A Polyamine-Based Dinitro-Naphthalimide Conjugate as Substrates for Polyamine Transporters Preferentially Accumulates in Cancer Cells and Minimizes Side Effects in vitro and in vivo

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Naphthalimides, such as amonafide and mitonafide in clinical trials, have been developed as antitumor agents for orthotopic tumor. However, the serious side effects in cancer patients limit their applications. Herein, a new class of polyamine-based naphthalimide conjugates 5a-5c, 7a-7b, and 11a-11b with and without the alkylation of the distant nitrogen in the polyamine chain were synthesized and the mechanism was determined. Compared with amonafide, dinitro-naphthalimide conjugate 5c with a 4,3-cyclopropyl motif preferentially accumulates in cancer cells and minimizes side effects in vitro and in vivo. More importantly, 5c at the dosage of as low as 3 mg/kg (57.97%) displays better antitumor effects than the positive control amonafide (53.27%) at 5 mg/kg in vivo. And a remarkably elevated antitumor activity and a reduced toxicity are also observed for 5c at 5 mg/kg (65.90%). The upregulated p53 and the apoptotic cells (73.50%) indicate that the mechanism of 5c to induce apoptosis may result from its enhanced DNA damage. Further investigation indicates that in addition to target DNA, 5c can modulate the polyamine homeostasis by upregulating polyamine oxidase (PAO) in a different way from that of amonafide. And also by targeting PTs overexpressed in most of cancer cells, 5c downregulates the contents of Put, Spd, and Spm, which are in favor of suppressing fast-growing tumor cells. Our study implies a promising strategy for naphthalimide conjugates to treat hepatic carcinoma with notable activities and reduced toxicities at a low dosage.

Keywords: dinitro-naphthalimide conjugate, polyamine, polyamine transporter, cancer, minimized side-effects
INTRODUCTION

As the sixth most prevalent malignancy, hepatocellular carcinoma (HCC) is a kind of cancer that is found too late and has a high mortality worldwide (Forner et al., 2012; Dou et al., 2016). Approximately 90% of cancer patients with HCC cannot survive for more than 5 years even after the treatment by anticancer drugs (Chen et al., 2016; Siegel et al., 2016). In spite of advances in the rational and combinatorial technologies for cancer therapy, current anticancer agents can only cure parts of recurrence and metastasis cells after tumor excision at high doses, which can lead to severe side effects. (Li et al., 2016) Therefore, it is urgent to develop novel drugs with enhanced activities and reduced toxicities at a relatively low dosage.

Recently, except for photophysical characters, a surge in the activities of naphthalimides has also been developed as versatile functional compounds for promising antitumor activities (Banerjee et al., 2013; Chen et al., 2013; Seifert et al., 2016; Mateusz and Krzysztof, 2018; Peddaboodi et al., 2018; Yulin et al., 2019). Amonafide and mitonafide in Figure 1 as the representative naphthalimides have entered clinical trials (Stone et al., 2015). One of the major reasons why amonafide and mitonafide cannot be used widely is because they can have serious side effects in cancer patients. Thus, the design of novel naphthalimide therapeutic agents with reduced toxicity represents an area that is in need of urgent attention.

Resurgence in the interest of natural polyamines as an anticancer strategy results from advances in our understanding of polyamine metabolism and their alterations in cancer (Casero and Marton, 2007; Casero et al., 2018; Phanstiel, 2018). Natural products with polyamine moieties have been found to be a promising strategy to enhance targeting properties and deduce the toxicities (Muth et al., 2013, 2014; Skruber et al., 2017). Polyamine analogs CHENSpm and CPENSpm in Figure 1 have entered in clinical trials. Our group (Wang et al., 2012; Li et al., 2016, 2018; Dai et al., 2017a; Ma et al., 2017a,b; Ma et al., 2018; Liu et al., 2019) focused on polyamine-based naphthalimide conjugates to achieve enhanced pharmacological effects. And polyamine-based naphthalimide conjugates are also used to treat HCC as mitochondria or lysosome targeting antitumor and antimetastatic agents. So far, the main modification of naphthalimides is focused on amino and nitro groups, which play an important role in the antitumor activity. Unfortunately, polyamine-based di-amino and di-nitro naphthalimide conjugates are virtually unexplored. Moreover, in the structure of CHENSpm and CPENSpm, the alkylation of the distant nitrogen in the polyamine chain plays an important role in increasing the activity in cancer cells and decreasing the toxicity in normal cells. Our group and others find that natural products with homospermidine moieties without the alkylation of the distant nitrogen in the polyamine chain showed good activities (Casero and Marton, 2007; Wang et al., 2012; Muth et al., 2013, 2014; Li et al., 2016, 2018; Dai et al., 2017a; Ma et al., 2017a,b, 2018; Skruber et al., 2017; Casero et al., 2018; Phanstiel, 2018; Liu et al., 2019). However, naphthalimide conjugates with the alkylation of the distant nitrogen in the polyamine chain are rarely reported.

Based on the key role of amino and nitro group in mediating antitumor efficacy of naphthalimide conjugates, we firstly designed and synthesized a new class of polyamine-based di-nitro and di-amino naphthalimide conjugates 5a-5c, 7a-7b, and 11a-11b with and without the alkylation of the distant nitrogen in the polyamine chain. For the first time, we summarized the structure-activity relationship (SAR) of 5a-5c, 7a-7b, and 11a-11b with and without the classical unsymmetrically-substituted polyamine analogs CHENSpm and CPENSpm (Casero and Marton, 2007; Casero et al., 2018; Phanstiel, 2018) (Figure 1 and Scheme 1). Chains with different lengths, such as 4,4 and 4,3-cyclohexyl or 4,3-cyclopropyl substituted diamine, or 3,3,3 and 3,4,3 substituted triamine motif are also selected for the construction of 5a-5c, 7a-7b, and 11a-11b to investigate different lengths on antitumor activity. Amonafide was selected as the positive control. We established feasible routes to 5a-5c, 7a-7b, and 11a-11b in Scheme 1.

EXPERIMENTAL SECTION

General Procedure for Obtaining Title Compounds, Using 5a as Example

The known intermediate 2 was prepared by a conventional method using concentrated nitric acid in glacial acetic acid (Gryshchenko et al., 2015; Soriano et al., 2016). A solution containing 3 (1 mmol) in CH3CN (5 mL) at 0°C was added to a solution of 2 (1 mmol) and K2CO3 in CH3CN (10 mL). Then the mixture was heated to 85°C for 5 h. After monitoring by Thin-Layer Chromatography (TLC), the reaction mixture was cooled to room temperature and concentrated under vacuum to give an oily residue. After extraction and purification by column chromatography with dichloromethane/methanol (100:1–100:3, v/v) as the elution solvent, 4 was obtained in a yield of 60%. At 0°C we added four molar HCl (2 mL), and then 4 in CH3CH2OH (2 mL) was stirred at room temperature overnight until a great amount of precipitate was generated. The filtered cake was washed by anhydrous CH3CH2OH, dried to give the target compound 5a as a hydrochloride salt in a yield of 56%. 1H NMR (300 MHz, DMSO-d6) δ = 9.76 (s, 2H), 9.09 (s, 2H), 2.97 (m, 2H), 2.50 (m, 7H), 1.64 (m, 16H), 13C NMR (75 MHz, D2O) δ = 163.75, 147.26, 131.96, 130.76,
SCHEME 1 | Chemical structures and synthetic route of polyamine-based naphthalimide conjugates 5a-5c, 7a-7b, and 11a-11b in yield of 25–30%. Reagents and conditions: (a) HNO$_3$, 50°C, 3 h; (b) K$_2$CO$_3$, CH$_3$CN, 85°C, 5 h; (c) EtOH, 4 M HCl, rt, overnight; (d) Pd/C, H$_2$, MeOH, rt, 2 h.

Dinitro-Naphthalimide Conjugate 5b

5b was obtained according to the procedure of 5a except for replacing 3 with 4,4′-cyclohexyl substituted diamine as shown in Scheme S1 in a yield of 65%. $^1$H NMR (300 MHz, D$_2$O) $\delta = 9.31$ (s, 2H), 9.09 (s, 2H), 4.03 (m, 2H), 3.01 (m, 7H), 2.04–0.94 (m, 19H). $^{13}$C NMR (75 MHz, D$_2$O) $\delta = 163.25$, 147.18, 131.79, 130.60, 126.95, 124.17, 57.20, 47.11, 46.78, 43.58, 40.19, 28.97, 24.45, 24.13, 23.86, 23.10, 22.99, 22.83. ESI-MS (positive ion mode): m/z [M]$^+$: calcd: 511.06; obsd: 512.06. Calcd for C$_{25}$H$_{33}$Cl$_2$N$_5$O$_6$: C 52.58%, H 5.73%, N 12.19%.

Dinitro-Naphthalimide Conjugate 5c

5c was obtained according to the procedure of 5a except for replacing 3 with 4,3-cyclopropyl substituted diamine as shown in Scheme S1 in a yield of 68%. $^1$H NMR (300 MHz, D$_2$O) $\delta = 9.39$ (s, 2H), 9.17 (s, 2H), 4.12 (s, 2H), 3.39–3.06 (m, 6H), 2.75 (d, J = 4.2, 1H), 2.30–2.01 (m, 2H), 1.81 (m, 4H), 0.90 (dd, J = 18.3, 7.2, 4H). $^{13}$C NMR (75 MHz, D$_2$O) $\delta = 163.28$, 147.16, 131.83, 130.72, 127.02, 124.09, 47.32, 44.96, 44.49, 40.22, 30.09, 24.15, 23.15, 22.47. ESI-MS (positive ion mode): m/z [M]$^+$: calcd: 497.07; obsd: 498.07. Calcd for C$_{25}$H$_{33}$Cl$_2$N$_5$O$_6$: C 52.64%, H 5.83%, N 12.28%. Found: C 52.58%, H 5.73%, N 12.19%.

Diamino-Naphthalimide Conjugate 7a

Intermediate 4 was obtained according to the procedure as described in 5a. Then Pd/C was added to the solution of 4 dissolved in MeOH (10 ml) and stirred in hydrogen at room temperature for 2 h to obtain 5 in a yield of 85%. Then,
Inhibitory Effects of 5c on Snu-368 and Snu-739 Cells With and Without Spd (50 µM) After 24-h Treatment

Inhibitory effects of 5c on Snu-368 and Snu-739 cells (5000 cells/well) were conducted similar to the MTT assay except that the Spd-containing RPMI medium was used for serial dilution of the compound-containing concentrated solutions.

Determination of the Contents of PAO (Polyamine Oxidase)

We measured the total PAO level using a PAO assay kit according to our previous report (Liu et al., 2019) (Hepeng Biotechnology, Cat. HEPENG150).

Western Blot Assay

We performed western blot analysis to determine the contents of p53 after treatment by 5c for 5, 10, and 15 µM for 24 h. After washing three times with PBS, we harvested and centrifuged the cells, which were lysed with a RIPA buffer (Beyotime, China). The total contents of protein were determined by a BCA assay kit (Beyotime, China). The total lysates were denatured in a 5 × SDS-loading buffer. Equal amounts of total proteins were separated by 12% SDS-PAGE. Dried skimmed milk (5%) was used to block the separated protein in Tris Buffered Saline Tween (TBST) at room temperature for 1 h. Then the corresponding primary antibodies and the appropriate HRP-conjugated secondary antibody were used to incubate with the separated protein. By ECL plus reagents (Beyotime, Jiangsu, China), we detected the expression of p53.

Polymamines Contents Assay

According to our previous report (Wang et al., 2012; Li et al., 2016, 2018; Dai et al., 2017a; Ma et al., 2017a,b, 2018; Liu et al., 2019), the contents of Put, Spd, and Spm were detected by a G1321A fluorescence detector in both Snu-368 and Snu-739 cells. Cells were harvested and dansyl chloride was used as the derivation reagent. After converting to the corresponding dansyl derivatives, Put, Spd, and Spm were separated by using High Performance Liquid Chromatography (HPLC) (Agilent 1260, Agilent Technologies, USA) with a C18 chromatographic column (25 × 4.6 mm, 5 µm). As the internal standard substance, 1,6-diaminohexane was used. The excitation and emission wavelengths were 340 and 515 nm, separately. The mobile phase was methanol–water from 65:35 to 100:0 within 30 min.

In vitro Cellular Cytotoxicity Assays

We incubated hepatic carcinoma (Snu-368 and Snu-739), breast carcinoma (MDA-MB-231 and MCF-7), cisplatin-sensitive lung cancer cells A549, and cisplatin-resistant lung cancer cells A549cisR in 96-well plates in a 5% CO₂ atmosphere at 37°C for 24 h (5,000 cells/well). After adding drugs in freshly prepared culture medium (100 µl) and incubating for another 48 h, we added 20 µl MTT (5 mg/ml) and incubated for another 3 h. At last, after the medium was removed, 150 µl DMSO was added. By using a Bio-Rad 680 microplate reader, the absorbance was measured at 570 nm and the IC₅₀ values were calculated using the GraphPad Prism software based on three parallel experiments.

Diamino-Naphthalimide Conjugate 7b

7b was obtained according to the procedure of 7a except for replacing 3 with 4,4-unsuobstituted diamine as shown in Scheme S1 in a yield of 69%. 1H NMR (300 MHz, D₂O) δ = 7.86–7.43 (m, 2H), 3.80 (d, J = 6.6, 2H), 3.18–2.84 (m, 6H), 2.61 (s, 1H), 1.90 (t, J = 29.2, 2H), 1.68 (m, 4H), 0.77 (t, J = 12.5, 4H). 13C NMR (75 MHz, D₂O) δ = 164.33, 133.95, 132.56, 124.45, 123.69, 123.25, 47.34, 47.09, 45.37, 37.56, 30.04, 24.27, 22.88, 22.61, 2.96. ESI-MS (positive ion mode): m/z [M]+: calcd: 395.04; obsd: 396.04. Calcd for C₂₃H₂₃Cl₂N₂O₃: C 53.98%, H 6.58%, N 14.85%.

Diamino-Naphthalimide Conjugate 11a

11a was obtained according to the procedure of 7a except for replacing 3 with 8 as shown in Scheme S2 in a yield of 62%. 1H NMR (300 MHz, D₂O) δ = 7.79 (d, J = 35.7, 2H), 3.91 (d, J = 6.1, 2H), 3.01 (m, 10H), 2.21–1.85 (m, 6H). 13C NMR (75 MHz, D₂O) δ = 166.53, 137.02, 134.91, 126.45, 126.28, 125.50, 125.28, 48.09, 47.25, 47.17, 47.11, 40.06, 39.08, 26.77, 26.26, 25.23. ESI-MS (positive ion mode): m/z [M]+: calcd: 398.05; obsd: 399.05. Calcd for C₂₃H₂₃Cl₂N₂O₃: C 49.66%, H 6.55%, N 16.55%. Found: C 49.56%, H 6.45%, N 16.45%.

Diamino-Naphthalimide Conjugate 11b

11b was obtained according to the procedure of 11a except for replacing 3 with 8 as shown in Scheme S2 in a yield of 62%. 1H NMR (300 MHz, D₂O) δ = 8.04–7.55 (m, 2H), 4.20–2.47 (m, 12H), 2.26–1.49 (m, 8H). 13C NMR (75 MHz, D₂O) δ = 164.35, 135.19, 132.56, 123.61, 122.78, 122.72, 122.61, 47.03, 45.45, 44.56, 37.52, 36.58, 24.25, 23.75, 22.98, 22.80. ESI-MS (positive ion mode): m/z [M]+: calcd: 412.06; obsd: 413.06. Calcd for C₂₂H₂₃Cl₂N₂O₂: C 50.63%, H 6.76%, N 16.10%. Found: C 50.58%, H 6.68%, N 15.98%.

In vitro Cellular Cytotoxicity Assays

We incubated hepatic carcinoma (Snu-368 and Snu-739), breast carcinoma (MDA-MB-231 and MCF-7), cisplatin-sensitive lung cancer cells A549, and cisplatin-resistant lung cancer cells A549cisR in 96-well plates in a 5% CO₂ atmosphere at 37°C for 24 h (5,000 cells/well). After adding drugs in freshly prepared culture medium (100 µl) and incubating for another 48 h, we added 20 µl MTT (5 mg/ml) and incubated for another 3 h. At last, after the medium was removed, 150 µl DMSO was added. By using a Bio-Rad 680 microplate reader, the absorbance was measured at 570 nm and the IC₅₀ values were calculated using the GraphPad Prism software based on three parallel experiments.

Inhibitory Effects of 5c on Snu-368 and Snu-739 Cells With and Without Spd (50 µM) After 24-h Treatment

Inhibitory effects of 5c on Snu-368 and Snu-739 cells (5000 cells/well) were conducted similar to the MTT assay except that the Spd-containing RPMI medium was used for serial dilution of the compound-containing concentrated solutions.
Annexin V-FITC/Propidium Iodide Staining

Annexin V-FITC/PI staining was used to detect the apoptosis of Snu-739 cells by FCM (flow cytometry). Firstly, we plated the cells in six-well plates (1 × 10⁵ cells/well). 5c 10 μM and amonafide 10 μM were added and incubated for 24 h. And then we harvested the cells and washed three times with PBS. Then the protocol was stained according to our previous report (Wang et al., 2012; Li et al., 2016, 2018; Dai et al., 2017a; Ma et al., 2017a,b; Ma et al., 2018; Liu et al., 2019) and was performed by FCM (BD Biosciences, San Jose, CA, USA).

In vivo Antitumor Assays

Healthy BALB/c mice (Cat. SCXK 2016-0006, Beijing, China) aged 5 weeks were used for the determination of anticancer activity. We obtained BALB/c mice from the Laboratory Animal Center, Academy of Military Medical Science. They were raised in compliance with the Guide for the Care and Use of Laboratory Animals. The weight of healthy BALB/c mice is 18–22 g.

We firstly injected HCC cells with 1 × 10⁵ cells via the tail vein every day for a total of seven treatments. Body weight was determined every day. And then the mice were sacrificed after 7 days. The tumor tissues were weighed and the inhibition rate [(average tumor weight of negative control group – average tumor weight of the drug treated or positive control group)/average tumor weight of control group] × 100] was calculated. The organ index [(organ weight/body weight) × 100%] was also counted including heart, liver, kidney, lung, and spleen at the last day. Both 5c and amonafide were dissolved in glucose injection. And they were used immediately after preparation.

The ethical committee approved the projects in vivo with the number HUSOM-2016-316, and we performed all animal procedures following the protocol approved by the Institutional Animal Care and Use Committee at Henan University.

### Table 1

| IC₅₀ (μM) | Snu-368 | Snu-739 | MDA-MB-231 | MCF-7 | A549 | A549cisR | RF[^4] |
|-----------|---------|---------|-------------|-------|------|----------|-------|
| 5a        | Cyclohexyl | 2 | 1 | 0.294 ± 0.06 | 2.16 ± 0.10 | 1.19 ± 0.12 | 2.3 ± 0.18 | 2.17 ± 0.20 | 3.62 ± 0.15 | 1.67 |
| 5b        | Cyclohexyl | 2 | 2 | 2.72 ± 0.25 | 3.89 ± 0.35 | 1.07 ± 0.10 | 1.25 ± 0.10 | 1.75 ± 0.15 | 2.27 ± 0.25 | 1.30 |
| 5c        | Cyclopropyl | 2 | 1 | 1.09 ± 0.10 | 0.76 ± 0.05 | 1.33 ± 0.12 | 1.35 ± 0.15 | 1.92 ± 0.23 | 0.83 ± 0.08 | 0.43 |
| 7a        | Cyclopropyl | 2 | 1 | >30 | 25.77 ± 2.90 | 26.91 ± 2.65 | 8.62 ± 0.85 | 13.98 ± 1.39 | 17.38 ± 1.68 | / |
| 7b        | H        | 2 | 2 | >30 | >30 | >30 | >30 | >30 | 17.30 ± 1.78 | / |
| 11a       | /        | 1 | 1 | 1 | >50 | 9.69 ± 0.98 | 25.96 ± 2.56 | 14.81 ± 1.45 | 28.3 ± 2.85 | 16.33 ± 1.69 | / |
| 11b       | /        | 2 | 1 | >30 | 23.17 ± 2.36 | >30 | 25.91 ± 2.56 | >30 | 15.81 ± 1.58 | / |
| Amonafide |          |     |     |      |      |      |      |      |      |      |      |
| Snu-368   | 13.98 ± 1.78 | 12.98 ± 1.56 | 14.89 ± 1.75 | 5.89 ± 0.56 | 6.89 ± 0.68 | 10.98 ± 1.02 | 1.59 |
| Snu-739   | 16.37 ± 1.26 | 10.02 ± 1.23 | 15.98 ± 1.59 | 9.60 ± 0.60 | 11.00 ± 0.15 | 40.36 ± 4.36 | 3.67 |
| MDA-MB-231 | 15.02 | 13.18 | 12.02 | 7.11 | 5.73 | 48.63 | 8.74 |
| MCF-7     | 100%     | 100% | 100% | 100% | 100% | 100% | 100% |

[^4]: The RF (resistance factor) is defined as the IC₅₀ value in A549cisR cells/IC₅₀ value in A549 cells.

[^5]: FI (fold increase) is defined as IC₅₀(amonafide)/IC₅₀(5c).

[^6]: FI (fold increase) is defined as IC₅₀(cisplatin)/IC₅₀(5c).

[^7]: An average of three measurements. ND = not determined.

### Table 2

| IC₅₀ (μM) | Snu-368 | Snu-739 | HL-7702 | Sf[^4] | Sf[^5] |
|-----------|---------|---------|---------|-------|-------|
| 5a        | 2.94 ± 0.06 | 2.16 ± 0.10 | 6.30 ± 0.23 | 2.14 | 2.92 |
| 5b        | 2.72 ± 0.25 | 3.89 ± 0.35 | 7.56 ± 0.43 | 2.78 | 1.94 |
| 5c        | 1.09 ± 0.10 | 0.76 ± 0.05 | 5.68 ± 0.83 | 5.21 | 7.47 |
| Amonafide | 13.98 ± 1.78 | 12.98 ± 1.56 | 11.56 ± 1.36 | 0.83 | 0.89 |
| Cisplatin | 16.37 ± 1.26 | 10.02 ± 1.23 | 8.60 ± 0.20 | 0.53 | 0.86 |

[^4]: Sf(selectivity index) is defined as IC₅₀ in HL-7702/IC₅₀ in Snu-368.

[^5]: Sf(selectivity index) is defined as IC₅₀ in HL-7702/IC₅₀ in Snu-739.
RESULTS

Synthesis and Characterization of Polyamine-Based Naphthalimide Conjugates 5a-5c, 7a-7b, and 11a-11b

Feasible routes to 5a-5c, 7a-7b, and 11a-11b (Scheme 1) were established and given in detail in the Supporting Information. The linkers 3 and 8 (Schemes S1, S2) were first prepared by the conventional reaction of 2-(4-bromobutyl)isoindoline-1,3-dione a or 2-(3-bromopropyl)isoindoline-1,3-dione i as the starting materials to obtain the hydrophobic polyamine chain varying connecting formats from 4,4 and 4,3-cyclohexyl or cyclopropyl substituted diamine, to 3,3,3 and 3,4,3 substituted triamine motif in a yield of 65%. We also established the detail on the feasible routes of the linkers 3 and 8 in the Supporting Information. Generated intermediate 2 by a conventional method using concentrated nitric acid in glacial acetic acid (Gryshchenko et al., 2015; Soriano et al., 2016). Generated intermediate 2 reacted with diverse protected polyamines 3 and 8 to give compounds 4 and 9, 6 and 10 were obtained by the reduction reaction with Pd/C in the presence of hydrogen in MeOH. 4, 6, and 10 were deprotected with 4 M HCl to provide target compounds 5a-5c, 7a-7b, and 11a-11b as hydrochloride salts in a yield of 25–30%.

By $^1$H, $^{13}$C NMR spectroscopy, ESI-MS (Figures S2–S24) and CHN elemental analysis, all new compounds were characterized. We confirmed the purity of platinum complexes 4-7 ≥ 95% by HPLC (Tables S1, S2).

In vitro Cytotoxicity Effects

In vitro assays were firstly conducted to evaluate the inhibitory effect of polyamine-based naphthalimide conjugates 5a-5c, 7a-7b, and 11a-11b on six cancer cell lines, namely, Snu-368 and Sun-739 (hepatoma cell line), MCF-7 (breast carcinoma), MDA-MB-231 (triple negative breast cancer), cisplatin-sensitive lung cancer A549, and resistant A549cisR cells by using traditional MTT tests. Two classic antitumor agents in clinic trials, amonafide and cisplatin, were chosen as the reference drugs.
The preliminary structure–activity relationship (SAR) can be obtained from the in vitro biological results. Compounds 5a–5c, 7a–7b, and 11a-11b, with a different methylene linker, exhibited different potency in the tested cancer cells, indicating that the linker in these polyamine conjugates plays an important role in their antitumor activities. Dinitro-naphthalimide conjugates 5a–5c displayed more potent antitumor activities than compounds 7a–7b and 11a–11b with diamino moieties. For dinitro-based scaffolds (5a–5c), 5c with a 4,3-cyclopropyl substituted diamine motif was the most active toward all six of the tested cancer cells, which was more potent than its 4,3-cyclohexyl (5a) and 4,4-cyclohexyl (5b) counterparts. Furthermore, 5c (FI 3.59–48.63) was significantly more active than amonafide and cisplatin with some potencies in the nanomolar range. Among these compounds, 5c displayed the highest cytotoxicity. And then 5c was focused for the following tests.

**In vitro Cytotoxicity Effects on Cancer Cells and the Matched Normal Cells**

The selectivity for cancer cells over normal healthy cells is important for ideal anticancer agents, thereby mitigating undesired toxic side effects associated with chemotherapy. The selectivity of polyamine-based dinitro-naphthalimide conjugates 5a–5c was evaluated by Snu-368 and Snu-739 (hepatic carcinoma), and the matched normal cells HL-7702 (normal liver cell). Moreover, dinitro-naphthalimide conjugates 5a–5c (SI 1.94–7.47) show lower cytotoxicity in normal cells. Notably, as presented in Table 2, the IC₅₀ values of 5c (SI 5.21–7.47) were significantly lower in the cancer cells compared to the matched normal cells, SI of which is 8–33-folds higher than that of amonafide (SI 0.83–0.89) and cisplatin (SI 0.53–0.86). A similar tendency was observed for the inhibition ratio of 5c, amonafide, and cisplatin with 10 µM of tested complexes (Figure 2).

For the first time, we found that polyamine-based dinitro-naphthalimide conjugate 5c displayed the highest cytotoxicity to cancer cells and reduced toxicity to normal cells in vitro. Therefore, we focus on 5c for the following tests. And also the results of antitumor activity in vitro prompted us to further test the in vivo antitumor activities.

**Tumor Growth Inhibition in vivo**

The lowest IC₅₀ of 5c (0.76 µM) in Snu-739 cells (Table 1) indicates its better therapeutic effects in HCC. The highest FI levels in Snu-739 also indicate a significant therapy for HCC. Therefore, HCC animal models were used to test the in vivo antitumor activities. Mice bearing HCC xenografts were treated with 5c (3 mg/kg), 5c (5 mg/kg), the positive control amonafide (5 mg/kg), and normal saline (negative control) once every day for 7 days by tail vein (Figure 3).

The tumor suppression of 5c at the dosage of 3 mg/kg (57.97%) and 5 mg/kg (65.90%) was better than that of amonafide (53.27%, 5 mg/kg). On day 7, the average tumor volume (900 mm³) for the control group was much higher than that of the 5c group (480 mm³), indicating enhanced antitumor activity in vivo. Meanwhile, the variations of organ weight indexes implied that 5c showed no obvious pathological changes in the experiments.
of toxicological profile in vivo (Figure 3D), which was consistent with the effects of 5c in vitro.

Induction of Apoptosis

Next, FCM experiments using Annexin V/PI double staining were conducted to further determine if apoptosis was induced by DNA damage (Figure 4). We can see that both the early apoptotic cells (35.00%) and late apoptotic cells (38.50%) of 5c (10 µM) were higher than that of amonafide (10 µM). This indicated that the enhanced toxicity of dinitro-naphthalimide conjugate 5c may result from the DNA damage.

Polyamine Transporters (PTs) Were Partially Involved in the Cellular Entrance of 5c

PTs, overexpressed in most of cancer cells, is vital to polyamine-conjugate. Next, cell viability of Snu-368 and Snu-739 cells was calculated with and without the PTs inhibitor Spd (Figure 6). We observed that in the presence of 20 µM Spd cell viability increased 20–30% in both Snu-368 and Snu-739 cells. Hypothesis was confirmed that in the cellular entrance of 5c PTs were at least partially involved.

5c Affects Polyamine Metabolism and Function by Upregulating Polyamine Oxidase (PAO)

PAO is a critical catabolism enzyme in polyamine metabolism. The upregulation of PAO can influence tumor high polyamine microenvironment to induce a significant accumulation of ROS, which can also promote apoptosis (Wang et al., 2013; Chen et al., 2017; Dai et al., 2017b). We found that the relative PAO activity in Snu-368 and Snu-739 cells upon 5c treatment is 1.5–2-folds higher than the control group (Figure 7).

The elevated PAO can upregulate oxidizing substances such as ROS and downregulate reducing substances such as GSH, which can lead to cisplatin resistance. Herein, the upregulated PAO is also believed to be among the major reasons for 5c to overcome cisplatin resistance.

And the contents of Put, Spd, and Spm can be decreased by the upregulated PAO to promote polyamine metabolism and suppress fast-growing tumor cells. Next, the contents of Spm, Spd, and Put in Snu-368 and Snu-739 cells were measured (Figure 8). Compared with the control group, 5c downregulated the contents of Put, Spd, and Spm in Snu-368 and Snu-739 cells.

The p53 protein expression in Snu-739 cells was tested to further determine the DNA damage in Figure 5. The upregulated p53 after treatment with 5c indicates that the mechanism of 5c to induce apoptosis may result from its enhanced DNA damage.

5c Affects Polyamine Metabolism and Function by Upregulating Polyamine Oxidase (PAO)
CONCLUSIONS

Taken together, our findings provide the first example of polyamine-based dinitro-naphthalimide conjugate 5c as substrates for PTs preferentially accumulate in cancer cells and minimize side effects in vitro and in vivo. By targeting polyamine catabolic enzyme PAO by PTs, 5c downregulates Put, Spd, and Spm to regulate tumor high polyamine microenvironment (Figure 9). And upregulating the p53 protein 5c causes significant DNA damage to induce apoptosis. The discovery of the potential role of dinitro-naphthalimide conjugate implies a promising strategy for naphthalimide conjugates with targeting properties to treat hepatic carcinoma with notable activities and reduced toxicities at a low dosage.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Henan University.

AUTHOR CONTRIBUTIONS

JM, CW, PW, JW, ZT, and SX contribute to the conception, design, analysis, and writing of the study against the collection of data and other routine work. HL, YL, LL, and KY contribute to the synthesis of compounds in this paper. LH, ZX, MS, SZ, QM, SL, SG, and JL contribute to the mechanism research and animal experiments in vitro and in vivo. LH contributed to the analysis, interpretation of data for this work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2020.00166/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.