Bioorganic & Medicinal Chemistry Letters 25 (2015) 363–366

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and biological evaluation of norcantharidin derivatives as protein phosphatase-1 inhibitors

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ARTICLE INFO

Article history:
Received 31 July 2014
Revised 27 October 2014
Accepted 10 November 2014
Available online 18 November 2014

Keywords:
Norcantharidin
Cytotoxicity
Protein phosphatase-1
Microtubules

ABSTRACT

Cantharidin and norcantharidin display anticancer activity against a broad range of tumor cell lines. In this study, we have synthesized a series of norcantharidin derivatives and evaluated their cytotoxic effects on four human tumor cell lines together with the genetically normal human diploid fibroblast line WI-38. One of our compounds (1*S*,4*R*)-3-((4-(4-(4-fluorophenyl)piperazin-1-ylsulfonyl) phenyl)carbamoyl)-7-oxa-bicyclo[2.2.1]heptane-2-carboxylic acid (**12**) exhibited potent cytotoxic effects on the tumor cell lines A-549, HepG2, HeLa, and HCT-8, whereas it was less toxic to WI-38 cells than its parent compound, norcantharidin. In addition, this compound inhibited protein phosphatase-1 activity and microtubule formation in HeLa cells, and it also interacts with calf thymus DNA.

Cantharidin (*exo*,*exo*-bicyclo[2.2.1]heptane 2,3-dicarboxylic acid anhydride) (CTD, **1**) is the principle active ingredient of *Epicanta gorhami* or *Mylabris* (blister beetles), a traditional Chinese medicine that has been used to treat liver, lung and digestive tract tumors. However its applications are limited by its severe nephrotoxic and inflammatory side effects, and it has mainly been used to treat the gastrointestinal tract, the ureter, and the kidney.[1,2] A series of bioactive analogues of cantharidin have been synthesized in an attempt to reduce its toxicity and thereby increase its use.[3,4] Norcantharidin (NCTD, **2**, Fig. 1), the demethylated analogue of cantharidin, appears to cause the least nephrotoxic and inflammatory side effects. NCTD is active in vitro against several tumor cell lines, including cervical, hepatoma, ovarian, laryngocarcinoma, colon, osteocarcinoma, and leukemia cell lines.[1,5,6] NCTD has also been used in vivo in the treatment of primary hepatoma, oesophageal, gastric and cardiac carcinomas.[1] The reported anticancer mechanisms of NCTD include interruption of DNA replication, retardation of cell cycle progression, and induction of apoptosis via the regulation of p53 and Bcl-2 gene expression.[7,8]

Serine/threonine protein phosphatases (S/T-PPs) are thought to be cancer suppressive, since the inhibition of the S/T-PPs can lead to an increased phosphorylation and hence the activation of substrate kinases. The activation of some of these kinases has been associated with the acceleration of cell growth.[9] The anticancer activity of CTD and NCTD are thought to come from the inhibition of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), two phosphatases that are known to be involved in many different cellular processes, including DNA damage, cell cycle arrest, and apoptosis.[10–12]

Recently, a series of NCTD analogues have been generated and shown to exhibit a greater antiproliferative potency, with only a modest PP1 inhibition, compared with that of NCTD.[13–15] In this study, which is part of our continuing effort to find new natural product-based compounds with potent activities and minimal side effects,[16–19] we have synthesized a series of norcantharidin derivatives and evaluated their cytotoxicities in vitro against four human tumor cell lines, together with a human lung fibroblast cell line. The most potent compound, **12**, was further evaluated for its suppression of protein phosphatase-1, its interaction with calf thymus DNA (CT DNA), and its inhibition of microtubule formation.

The synthesis of compounds **6a–c** and **9a–c** proved to be straightforward by following standard procedures for sulfonamide analogues, as illustrated in Scheme 1. For example, a combination of 4-nitro-benzenesulfonyl chloride with compounds **4a–c** afforded the 4-nitro-benzenesulfonamides **5a–c**. These were reduced by hydrogenation over 10% Pd/C to provide the 4-amino-benzenesulfonamides **6a–c**, with good yields.[20]

The synthesis of compounds **11–20** was carried out according to the procedure outlined in Scheme 2. First, the key starting material in this work was the readily synthesizable 5,6-dehydronorcantharidin **10**, which was prepared on a large scale through the

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http://dx.doi.org/10.1016/j.bmcl.2014.11.032
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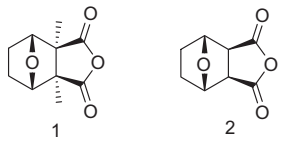
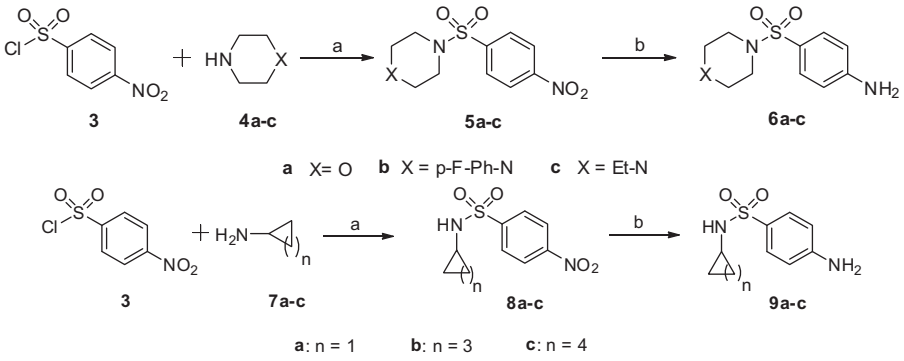


Figure 1. The structures of cantharidin (**1**) and norcantharidin (**2**).

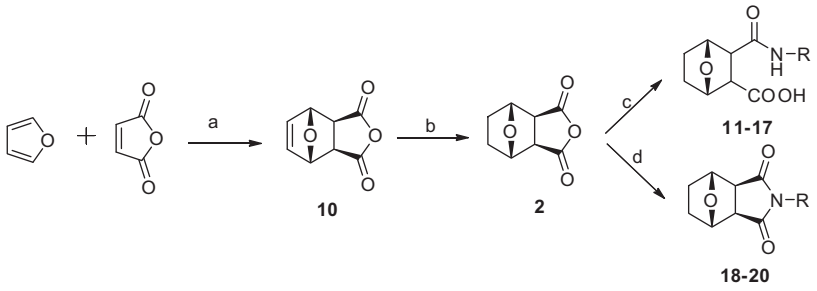
exo-selective Diels–Alder addition of furan to maleic anhydride.[21,22] Subsequently, NCTD was obtained from 5,6-dehydronorcantharidin via simple hydrogenation, as described in our previous publication.[22] Additionally, treatment of NCTD, in THF at room temperature, with a series of substituted benzenesulfonic amides afforded the corresponding amide–acid analogues **11**–**17** with good yields.[14c] Simultaneously, the treatment of NCTD with an appropriate primary amine and triethylamine facilitated a condensation reaction to provide a series of cyclic imides **18**–**20** with moderate yields.[23] The structures of compounds **11**–**20** were identified by IR, ^{1}H NMR spectra, ^{13}C NMR spectra and high resolution mass spectrometry (HRMS).

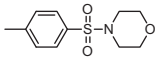
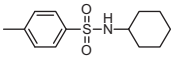
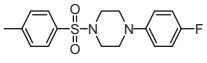
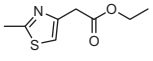
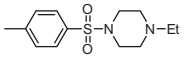
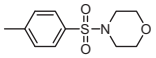
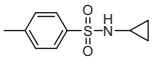
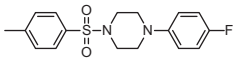
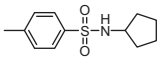
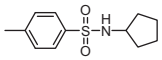
The in vitro cytotoxicity of the norcantharidin derivatives **11**–**20** were evaluated against a panel of four human cancer cell lines (A-549, HepG2, HeLa, and HCT-8) together with the human lung fibroblast cell line WI-38, using NCTD as a reference compound. The screening procedure was based on the standard MTT method,[24] and the IC_{50} values are summarized in Table 1.

Generally, most of the target compounds exhibited moderated cytotoxicities in vitro against the tumor cell lines A-549, HepG2, HeLa, and HCT-8. As previously published results,[8] the ring-opened acid amides (**11**, **12** and **15**) were more cytotoxic than the ring-closed norcantharimides (**18**, **19** and **20**). The IC_{50} values for compound **12**, which was the most promising of the ring-opened acid amides, were 18.4 ± 1.5, 14.4 ± 0.9, 6.3 ± 1.7, and 16.6 ± 1.2 μM



Scheme 1. Synthesis of compounds **6a**–**c** and **9a**–**c**. Reagents and conditions: (a) Py/CH_2Cl_2; (b) Pd/C (10%), H_2 (50 psi), 1 h.



Compds	R	Compds	R
11		**16**	
12		**17**	
13		**18**	
14		**19**	
15		**20**	

Scheme 2. Synthesis of compounds **11**–**20**. Reagents and conditions: (a) Et_2O, rt, 48 h; (b) H_2, 10% Pd–C, rt, EtOH; (c) RNH_2, THF, rt, overnight; (d) RNH_2, toluene, reflux, 36 h.

Table 1
The cytotoxicity (IC_{50}, μM)[a] and log*P* of compounds **2** and **11**–**20**

Compds	Tumor cell				Normal cell	Log*P*
	A549[b]	HepG2[b]	HeLa[b]	HCT-8[b]	WI-38[b]	
2	12.1 ± 0.8	24.7 ± 1.2	12.4 ± 0.6	28.4 ± 0.9	26.7 ± 2.1	0.7
11	24.2 ± 2.1	19.3 ± 0.5	17.2±1.5	31.3 ± 1.6	48.8 ± 2.6	0.4
12	18.4 ± 1.5	14.4 ± 0.9	6.3 ± 1.7	16.6 ± 1.2	50.2 ± 3.2	0.6
13	28.4 ± 1.6	20.2 ± 1.5	15.7 ± 2.8	27.4 ± 2.5	73.1 ± 2.2	0.4
14	34.3 ± 1.0	26.2 ± 2.6	20.9 ± 1.5	28.3 ± 2.0	52.1 ± 1.4	0.2
15	20.1 ± 3.2	22.3 ± 2.1	17.6 ± 0.9	30.2 ± 3.5	59.0 ± 3.3	0.2
16	36.2 ± 1.7	22.5 ± 1.3	18.7 ± 2.2	31.0 ± 2.6	62.5 ± 4.0	0.2
17	>100	>100	>100	>100	>100	0.8
18	39.2 ± 2.2	28.7 ± 1.3	16.3 ± 2.1	43.5 ± 3.5	52.1 ± 3.3	0.9
19	25.8 ± 1.6	25.6 ± 1.8	18.6 ± 1.8	32.1 ± 1.0	51.8 ± 3.3	1.4
20	32.5 ± 1.3	23.5 ± 1.6	20.67 ± 3.2	32.8 ± 2.2	40.3 ± 2.3	1.1

[a] IC_{50} values are presented as the means ± SD of triplicate experiments.
[b] MTT method.

for the A-549, HepG2, HeLa, and HCT-8 tumor cells, respectively. Whereas compound **12** was less toxic than its parent compound NCTD to the normal human lung fibroblast cell WI-38, the CC_{50} values for compound **12** and NCTD were 50.2 ± 3.2 and 26.7 ± 2.1 μM for WI-38, respectively. The selectivity index (SI = CC_{50} for WI-38 cell line/IC_{50} for HeLa cell line) for compound **12** was 8.0. Interestingly, we found that compound **17**, in which the 4-amino-benzenesulfonamides was substituted with ethyl 2-aminothiazole-5-carboxylate, showed much less active than its parent compound NCTD for these four tumor cell lines. Furthermore, the octanol–water partition coefficients of **11**–**20** were also determined to evaluate the water-solubility of target compounds. The partition coefficient log*P* (Table 1) indicated that most compounds exhibited better water-solubility than that of NCTD, and that the ring-closed compounds **18**–**20** showed less water-solubility than those of ring-opened acid amides **11**–**17**. These results indicated that compound **12** might serve as a potential anticancer drug, and we choose the HeLa cell line to further investigate the mechanism of its cytotoxic effect.

CTD and NCTD are potent inhibitors of PP1 and PP2A.[12] To determine whether compound **12** inhibits the PP1, we evaluated the suppressive effects of compounds **12**, **19** and NCTD on PP1.[16] As shown in Figure 2A, compound **12** displayed more potent inhibitory activity against PP1 than compound **19** or NCTD, the IC_{50} values of compounds **12**, **19** and NCTD for PP1 were 0.2 ± 0.6, 2.3 ± 1.0 and 2.6 ± 0.7 μM, respectively. We also observed that the inhibition of PP1 by compound **12** was in dose-dependent effect (Fig. 2B). These data indicate that compound **12** may represent a potential PP1 inhibitor. The cyclic imide compound **19** may bound to the catalytic domain of the protein phosphatase 5 (PP5c) other than PP1 inhibitory activity.[25]

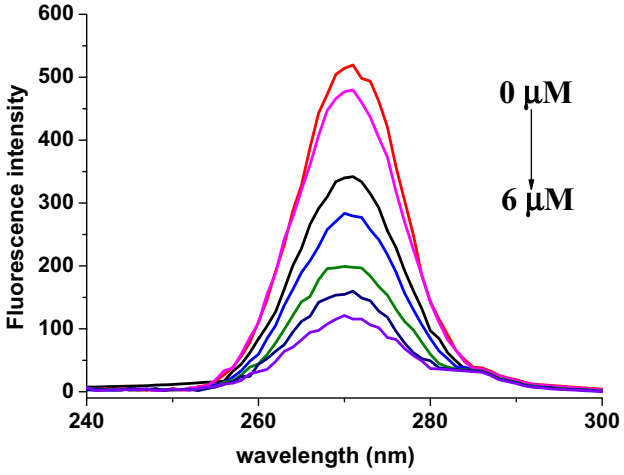


Figure 3. Effects of compound **12** on CT DNA. Fluorescence spectra of **12** (1 μM) by increasing concentrations of CT DNA (0–6 μM).

DNA is the important pharmacological target of antitumor drugs. It is therefore essential to the development of effective chemotherapeutic agents to explore their interaction with DNA. A binding assay of compound **12** with CT DNA was performed by monitoring changes in the fluorescence spectroscopy emission pattern of compound **12** (1 μM, excited at 264 nm) with increasing concentrations of CT DNA (0–6 μM) in pH 7.2 PBS.[26] As shown in Figure 3, the fluorescence intensity of the complex decreases progressively with increasing concentration of DNA, suggesting that compound **12** is bound to DNA.

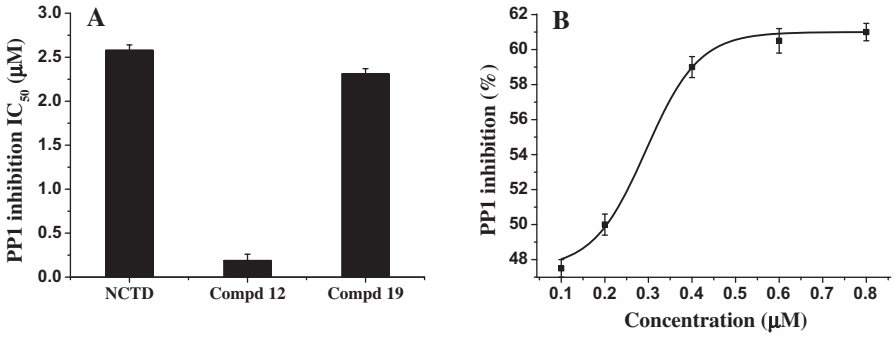


Figure 2. (A) Inhibition of protein phosphatase-1 (PP1) by NCTD, **12** and **19**, data are presented as the means ± SD of triplicate experiments; (B) PP1 was treated by compound **12** with different concentration (0.1, 0.2, 0.4, 0.6, 0.8 μM), a dose–response curve of percentage enzyme inhibition activity versus drug concentration. $P < 0.01$ versus DMSO group.

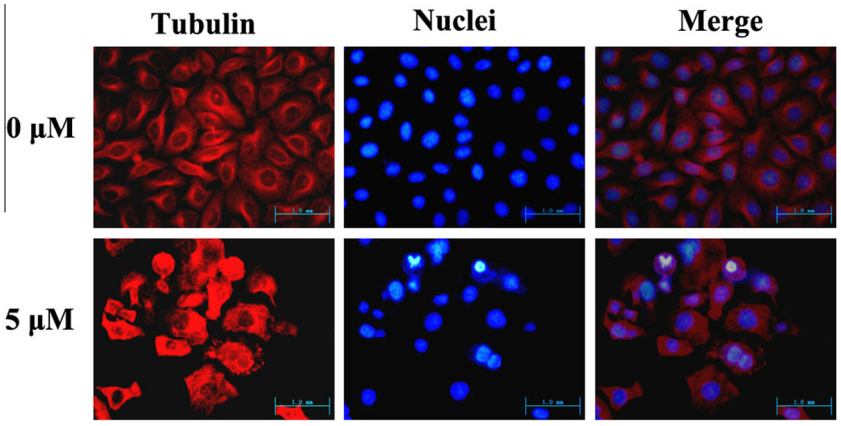


Figure 4. Effects of compound **12** on the formation of microtubule. HeLa cells were treated with **12** (0 μM and 5 μM) for 24 h. Microtubules (red) were stained with an anti-β-tubulin antibody and a Rhodamine (TRITC)-conjugated secondary antibody. Chromosomal DNA (blue) was stained with DAPI.

Cytoskeleton proteins, such as actin filaments and microtubules, are potential targets for cancer chemotherapy.[27] The morphology of non-treated and compound **12**-treated HeLa cells was examined by means of indirect immunofluorescence using an anti-β-tubulin antibody to stain microtubules and a 4′,6-diamidino-2-phenylindole (DAPI) staining of the nuclei.[28] As shown in Figure 4, the non-treated cells were elongated, and staining of thin bundles of microtubules was observed throughout the cytoplasm. In contrast, cells treated with compound **12** for 24 h became round and contained short, dense microtubule networks; we also observed cell shrinkage and nuclear fragmentation and condensation dispersed throughout the cytoplasm, hallmarks of apoptosis.

In summary, we introduced benzene sulfonamides groups into the norcantharidin chemotype for the design and discovery of novel anticancer drugs. The biological results revealed that a 4-(4-(4-fluorophenyl)piperazin-1-ylsulfonyl)benzenamine group was favored for increased cytotoxicity and decrease toxicity of the ring-opened acid of norcantharidin. Taken together, the cytotoxicity, protein phosphatase-1 inhibition, DNA interaction and the inhibition of microtubule formation in HeLa cells suggest that compound **12** might be suitable as a potential drug candidate, a PP1 inhibitor for cancer chemotherapy.

Acknowledgments

Financial support from National Natural Science Foundation of China (Nos. 21372110, 81372177), and Natural Science Foundation of Gansu Province (No. 1208RJZA247) are gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.11.032.

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