Synthesis, Biological Evaluation, and Autophagy Mechanism of 12N-Substituted Sophoridinamines as Novel Anticancer Agents

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Supporting Information

ABSTRACT: A series of 12N-substituted sophoridinamine derivatives were synthesized and evaluated for their cytotoxic activities in human HepG2 hepatoma cells. Structure–activity relationship revealed that introduction of a suitable arylidene or aryethyl at the N'-end could greatly enhance antiproliferation potency. Among them, compound **6b** possessing a N'-trimethoxyphenyl methylene exhibited potent antiproliferation effect against three human tumor cell lines including HepG2, leukemia (K562), and breast cancer (HMLE), with IC₅₀ between 0.55 and 1.7 μ M. The underlying mechanism of **6b** against tumor cells is to block autophagic flux, mainly through neutralizing lysosomal acidity. Our results indicated that compound **6b** is a potent lysosomal deacidification agent and is accordingly able to block autophagic flux and inhibit tumor cell growth.

KEYWORDS: Sophoridinamine, anticancer, structure–activity relationship, autophagy, lysosomes

Fufang Kushen injection, a traditional Chinese medicine, was approved by China Food and Drug Administration (CFDA) in 1995 for treatment of nonsmall cell lung carcinoma, liver cancer, gastric cancer.^{1–4} It has been widely used for the treatment of diverse cancer patients in combination with other anticancer drugs, such as vinorelbine, cisplatin, and taxol.^{5–10} Sophoridine (Figure 1), one of the key active ingredients in *Fufang Kushen injection*, was also approved by CFDA in 2005 to treat cancer patients with malignant trophoblastic tumors. Sophoridine, as a new chemical entity, exhibits multiple druglike properties such as structural flexibility, high solubility, and good pharmacokinetic (PK) profiles.¹¹

Figure 1. Chemical structures of sophoridine, 12N-*p*-cholobenzyl sophoridinol (**1**), colchicine, and podophyllotoxin.

Recently, we took sophoridine as the lead compound and successfully identified that the 12N-substituted sophoridinols with a tricyclic core were more favorable than sophoridine with a 4-ring scaffold.^{12,13} The representative compound **1** (Figure 1), 12N-*p*-cholobenzyl sophoridinol, exerted a significantly enhanced anticancer activity in human HepG2 hepatoma cells with an IC₅₀ of 9.3 μ M,^{12,13} much better than sophoridine with an IC₅₀ > 80 μ M. The mechanism of compound **1** was to inhibit DNA topoisomerase I activity and arrest the cell cycle at the G₀/G₁ phase,^{12,13} which is consistent with its parent compound. Especially, this kind of compounds displayed a promising potency against both wild-type and drug-resistant carcinoma cell lines. In addition, it is well-known that autophagy plays a crucial role in the regulation of cancer cell survival. However, it is still unclear how to take effect in tumor cells of this type of compounds through the autophagy mechanism.

In this letter, structure–activity relationship (SAR) study was moved on the variations of the 11-side chain, while 12N-*p*-cholobenzyl was retained as a required group for activity. As depicted in Figure 1, the hydroxyl located at the 11-attachment was replaced with amine group as isosterism of hydroxyl, by which a series of new sophoridinamine derivatives were designed, synthesized, and biologically evaluated for their anticancer activities. In addition, it is well-known that a trimethoxyphenyl or methylenedioxybenzyl group (Figure 1) is

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Taking commercially available sophoridine as the starting material, 19 target compounds were synthesized as described in Schemes 1 and 2, respectively. The sophoridinic ester 2 was

Reaction scheme for the synthesis of 1,2,3,4-tetrahydro-1,4-methanonaphthalene derivatives:

Sophoridine is converted to compound **2** (53-61% yield) via path **b**, or to compounds **7a-b** (35-42% yield) via path **f**.

Compound **2** is converted to compound **9** (49% yield) via path **h**, or to compounds **3a** ($X = \text{CH}_2$, 53-61% yield) and **3b** ($X = \text{SO}_2$, 53-61% yield) via path **b**.

Compound **9** is converted to compound **10** (57% yield) via path **i**.

Compound **3a** is converted to compound **4a** ($X = \text{CH}_2$, 83-86% yield) via path **c**, and compound **3b** is converted to compound **4b** ($X = \text{SO}_2$, 83-86% yield) via path **c**.

Compound **4a** is converted to compound **5a** ($X = \text{CH}_2$, 56-76% yield) via path **d**, and compound **4b** is converted to compound **5b** ($X = \text{SO}_2$, 56-76% yield) via path **d**.

Compound **5a** is converted to compound **6a-h** (61-71% yield) via path **e**.

Compound **7a-b** is converted to compound **8a-b** (35-42% yield) via path **g**.

Definitions of substituents:

- $R_1 = \text{Ph-Cl-p}$ or tert-butyl
- $R_2 = \text{Ph-Cl-p}$, $X = \text{CH}_2, \text{SO}_2$
- $R_3 =$ $n = 0, 1, 2$

However, sophoridinamine **18a–c** could not be directly obtained from **8a** using the similar methods due to the instability of the amide bond at the 12-position. Alternatively, as depicted in [Scheme 2](#), *t*-butyloxycarbonyl was first introduced on the 12*N*-atom of **2** as a protective group to afford **11** with a yield of 83%, which was used in the following

Chemical reaction scheme showing the synthesis of compounds 17a-c and 18a-c from compound 2.

Reaction sequence:

- 2 → 11 (83% yield)
- 11 → 12 (45% yield)
- 12 → 13 (51% yield)
- 13 → 14a-c (39-51% yield)
- 13 → 15a-c (85-91% yield)
- 14a-c → 16a-c (76-82% yield)
- 16a-c → 17a-c (53-72% yield)
- 17a-c → 18a-c (34-51% yield)

Substituents:

- $R_1 = \text{Ph-Cl-IPr}$
- $R_2 =$ (3,4,5-trimethoxyphenyl) group with substituent X

Next, replacing 12*N*-benzyl with 12*N*-benzoyl, a group of 12*N*-*p*-cholobenzoyl sophoridinic ester (**7a**), sophoridinol (**8a**), and sophoridinic amines (**18a–c**) were made and examined. As

Table 1. SAR of All the Compounds for Their Antiproliferative Activities in HepG2 Cells

Compd	R ₁	R ₂	IC ₅₀ (μM)
1	CH ₂ PhCl- <i>p</i>	CH ₂ OH	9.3 ± 0.4
3b	SO ₂ PhCl- <i>p</i>	COOCH ₃	15.8 ± 3.2
4b	SO ₂ PhCl- <i>p</i>	CH ₂ OH	28.8 ± 5.6
6a	CH ₂ PhCl- <i>p</i>		6.4 ± 1.3
6b	CH ₂ PhCl- <i>p</i>		1.7 ± 0.7
6c	CH ₂ PhCl- <i>p</i>		10.6 ± 0.8
6d	CH ₂ PhCl- <i>p</i>		2.6 ± 0.3
6e	CH ₂ PhCl- <i>p</i>		2.6 ± 0.1
6f	SO ₂ PhCl- <i>p</i>		7.9 ± 2.0
6g	SO ₂ PhCl- <i>p</i>		3.8 ± 1.6
6h	SO ₂ PhCl- <i>p</i>		5.5 ± 1.2
7a	COPhCl- <i>p</i>	COOCH ₃	> 50
7b	CO(CH ₃) ₃	COOCH ₃	> 50
8a	COPhCl- <i>p</i>	CH ₂ OH	> 50
8b	CO(CH ₃) ₃	CH ₂ OH	> 50
9		COOCH ₃	> 50
10		COOCH ₃	38.3
18a	COPhCl- <i>p</i>		> 20
18b	COPhCl- <i>p</i>		16.5 ± 5.2
18c	COPhCl- <i>p</i>		> 20
Taxo			
1			15.6 ± 2.3

shown in Table 1, all of them exhibited partially or completely abolished activities, indicating that 12N-benzoyl might not be beneficial for keeping good potency. Then, another group of 12N-benzenesulfonyl compounds, such as 12N-*p*-cholo benze-

nesulfonyl sophoridinic ester (**3b**), sophoridinol (**4b**), and sophoridinic amines (**6f–h**), were also prepared and measured. Compared with the lead compound **1**, compounds **3b** and **4b** showed decreased activity with an IC₅₀ of 15.8 and 28.8 μM, respectively, while compounds **6f–h** possessing an arylidene or arylethyl group at the N'-end exhibited an increased activity with an IC₅₀ from 3.8 to 7.9 μM. Therefore, replacing the CH₂ group in compound **1** with a sulfonyl group, compound **4** attenuated the anticancer potency; while introduction of an arylidene or arylethyl group at the N'-end, compounds **6f–h** offset the negative effect of sulfonyl and showed an increased activity reversely. Therefore, the group at the N'-end is replaceable and introduction of a suitable functional group will enhance their anticancer activity, which is a good strategy for future modification and SAR study.

In addition, *N*-(4-*tert*-butylphenyl)-*N'*-(2-chloroethyl)urea (Figure 1) shows potent anticancer activity, and the chloroethyl urea moiety is the essential functional group for its good activity.¹⁵ Hence, this active group was introduced on the 12-Nitron atom, which offered two new sophoridinic esters **9** and **10**. Unfortunately, both of them lost the activity completely, indicating that the R₁ group may prefer a big bulky group, such as a substituted benzyl group.

As compounds **6b**, **6d**, and **6e** displayed potential inhibition effect on HepG2 hepatoma, their antiproliferative activities were further measured in other solid and liquid human tumor cell lines taking topotecan (TPT) as reference control. As described in Table 2, all of them showed promising potency

Table 2. Antiproliferative Activities (IC₅₀, μM) of the Key Compounds in Two Human Tumor Cell Lines

compd	K562	HMLE
6b	1.03 ± 0.01	0.55 ± 0.04
6d	1.14 ± 0.01	0.64 ± 0.01
6e	1.09 ± 0.02	0.68 ± 0.02
TPT	1.15 ± 0.04	0.75 ± 0.05

against breast cancer (HMLE) and leukemia (K562) with IC₅₀ values in the range of 0.55–1.14 μM. Therefore, all three compounds were chosen for *in vivo* PK investigation to evaluate their drug-like properties.

Single dose PK investigation for compounds **6b**, **6d**, and **6e** was performed in adult male Sprague–Dawley (SD) rats. The tested compound was administered via oral route (25 mg/kg), and nine blood samples (0.3 mL) were collected over a 24 h period. As described in Table 3, compound **6b** exhibited a favorable PK profile with a moderated half-life of 13.0 h and a mean residence time (MRT) of 11.6 h. The area under the concentration–time curve (AUC) and C_{max} of **6b** were 10.8 μM·h and 4.9 μM, respectively. Considering the excellent

Table 3. PK Parameters^a of the Key Compounds in Rats after Single Oral Dosing (n = 3)

compd	t _{max} (h)	C _{max} (μM)	AUC _{0–t} (μM·h)	AUC _{0–∞} (μM·h)	MRT (h)	t _{1/2} (h)
6b	1.6	4.9	10.8	13.3	11.6	13.0
6d	1.2	2.7	5.7	6.8	9.1	11.2
6e	1.8	2.9	6.1	11.7	10.8	21.9

^aPK parameters were calculated by noncompartmental analysis using WinNonlin, version 5.3.

anticancer activity and good PK profiles of compound **6b**, it was selected as the top candidate for further investigation.

To further understand the effect of compound **6b** on autophagy in tumor cells, intracellular autophagosome formation in HepG2 cells was detected using the enhanced green fluorescent protein–microtubule associated protein 1 light chain 3 (EGFP-LC3) as a specific indicator of autophagic vacuoles.²² As shown in Figure 2A, in HepG2 cells transfected

Figure 2. Compound **6b** induces autophagosome accumulation in tumor cells. (A) HepG2 cells were transfected with the EGFP-LC3 plasmid. After 24 h, the cells were incubated without or with **6b** (5 μM) for 6 h and visualized with fluorescence microscopy (upper panel; scale bars, 20 μm). The number of punctate EGFP-LC3 in each cell was counted, and at least 100 cells were included for each group (lower panel). (B) A549 and HepG2 cells were incubated with the indicated concentration of **6b** for 24 h or treated with **6b** (5 μM) for the indicated time. The lipidation of LC3 and the levels of SQSTM1/p62 in both cells were detected by Western blot.

with EGFP-LC3, treatment of **6b** (5 μM) resulted in significant punctate dots in cells, while control cells showed a relative diffuse green fluorescence in the cytosol. Similar results were also obtained in **6b**-treated A549 cells (data not shown). To further confirm the formation of autophagic vesicles, we determined the levels of LC3-II, which is an indicator of autophagosome formation. As expected, LC3-II levels in A549 and HepG2 cells were increased in a dose- and time-dependent manner as depicted in Figure 2B. Notably, **6b** markedly increased the expression of LC3-II as early as 6 h of treatment. These results demonstrated that compound **6b** significantly increased the amount of autophagosomes in tumor cells.

The amount of autophagosomes can be increased by either upstream processes or blockade of lysosomal degradation at a later stage. To discriminate between these two possibilities, we measured the effect of **6b** on autophagic flux in tumor cells, taking p62/SQSTM1 level as a marker for autophagic flux.²³ As shown in Figure 2B, p62/SQSTM1 levels increased in a concentration- and time-dependent manner in **6b**-treated A549 and HepG2 cells, indicating **6b**-induced accumulation of autophagosomes resulted from inhibition of their degradation. These results were further confirmed by LC3 turnover assays in A549 and HepG2 cells with rapamycin (200 nM) as a positive control. As expected (Figure 3A), we observed that chloroquine (CQ) treatment had no effect on LC3-II levels in **6b**-treated

Figure 3. Compound **6b** blocks autophagic flux in tumor cells. (A) A549 and HepG2 cells were pretreated with CQ (10 μM) or rapamycin (200 nM) for 2 h, followed by **6b** (5 μM) treatment for 8 h. The LC3 turnover in both cells was detected by Western blot. (B) HepG2 cells were transfected with mCherry-EGFP-LC3 plasmid, followed by treatment with **6b** (5 μM) or CQ (50 μM) for 6 h. Representative fluorescent images are visualized with confocal microscopy (original magnification ×63, scale bars, 20 μm). DAPI was used to visualize the nuclei. (C) Quantification of GFP/mCherry double-positive and mCherry single-positive puncta per cell in control or cells treated with CQ or **6b**. Data were the mean value of three independent experiments with each count of no less than 100 cells. Values are expressed as the mean ± SD, *p < 0.05, **p < 0.01 vs untreated control.

cells. However, in rapamycin-treated cells, cotreatment with **6b** increased LC3-II levels.

To further demonstrate that compound **6b** blocks autophagic flux in tumor cells, we transfected HepG2 cells with a marker protein, mCherry-EGFP-LC3 tandem tagged fluorescent protein, and analyzed them by confocal microscopy. After treatment of the cells with **6b** (5 μM) for 6 h, yellow punctate fluorescence was markedly increased, indicating a blockade of autophagy in **6b**-treated cells (Figure 3B,C). Taken together, these results suggested that **6b** suppressed the degradation of autophagosomes, resulting in their accumulation in the cytosol.

As compound **6b** inhibited the degradation stage of autophagy, it may be involved in the function of the lysosomes.²⁴ Since acidic pH is required for lysosomal activity, we therefore evaluated lysosomal acidification upon **6b** treatment using LysoTracker Red. As shown in Figure 4A,B, the LysoTracker Red signal in **6b**-treated or CQ-treated HepG2 cells was significantly reduced, compared with that in untreated control, indicating **6b** decreased lysosomal acidification. To further explore the link between the inhibition of autophagy flux and cell viability by **6b**, we next determined whether lysosomal acidification manipulated cell growth. As depicted in Figure 4C, both CQ and **6b** reduced cell viability by about 50%, suggesting that compound **6b** is a potent lysosomal deacidification agent and is accordingly able to inhibit tumor cell growth. Targeting autophagy flux by **6b** may thus provide a novel therapeutic option in the treatment of cancer.

A total of 19 new sophoridinamine derivatives were synthesized and evaluated for their antitumor activities in human HepG2 cells. SAR analysis indicated that introduction of a suitable substituent on the N'-end, such as trimethoxybenzyl and methylenedioxybenzyl, could greatly improve

Figure 4. Compound **6b** prevents lysosomal acidification in tumor cells. (A) HepG2 cells were incubated with **6b** (5 μ M) or CQ (50 μ M) for 6 h, followed by staining with LysoTracker Red (Lys), and cells were imaged by confocal microscopy (original magnification $\times 63$, scale bars, 20 μ m). White arrows indicate acidified lysosomal puncta. DAPI was used to visualize the nuclei. (B) Quantification of LysoTracker Red signal per cell in control or cells treated with **6b**. (C) HepG2 cells were treated with CQ (50 μ M) or **6b** (5 μ M) for the indicated time, cell viability was determined by MTT assay. ** $p < 0.01$ vs untreated control.

activity. Among them, compound **6b** displayed a potent antitumor activity against three human carcinoma cell lines with IC₅₀ ranging from 0.55 to 1.7 μ M. The underlying antitumor mechanism showed that compound **6b** severely suppressed lysosomal acidification, impaired the lysosomal function of cancer cells, and caused a blockade of autophagic flux, thereby leading to tumor cell death. Our results indicated that **6b** is a potent lysosomal deacidification agent that blocks autophagic flux for developing new candidates against tumor.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00466.

Synthetic procedure, analytical data, biological assay, and PK evaluation (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PK, pharmacokinetic; SAR, structure–activity relationship; MTT, tetrazolium bromide; CQ, chloroquine

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