at $m/z = 443$ [M + H]$^+$ at retention time (RT) = 44.3 min (Figure 6a). PI fragmentation of $m/z = 443$ gave two main fragment ions at $m/z$ 425 and 190. Fragment ion at $m/z$ 190 showed 16 $m/z$ more units compared with compound 1 fragments, which revealed a hydroxylation metabolic reaction. Fragment ion at $m/z$ 425 showed water loss that confirmed compound 1 hydroxylation (Figure 6b and Scheme 5).

### 3.3.3 Fragmentation pattern for M3 metabolite of compound 1

Mass spectrum of M3 manifested a molecular ion peak at $m/z = 413$ [M + H]$^+$ at retention time (RT) = 41.9 min...
Pl fragmentation at m/z 413 gave one fragment ion at m/z 160 that showed 14 m/z less units compared with compound 1 fragments, which revealed an O-demethylation metabolic reaction (Figure 7b and Scheme 6).

3.4 In silico toxicity prediction using StarDrop DEREK module

In silico toxicity assessment of the isatin–indole conjugates 1–7 and the metabolites of compound 1 were carried out using DEREK software (Figures 8 and 9). Compounds 1–7 and the metabolites M1–M3 showed skin sensitization (due to hydrazine moiety), chromosome damage and mutagenicity (due to arylhydrazine moiety). Also, M1 showed hepatotoxicity due to 4-aminophenol part, while compounds 2, 3 and 6 showed nephrotoxicity due to halogenated benzene moiety. Table 3 presents a complete list of the predicted toxicities for compounds 1–7 and the metabolites M1–M3 using DEREK software.

4 Conclusion

This study involves in silico, in vitro (RLMs) and fragmentation pattern for certain isatin–indole conjugates 1–7. Fragmentation patterns of compounds 1–7 were studied and were used as a clue for metabolic profiling of the potent antiproliferative agent, compound 1, in RLMs using LC-MS/MS. Compound 1 was biotransformed into three possible metabolites by cleavage reduction, hydroxylation and O-demethylation in RLMs in the presence of NADPH. Structures of those possible metabolites were elucidated by comparing their fragmentation behavior with the parent compound 1. The StarDrop WhichP450™ module efficiently characterized the vulnerable sites for metabolism in compound 1, and the results are consistent with the experimental ones. Structural alerts and the predicted toxicological parameters were also identified using the StarDrop DEREK module. Further drug discovery experiments can be performed depending on this concept allowing the establishment of new drugs with the elevated safety profile without affecting their pharmacological action. This study could support the development of new potent antiproliferative agents in the preclinical trials aiming to get future potential drug-like anticancer candidates.

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References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87–108.
[2] Padma VV. An overview of targeted cancer therapy. Biomedicine (Taipei). 2015;5(4):19–24.

[3] Flynn BL, Hamel E, Jung MK. One-pot synthesis of benzo[b]furan and indole inhibitors of tubulin polymerization. J Med Chem. 2002;45(12):2670–3.

[4] Leboho TC, Michael JP, van Otterlo WA, van Vuuren SF, de Koning CB. The synthesis of 2-and 3-ary indoles and 1,3,4,5-tetrahydropryanol[4,3-b]indoles and their antibacterial and antifungal activity. Bioorg Med Chem Lett. 2009;19(17):4948–51.

[5] Vine KL, Indira Chandran V, Locke JM, Matesic L, Lee J, Skropeta D, et al. Targeting urokinase and the transferrin receptor with novel, anti-mitotic N-alkylisatin cytotoxic conjugates causes selective cancer cell death and reduces tumor growth. Curr Cancer Drug Targets. 2012;12(1):64–73.

[6] Attia MI, Eldehna WM, Afifi SA, Keeton AB, Piazza GA, Abdel-Aziz HA. New hydrazonoinodolin-2-ones: synthesis, exploration of the possible anti-proliferative mechanism of action and encapsulation into PLGA microspheres. PLoS one. 2017;12(7):e0181241.

[7] Roberts SA. High-throughput screening approaches for investigating drug metabolism and pharmacokinetics. Xenobiotica. 2001;31(8–9):557–89.

[8] Kumar GN, Surapaneni S. Role of drug metabolism in drug discovery and development. Med Res Rev. 2001;21(5):397–411.

[9] Leinonen A, Kuuranne T, Kostlainen R. Liquid chromatography/mass spectrometry in anabolic steroid analysis–optimization and comparison of three ionization techniques: electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization. J Mass Spectrom. 2002;37(7):693–8.

[10] Attwa MW, Kadi AA, Alrabiah H, Darwish HW. LC-MS/MS reveals the formation of iminium and quinone methide reactive intermediates in entrectinib metabolism in vivo and in vitro metabolic investigation. J Pharm Biomed Anal. 2018;160:19–30.

[11] Del Boccio P, Di Deo A, De Curtis A, Celli N, Iacoviello L, Rotilio D. Liquid chromatography-tandem mass spectrometry analysis of oleuropein and its metabolite hydroxytyrosol in rat plasma and urine after oral administration. J Chromatogr B AnalTechnol Biomed Life Sci. 2003;785(1):47–56.

[12] Amer SM, Kadi AA, Darwish HW, Attwa MW. Liquid chromatography tandem mass spectrometry method for the quantification of vandetanib in human plasma and rat liver microsomes matrices: metabolic stability investigation. Chem Cent J. 2017;11(1):45.

[13] Attwa MW, Kadi AA, Abdelhameed AS. Reactive intermediates and bioactivation pathways characterization of avitinib by LC–MS/MS: in vitro metabolic investigation. J Pharm Biomed Anal. 2019;164:659–67.

[14] Kadi AA, Attwa MW, Rahman AF. A preliminary study of arecoline and guvacoline presence in the saliva of a “betel-quid” chewer using liquid chromatography ion trap mass spectrometry. Eur J Mass Spectrom (Chichester). 2013;19(5):391–7.

[15] El-Subbagh H, El-Kashef HA, Kadi AA, Abdel-Aziz AA, Hassan GS, Tettey J, et al. New ultra-short acting hypnotic: synthesis, biological evaluation, and metabolic profile of ethyl 8-oxo-5,6,7,8-tetrahydro-thiazolo[3,2-a][1,3]diazepin-3-carboxylate (HIE-124). Bioorg Med Chem Lett. 2008;18(1):72–7.

[16] Attwa MW, Kadi AA, Darwish HW, Amer SM, Al-shakliah NS. Identification and characterization of in vivo, in vitro and reactive metabolites of vandetanib using LC-ESI-MS/MS. Chem Cent J. 2018;12(1):99.

[17] Islam MS, Park S, Song C, Kadi AA, Kwon Y, Rahman AFMM. Fluorescence hydrazones: a series of novel non-intercalating topoisoenzyme lxa catalytic inhibitors induce G1 arrest and apoptosis in breast and colon cancer cells. Eur J Med Chem. 2017;125:49–67.

[18] Abdelhameed A, Bakheit A, Mohamed M, Eldehna W, Abdel-Aziz H, Attia M. Synthesis and biophysical insights into the binding of a potent anti-proliferative non-symmetric bis-isatin derivative with bovine serum albumin: spectroscopic and molecular docking approaches. Appl Sci. 2017;7(6):617.

[19] Almutairi MS, Zakaria AS, Ignasius PP, Al-Wabli RI, Joe IH, Attia MI. Synthesis, spectroscopic investigations, DFT studies, molecular docking and antimicrobial potential of certain new isatin molecular hybrids: experimental and theoretical approaches. J Mol Struct. 2018;1153:333–45.

[20] Marchant CA, Briggs KA, Long A. In silico tools for sharing data and knowledge on toxicity and metabolism: derek for windows, meteor, and vticl. Toxicol Mech Methods. 2008;18(2–3):177–87.

[21] T’Jolyn H, Boussery K, Mortishire-Smith RJ, Coe K, De Boeck B, Van Boeckel JF, et al. Evaluation of three state-of-the-art metabolite prediction software packages (Meteeor, MetaSite, and StarDrop) through independent and synergistic use. Drug Metab Dispos. 2011;39:2066–75.

[22] Attwa MW, Kadi AA, Darwish HW, Amer SM, Alrabiah H. A reliable and stable method for the determination of forinetin in human plasma by LC-MS/MS: application to metabolic stability investigation and excretion rate. Eur J Mass Spectrom. 2018;24(4):344–51.

[23] Attwa MW, Kadi AA, Abdelhameed AS. Phase I metabolic profiling and unexpected reactive metabolites in human liver microsome incubations of X-376 using LC-MS/MS: bioactivation pathway elucidation and in silico toxicity studies of its metabolites. RSC Adv. 2020;10(9):5412–27.

[24] Al-Wabli RI, Zakaria AS, Attia MI. Synthesis, spectroscopic characterization and antimicrobial potential of certain new isatin–indole molecular hybrids. Molecules. 2017;22(11):1958.