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Discovery of degradable niclosamide derivatives able to specially inhibit small cell lung cancer (SCLC)

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ABSTRACT

Small cell lung cancer (SCLC) is exceedingly tough to treat and easy to develop resistance upon long use of the first-line drug carboplatin or radiotherapy. Novel medicines effective and specific against SCLC are greatly needed. Herein, we focused on the discovery of such a medicine by exploring a drug niclosamide with repurposing strategy. Initial screening efforts revealed that niclosamide, an anthelmintic drug, possessed the *in vitro* anticancer activity and an obvious sensitivity towards SCLC. This observation inspired the evaluation for two different kinds of niclosamide derivatives. **2** with a degradable ester as a linker exhibited the comparable activity but slightly inferior selectivity to SCLC, by contrast, the cytotoxicities of **4** and **5** with non-degradable ether linkages completely disappeared, clearly validating the importance of 2-free hydroxyl group or 2-hydroxyl group released in the antitumor activity. Mechanism study unfolded that, similar to niclosamide, **2** inhibited growth of cancer cells *via* p 53 activation and subsequent underwent cytochrome *c* dependent apoptosis. Further structural modification to afford phosphate sodium **8** with significantly enhanced aqueous solubility (22.1 mg/mL) and a good selectivity towards SCLC demonstrated more promising druggability profiles. Accordingly, niclosamide as an attractive lead hold a huge potential for developing targeted anti-SCLC drugs.

1. Introduction

Small cell lung cancer (SCLC), a heterogeneous and genetically complex disease, is an aggressive neuroendocrine lung neoplasm, accounting for approximately 15–20% of all lung cancers [1]. It comprises limited stage (LS) disease with tumor confined to one hemithorax, and extensive stage (ES) disease with metastasis beyond one hemithorax [2]. Clinically, concurrent chemo-radiotherapy is a main remedy for LS disease, while chemotherapy alone is used to treat ES disease [2]. In comparison to overall 5-year survival rates of 16% for non-small cell lung cancer (NSCLC), the best currently available therapies for patients suffering from SCLC achieve only 6%, [3] emphasizing the complexity of SCLC and the urgent need for developing anti-SCLC drugs.

Platinum-containing agents always served as the first-line treatment drugs for SCLC. Long exposure to carboplatin posed a “refractory” SCLC with an extremely poor prognosis, demanding participant of the second-line chemotherapy [4]. Toptecan, clophosphamide, doxorubincin and vincristine were reported as the second-line SCLC’s drugs [5]. Additionally, a plethora of targeted agents alone or in combination with conventional chemotherapy in the treatment of SCLC were widely studied. However, various targeted tyrosine kinase inhibitors (TKIs) including EGFR TKIs, BCR-ABL TKIs, as well as targeted mTOR inhibitors, had failed to demonstrate a survival advantage in SCLC [2,6]. More disappointingly, clinical antiangiogenic agents, such as bevacizumab, thalidomide and sorafenib, had no improvements on overall survival (OS) [7]. Therefore, the limitations of current chemotherapy drugs necessitated the continuous exploration of novel chemotherapeutic agents.

Niclosamide (5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide, **1**) was an FDA-approved salicylanilide derivative to treat

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tapeworm infections [8]. With an increase in drug repurposing initiatives, niclosamide had emerged as a true lead in the screening campaign against a large cohort of human cancers [9–13]. Growth inhibition of various cancer cells and reversal of drug resistance [12] were results of niclosamide's ability to regulate a selection of signaling pathways [14–18] and to induce mitochondrial-dependent uncoupling apoptosis [19]. Niclosamide eliminated radio-resistance in the lung cancer cells and the corresponding xenograft tumors *via* the inhibition of signal transducer and activator of transcription 3 (STAT3) and nuclear localization [12]. The similar result was observed that niclosamide exhibited synergistic effects on cisplatin-resistant lung cancer cells [20–22]. Recently, mitochondrial-dependent uncoupling apoptosis imposed by niclosamide rendered an unexpected metabolic vulnerability to selectively killing p53-defective cancer cells, [19] suggesting that p53 was regarded as a new potential biomark for precision treatment of niclosamide and its derivatives. To be taken together, these observed anticancer activities and resistance reversal supported the fact that nicosamide was an attractive anticancer therapeutic agent implicated in the inhibition of tumor growth and elimination of drug resistance. [23,24]

Although niclosamide hold a great potential in inhibiting the sensitive/resistant NSCLC[14–19], little attention was paid in the treatment of SCLC. As reported, [15] underlying side effects and very poor water solubility largely compromised the development of niclosamide as an antitumor drug. It had been demonstrated that niclosamide exhibited many drug repurposes, [15] hinting other unpredictable bioactivities probably leading to severe side effects. Besides, extremely poor aqueous solubility of niclosamide was only amendable to treating tapeworm infections in the gut, but seriously impeded the possibility of developing an antitumor drug. In this study, we proposed a new paradigm for targeting SCLC according to niclosamide's molecular structure in the anticipation of potentially maintaining the antitumor activity and selectivity but improving water solubility. Attachment of three typical substituents to 2-hydroxyl group of niclosamide, bearing an ester or an ether as a linkage, was used to explore a potential modification direction, and then their *in vitro* and *in vivo* antitumor activities towards SCLC were evaluated, accompanying with the explanation for a possible mechanism, and ultimately various water-soluble niclosamide derivatives with a good selectivity toward SCLC were developed by further structural modifications.

2. Results and discussion

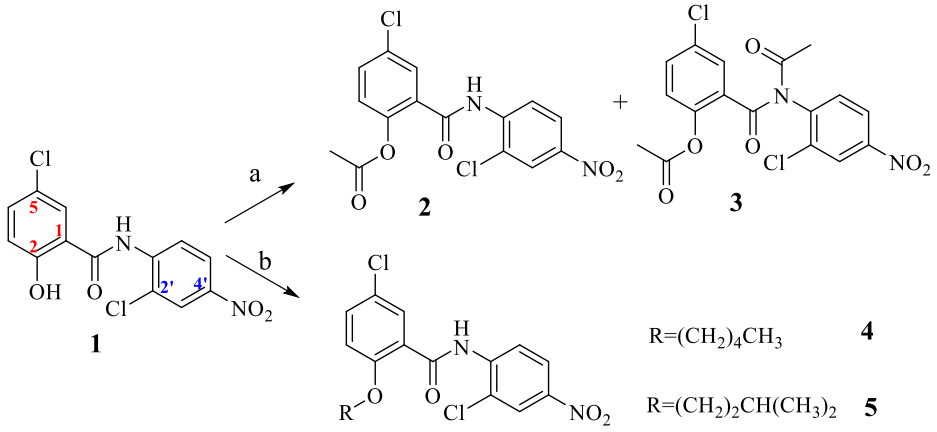
2.1. Chemistry

In the synthesis of three typically modified niclosamide derivatives **2**, **4** and **5** (Scheme 1), commercially available niclosamide **1** as the starting material was converted into acetate **2** in the presence of acetyl chloride[25] or acetic anhydride, [26] but the yield was unsatisfactory due to the generation of diacetate **3**. A modified procedure for synthesizing **2** was conducted that niclosamide was acylated with excessive acetic anhydride in anhydrous DMF at 0 °C, and only little diacetate **3** was observed. Ethers **4** and **5** were prepared by condensation of 2-hydroxyl group of niclosamide with the corresponding brominated alkanes [27]. As illustrated in Scheme 1, in the presence of potassium carbonate, potassium iodide and tetrabutyl ammonium bromide (TBAB), etherification of niclosamide with 1-brompentane and isoamylbromide afforded ethers **4** and **5** in 35.4% and 36.6% yields, respectively.

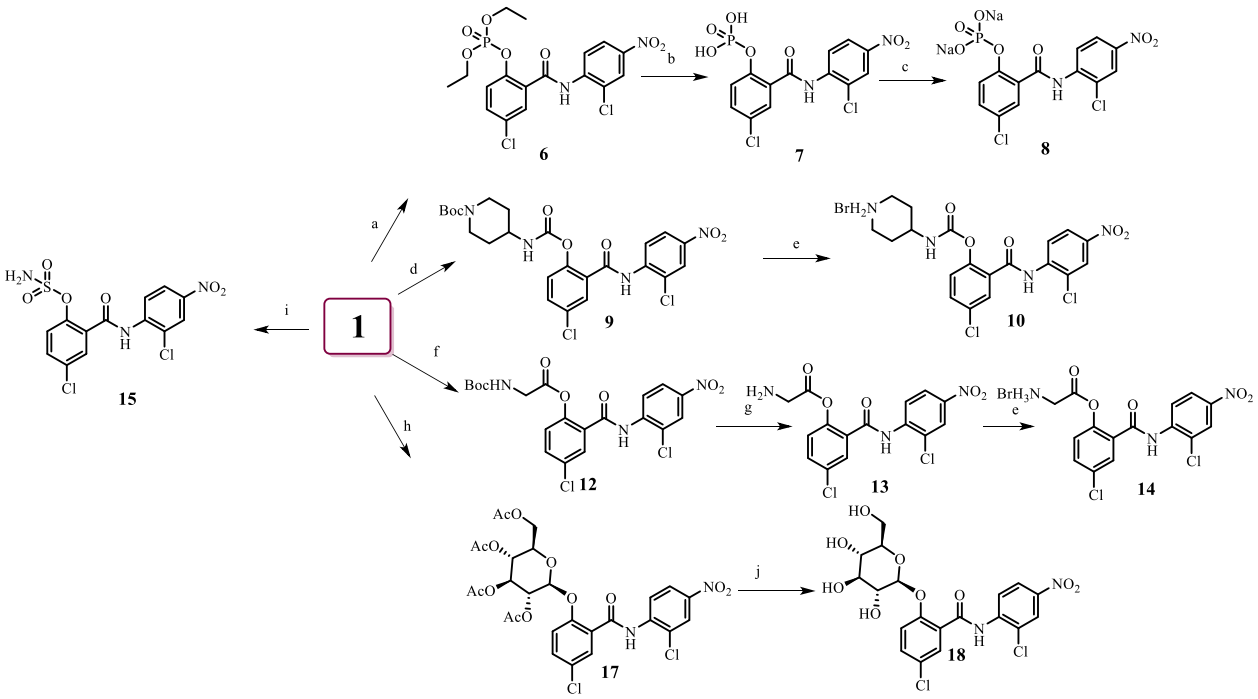
To solve the issue of niclosamide's poor aqueous solubility and its resulting low bioavailability, five kinds of widely accepted strategies towards improvements on drug's water solubility listed in Scheme 2 were studied. Diethyl **6** was obtained by condensation of niclosamide with diethyl phospite followed by hydrolysis with Me_3SiBr to provide phosphate **7** in a high yield, and subsequent treatment of **7** with sodium hydroxide in anhydrous methanol yielded disodium **8** [28,29]. Exposure to diisopropylethylamine (DIPEA) and dimethylaminopyridine (DMAP), incorporation of commercially available tertbutyl-4-aminopiperidine-1-carboxylate hydrochloride to niclosamide was activated by triphosgene to produce construct **9**, which was further converted into bromide **10** with 33.0% HBr-AcOH in a 72.0% yield [30]. In the presence of dicyclohexylcarbodiimide (DCC) and DMAP, tertbutyl (2-hydroxyethyl)-carbamate **11** prepared previously, [31] was coupled with niclosamide to afford construct **12**, and subsequent deprotection with trifluoroacetic acid (TFA) in DCM produced amine **13**. The similar operation as **10** was introduced to synthesize bromide **14**. Direct reaction of niclosamide with chlorosulfonyl isocyanate yielded sulfanilamide **15** at 50 °C [32]. Coupling of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **16** with niclosamide generated acetyl-protected **17** with a yield of 56.2%, [33] and deacetylation of **17** with a catalytic amount of sodium methoxide in anhydrous methanol followed by acidification with Dowex 50 (H^+) resins afforded glucose-coupled **18**, [34] with the yield demonstrating absolutely advantages over the reported synthetic method. [35]

2.2. Biological evaluation

The *in vitro* antitumor activities of all the newly synthesized niclosamide derivatives were assessed in comparison with a first-line drug carboplatin and the lead niclosamide **1** against three cancer cell lines including NSCLC (A549), SCLC (NCI-H446), human T lymphoblasts cell line (Jurkat) and one normal cell line (human bronchial epithelial cell, HBE) (Table 1). Carboplatin, a first-line therapy for SCLC, was used a



Scheme 1. Reagents and Conditions: a). Ac2O, DMAP, DMF, 0 oC, yield, 88.2% for 2, 3.3% for 3; b).K2CO3, KI, (Bu)4NBr, 1-brompentane for 4, yield, 35.4%, isoamyl bromide for 5, yield, 36.6%.



Scheme 2. Reagents and Conditions: a). i, diethyl phosphite, DIPEA, DMAP, ii, CCl4, DMF, 0 oC, 96.2%; b) Me3SiBr, DCM, 0 oC, yield, 99.6%; c). NaOH, CH3OH, yield, 86.5%; d). i, tertbutyl-4-aminopiperidine-1-carboxylate hydrochloride, DIPEA, ii, triphosgene, 0 oC, 21.5%; e). 33% HBr-AcOH, DCM, 72.0%; f). tertbutyl(2-hydroxyethyl)-carbamate 11, DCC, DMAP, yield, 57.0%; g) TFA, DCM, yield, 81.0%; i) chlorosulfonyl isocyanate, 50 oC, yield, 76.0%; h)0.2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (16), TBAB, Cs2CO3, yield, 56.2%; j) NaOMe, anhydrous MeOH, yield, 82.7%.

Table 1
The *in vitro* antitumor activities of niclosamide derivatives against three cancer cell lines (A549, NCI-H446, Jurkat) and one normal cell line (HBE).

Compds. no.	R	IC_{50} (μM)[a]				
		A549	NCI-H446	Jurkat	HBE	SI[c]
1	H	7.70	0.96	1.8	6.42	8.0
2	$COCH_3$	4.13	0.92	1.5	5.76	4.5
4	$(CH_2)_4CH_3$	>100[b]	>100	>100	>100	/
5	$(CH_2)_2CH(CH_3)_2$	>100	>100	>100	>100	/
	Carboplatin	22.2	37.40	44.66	>100	0.59

[a] indicates the average value of the three independent experiments
[b] indicates that more > 100 μM is ineffective, SI means selectivity index
[c] indicates that SI is a ratio of IC_{50} (A549)/IC_{50} (NCI-H446).

positive compound [4]. An IC_{50} value was the expression for the *in vitro* antitumor activity, and it meant the half-maximal inhibitory concentration of a compound to inhibit cell growth.

To ascertain whether 2-hydroxyl group of niclosamide as a potential modification direction or not, three typical niclosamide derivatives (**2**, **4** and **5**) were explored using a MTT-based cell assay. Noticeably, although acetate **2** was reported be effective in inhibiting plentiful of cancer cells [36,37], the involvement of its specificity toward SCLC and its selectivity between SCLC and NSCLC remained blank. As listed in Table 1, among three cancer cell lines and one normal cell line, the lead compound niclosamide displayed obvious selectivity toward SCLC cells NCI-H446 (IC_{50} = 0.96 μM) with a SI (selectivity index) of 8.0, indicating the great potential of niclosamide as a true lead in developing anti-SCLC drugs with high specificity. However, the first-line drug carboplatin exhibited a preference for A549 cells (SI = 0.45). Modification on 2-OH of niclosamide with acetyl (**2**), had no influence on anti-SCLC, whereas the cytotoxicity against A549 (IC_{50} = 4.13 μM) increased by twofold in comparison with niclosamide (IC_{50} = 7.70 μM), giving rise to a certain decrease in the SI of **2** (SI = 4.5). When acetyl was replaced with pentyl (**3**) and isoamyl (**4**), respectively, the cytotoxicities against all the tested cancer cell lines sharply disappeared (IC_{50} > 100 μM). This obvious difference

was possibly ascribed to the linkage modes: **2** with a degradable ester was able to release the parent compound niclosamide in the presence of a certain esterase, while **4** and **5** bearing a non-degradable ether fail. From these *in vitro* observations, niclosamide indeed hold a great sensitivity toward SCLC and free 2-hydroxyl was an essential pharmacophore to the antitumor activities, obviously implying the importance of 2-OH released, or not, in the antitumor activity.

To confirm the *in vivo* efficacy of **2** whether was in accordance with *in vitro* observation, BALB/c mice suffering from SCLC NCI-H69 was used as an animal evaluation model. Twenty-five female mice tested were randomly divided into five groups, and each group had five mice. The first-line drug carboplatin and PBS were served as a positive control and a negative one, respectively. As shown in Table 2, although, compared with the positive compound carboplatin (47.3%), **2** administrated with 50 mg/kg/d intragastrically exhibited a slightly inferior antitumor activity with a TGI of 35.6%, no overt toxicity was apparently observed. Moreover, at the same dosing (50 mg/kg/d), the antitumor potency of **2** was comparable to that of the lead niclosamide **1** in inhibiting growth of

Table 2
In vivo antitumor activity of niclosamide derivative **2** and carboplatin.

Group Name	Administration route	Size of animals	Dosage (mg/kg/d)	Animal numbers left on Day 29	TGI[a] (%)
N.C	i.g.	5	/	5	0
Carboplatin	i.g.	5	6	5	47.3
2	i.g.	5	50	5	35.6
1	i.g.	5	50	5	32.4
2	i.p.	5	50	4	/

i.g. means intragastrically; i.p. means intraperitoneally; TGI means tumor growth inhibition, which is equal to a ratio of weight of treatment group to weight of control group.

tumor. Anatomically, most of **2** or **1** was accumulated in the gut as solid powders. This was a good explanation for high dosing (50 mg/ kg/d) but unintended effect. When administrated intraperitoneally, one mouse died due to excessive insoluble solid **2** accumulated in all the organs of abdomen. Noticeably, no efficacy was found when treated with compound **4** or **5** at the same dose (data not offered). Collectively, **2**

displayed the *in vivo* antitumor activity in line with its *in vitro* one, but it was severely compromised by poor aqueous solubility, even leading to unexpected toxicity when administrated intraperitoneally.

Mutation in p53 prevailed across a variety of human cancers. Niclosamide was showed to trigger optimal protective p53 activation while inducing cytotoxicity, as a result, growth of p53-deficient cells and of p53 mutant patient-derived ovarian xenografts was significantly impaired[19] To determine whether **2** induced p53 activation and exerted the anti-SCLC activity or not, the expression of p53 protein in SCLC (NCI-H446) as a tested cell model was explored. As demonstrated in Fig. 1, NCI-H446 cells did not belong to p53-deficient cells, but a significant increase of p53 protein was observed when to be fully exposed to **2**, possibly indicating the direct association of p53 activation and antitumor activity. Moreover, the inducing effect on p53 in NCI-H446 was comparable to that of the template compound niclosamide **1**. As reported [19], 2-free hydroxyl group of niclosamide was an essential pharmacophore in sensitizing p53 knockout cells. A niclosamide derivative with a methoxy group (–OCH_3) instead of a hydroxyl one (2-OH) had no effect on growth of either wildtype or p53-deficient cells even at high concentrations [19]. This was a reasonable explanation for our results that compounds **4** and **5** failed to release 2-OH and sharply damaged the antitumor activities (Table 1). Moreover, p53 activation of **2** was probably due to cleavage of an acetyl group from **2**. In addition, we further extended our analysis to cytochrome *c* (Cyt *c*) associated with cell apoptosis and heat shock protein (Hsp90) responsible for controlling distinct client proteins *via* protein folding [19,38]. A significant increase in Cyt *c* release in response to **2** and **1** was observed (Fig. 1), but leaving Hsp90 minimally affected. This supported the possibility that **2** inhibited growth of NCI-H446 *via* p53 activation and subsequent underwent cytochrome *c* dependent cell apoptosis, without participation of molecular chaperone Hsp90.

From the *in vivo* biological assessment of **2**, the hypothesis was further supported that the low aqueous solubility placed severe limits on its antitumor efficacy. Poor aqueous solubility and the resulting low bioavailability indeed posed a huge obstacle to developing anticancer medications from niclosamide as a lead. To exert their bioactivities of niclosamide and its derivatives, it was very urgent to facilitate the dissolution. Several approaches to increases in the water solubility of niclosamide had been reported, including crystal particle size milled to nanoscale, [39] co-crystallization with safe materials, [40] complexation with *O*-phosphorylated calixarene, [41] prodrug formed by incorporation of a hydrophilic functional group [42] and solublization *via* attachment of octenylsuccinate hydroxypropyl phytoglycogen. [43] Of note, prodrug design hold attractive advantages over other strategies, including decreased toxicity, enhanced water solubility, and site-specific release by a certain enzyme at a targeted location.

In search of the optimal moiety possessing high hydrophilia or contributing to the formation of organic salt with base or acid, five widely accepted strategies were selected to improve water solubility of niclosamide. According to the procedure for measuring aqueous solubility reported previously, [44] the data were summarized in Table 3. Niclosamide and its acetyl derivative **2** were very poor soluble in aqueous solution at pH = 7.4 (0.06 mg/mL for **1** and 0.05 mg/mL for **2**). Niclosamide phosphate was beneficial to an increase in water solubility, but no data was available for the antitumor activity [45]. As expected, the incorporation of phosphate (**7**, 7.2 mg/mL**)** and disodium phosphate (**8**, 22.1 mg/mL) resulted in 120-fold and 368-fold increases in aqueous solubility, respectively. In contrast, **15** carrying sulfanilamide, **14** with glycine and **18** endowed with glucose exhibited limited enhancements in water solubility compared with niclosame **1**. Unfortunately, aqueous solubility of **10** can be unmeasurable due to its instability exposed to methanol contained an eluent in HPLC analysis. Taken together, among five strategies we used, niclosamide modified with phosphate was the optimal option to solve aqueous solubility.

Since all the newly synthesized compounds displayed by far better water solubility over the lead niclosamide and acetyl derivative **2**, release of niclosamide was crucial in the antitumor activities and a selectivity towards SCLC. **2** was selected as a positive control, and A549 cells (NSCLC) and NCI-H 446 cells (SCLC) were served as cell evaluation models. Seen in Fig. 2, compounds **8** and **10** exhibited the comparable antitumor activities to **2**, possibly suggesting similar release of the lead niclosamide from **8** and **10** as **2**. The antitumor activity of **14** was inferior to those of **8** and **10**, which was possibly due to the difference of releasing ability. Moreover, three derivatives were sensitive towards NCI-H446 with the non-differential selectivity (SI = 2.90 for **8**, 2.3 for **10**, 2.7 for **14**). By contrast**,** A549 cells and NCI-H 446 cells were insensitive to **15** with sulfanilamide and **18** carrying glucose, emphasizing that sulfamide and glycosidic bond were tolerant to SCLC and NSCLC. The difference in the antitumor activities of these tested compounds possibly originated from the releasing ability. Therefore, from the angels of antitumor activity and water solubility, **8** with disodium phosphate exhibited better physicochemical property and a good selectivity towards NCI-H 446 cells, possibly contributing to potential bioavailability as an anti-SCLC drug.

3. Conclusion

In this study, the antitumor properties of the anti-helminthic drug niclosamide sensitive to SCLC encouraged synthesis and evaluation of niclosamide-derived agents. The crucial observation from the initial *in vitro* screen highlighted the importance of 2-hydroxyl group of niclosamide in the antitumor activity. 2-hydroxyl group capped with alkane chains including pentyl **4** and isopentyl **5** resulted in the disappearance of the antitumor activities, while compound **2** with an acetyl group remained the potent antitumor activity at a nanomolar concentration and displayed a good selectivity towards SCLC, implying the cleavage of

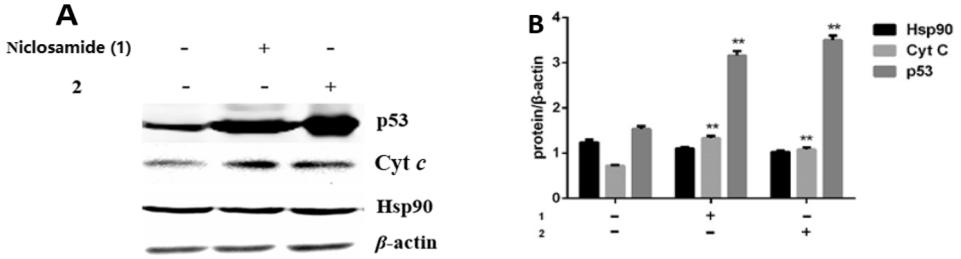


Fig. 1. Effects of niclosamide (**1**) and its derivative (**2**) on several proteins of SCLC (NCI-H446). A. p53, Cyt *c* and Hsp90 expressions measured by western blotting analysis. B. Quantification of p53, Cyt *c* and Hsp90 proteins (Error bars in B indicate SD of the three independent experiments. The significance of difference between the treated group and the control one was analyzed by Student's *t* test, $^{*}p < 0.05$, $^{**}p < 0.01$).

Table 3
Aqueous solubility of niclosamide and its derivatives at pH 7.4.

Compds.	Strategies towards water solubility	Solubility[a,b] (mg/mL)	Compds.	Strategies towards Water solubility	Solubility[a,b] (mg/mL)
1		0.06	**2**		0.05
7	Phosphate	7.2	**8**	Disodium phosphate	22.1
10	Piperidine salt	/[c]	**14**	Primary amine salt	1.2
15	Sulfanilamide	0.2	**18**	Glycosylation	1.4

[a] Aqueous solubility was measured in mg/mL of a compound tested in 0.1 M Na_2HPO_4 solution (pH = 7.4 and 37 °C)
[b] means the mean value of determinations in triplicate.
[c] indicates unmeasurable due to instability to methanol contained as an eluent.

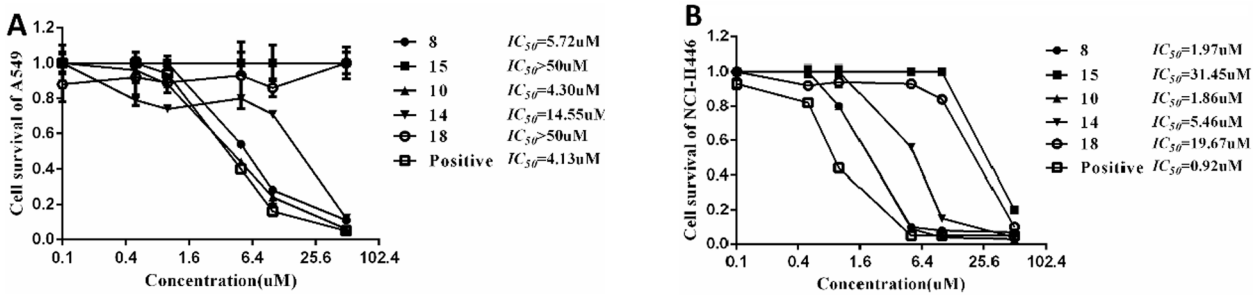


Fig. 2. Cytotoxicities of all the newly-synthesized water-soluble niclosamide derivatives against NSCLC A549 (**A**) and SCLC NCI-H446 (**B**).

ester **2** to release 2-hydroxyl group, but failure to ethers **4** and **5** in the cell culture medium. *In vivo* antitumor activity of **2** further supported the possibility that 2-hydroxyl group was an essential pharmacophore. Mechanism study showed that **2** induced Cyt *c* release dependent cell apoptosis *via* p53 activation. Of five strategies to improve niclosamide's aqueous solubility, structural modification with phosphate to afford **8** exhibited better water solubility, comparable anti-SCLC activity to **2** and an obvious preference in the inhibition of SCLC. The failure to inhibit *in vitro* antitumor activity of other derivatives suggested release of 2-hyroxyl group was a promising characteristic of novel niclosamide analogues. Identification of the molecular target of these compounds would provide a guiding template for structural optimization in the development of specific SCLC inhibitors.

4. Experiential section

4.1. Chemistry

All chemicals were of reagent grade quality or better, which were purchased from commercial suppliers and used without further purification. Solvents were used as received or dried over molecular sieves. Column chromatography was performed on silica gel (100–200 mesh) purchased from Qingdao Ocean Chemical Factory. All the reaction processes were monitored by TLC (HSGF 254) from Yantai Jiangyou Silica Gel Development Co. LTD (Yantai, China). All the key intermediates and final products were confirmed by ^{1}H NMR and ^{13}C NMR, recorded in a Bruker Avance 400 or 500 (^{1}H at 400 MHz or 500 MHz, ^{13}C at 100 MHz or 125 MHz), and chemical shifts were reported in parts per million using the residual solvent peaks as internal standards ($CDCl_3$ = 7.26 ppm for ^{1}HNMR and 77.16 ppm for ^{13}CNMR, CD_3SOCD_3 = 2.50 ppm for ^{1}H NMR and 39.52 ppm for ^{13}C NMR, CD_3OD = 3.31 ppm for ^{1}H NMR, and 49.00 ppm for ^{13}C NMR). Also, several key intermediates and final products were determined by electrospray ionization high resolution mass spectrometry (ESI-HRMS), recorded on AB Sciex triple TOF 5600 + system. The purity was>95.0%, and it was determined with C_{18}-column (250 × 4.6 mm, 5 μm, Agilent Eclipse Plus) run on Agilent Technologies 1260 infinity II.

4.1.1. Synthesis of 4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl acetate (2) and 2-(acetyl(2-chloro-4-nitrophenyl)carbamoyl)-4-chlorophenyl acetate (3)

To a solution of niclosamide **1** (0.13 g, 0.4 mmoL) and pyridine (0.036 mL, 0.44 mmoL) dissolved in dry THF (5 mL) under an argon atmosphere at room temperature was added acetyl chloride (0.034 g, 0.44 mmoL), and a white precipitate was formed over 3 hrs. The reaction mixture was neutralized with diluted acid, and then extracted with ethyl acetate (EtOAc, 100 mL). The organic layer was washed with brine once, and dried over anhydrous Na_2SO_4, and filtered, and then concentrated under reduced pressure to produce the crude residue, which was further chromatographed over silica gel with a mixture of EtOAc/petroleum ether (V/V = 1/20) as an eluent to give 38.4 mg of the desired **2** and 84.9 mg of by-product **3**, respectively.

Compound **2**: Yield, 25.2%, ^{1}H NMR (400 MHz, DMSO) δ 10.48 (s, 1H), 8.41 (s, 1H), 8.29 (d, 1H, J = 8.0 Hz), 8.09 (d, 1H, J = 8.0 Hz), 7.85 (d, 1H, J = 4.0 Hz), 7.71 (d, 1H, J = 4.0 Hz), 7.37 (d, 1H, J = 8.0 Hz), 2.36 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.2, 163.6, 147.4, 145.0, 141.2, 132.5, 130.5, 130.2, 129.7, 127.7, 126.5, 126.0, 125.5, 123.5, 21.2. TOF-MS, *m/z*: [M−H$^-$], Calcd for $C_{15}Cl_2H_9N_2O_5^-$, 366.9894, Found, 366.9885.

Compound **3:** Yield, 51.6%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.35 (d, 1H, J = 3.5 Hz), 8.11 (dd, 1H, J = 8.5, 3.5 Hz), 7.46 (d, 1H, J = 8.5 Hz), 7.40 (d, 1H, J = 2.5 Hz), 7.37 (dd, 1H, J = 8.5, 3.5 Hz), 7.08 (d, 1H, J = 8.5 Hz), 2.45 (s, 3H), 2.33 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.6, 168.6, 166.4, 148.0, 146.4, 141.9, 134.3, 132.4, 131.6, 131.3, 129.1, 127.8, 125.7, 124.9, 123.0, 25.9, 20.9.

4.1.2. Modified synthetic procedure for compound 2

To a solution of niclosamide (0.65 g, 2.0 mmoL) and DMAP (0.12 g, 1.0 mmoL) dissolved in anhydrous DMF was added acetic anhydride (0.5 mL, 4.8 mmoL) dropwise at 0 °C. The mixture was stirred at the same temperature overnight. After finished, the reaction mixture was diluted with double distilled water (50.0 mL), extracted with EtOAc (100.0 mL) twice. The combined organic layer was washed with water (50.0 mL) and brine (100.0 mL), and dried over anhydrous Na_2SO_4, and then concentrated to afford the crude residue, which was purified by silica gel-based chromatography with a mixture of petroleum ether and EtOAc (V/V = 20/1) to provide 0.65 g of **2** as a white solid in an 88.2% yield.

4.1.3. 5-chloro-N-(2-chloro-4-nitrophenyl)-2-(pentyloxy)benzamide (4)

Niclosamide (0.65 g, 2.0 mmoL), KI (0.16 g, 1.0 mmoL), Bu_4 NBr (0.16 g, 0.5 mmoL) and K_2CO_3 (0.84 g, 12.0 mmoL) were taken up in 1,4-dioxane (50.0 mL) in order, and 1-brompentane (1.22 g, 16.0 mmoL) was added dropwise. The resultant mixture was heated to 85 ℃ and stirred overnight. After completion of the reaction, 1,4-dioxane was removed under reduced pressure. The obtained residue was re-dissolved with dichloromethane (DCM) and washed with water twice. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to give the crude product, which was purified over silica gel column with an eluent containing petroleum ether and EtOAc (V/V = 15/1) to provide 0.25 g of **4** as a white solid.

Yield, 35.4%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.57 (s, 1H), 8.87 (d, 1H, J = 8.0 Hz), 8.45 (s, 1H), 8.31 (d, 1H, J = 4.0 Hz), 8.21 (d, 1H, J = 4.0 Hz), 8.19 (d, 1H, J = 8.0 Hz), 4.27 (t, 2H, J = 8.0 Hz), 1.88–1.95 (m, 2H), 1.37–1.40 (m, 4H), 0.88 (s, 3H).

4.1.4. 5-chloro-N-(2-chloro-4-nitrophenyl)-2-(isopentyloxy)benzamide (5)

The synthetic procedure for compound **5** was similar as that of **4**, only differing in isoamyl bromide as a starting material instead of 1-brompentane.

Yield, 36.7%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.54 (s, 1H), 8.88 (d, 1H, J = 8.0 Hz), 8.45 (s, 1H), 8.30 (d, 1H, J = 4.0 Hz), 8.21 (d, 1H, J = 4.0 Hz), 8.19 (d, 1H, J = 8.0 Hz), 4.30 (t, 2H, J = 8.0 Hz), 1.87 (t, 2H, J = 8.0 Hz), 1.23 (m, 1H), 0.96 (s, 3H), 0.94 (s, 3H).

4.1.5. 4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl diethyl phosphate (6)

To a stirred solution of niclosamide (2.0 g, 6.12 mmoL) dissolved in a mixture of dry *N,N*-dimethylformamide (20.0 mL) and carbon tetrachloride (3.0 mL) were slowly added *N,N*-diisopropylethylamine (2.0 mL, 11.5 mmoL) and a catalytic amount of DMAP at 0 °C. Subsequently, diethyl phosphite (850.0 μL, 12.3 mmoL) was added dropwise. The reaction mixture was stirred for 2.0 hrs at 0 °C. After done, the solution was quenched with 0.50 M K_2HPO_4 and warmed to r.t., and then stirred for another 20 mins. The resultant mixture was extracted with EtOAc (100 mL). The organic layer was washed with brine (30.0 mL), and dried by anhydrous Na_2SO_4, and filtered, and then concentrated under reduced pressure to offer the crude product, which was purified by silica gel-based chromatography with an eluent containing a mixture of EtOAc and petroleum ether (V/V = 1/2) to yield 2.78 g of **6** as a white powder.

Yield, 96.2%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.51 (s, 1H), 8.81 (d, 1H, J = 9.5 Hz), 8.32 (s, 1H), 8.19 (d, 1H, J = 9.5 Hz), 8.03 (s, 1H), 7.51 (d, 2H, J = 9.5 Hz), 4.20 (m, 4H), 1.29 (t, 6H, J = 7.5 Hz). ^{31}P NMR (200 MHz, $CDCl_3$) δ −6.06. ^{13}C NMR (125 MHz, $CDCl_3$) δ 162.1, 146.7, 143.4, 140.5, 133.6, 131.9, 131.6, 126.6, 124.9, 123.6, 123.2, 122.7, 121.2, 65.7, 65.6, 16.2, 16.1.

4.1.6. 4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl dihydrogen phosphate (7)

6 (1.4 g, 3.05 mmoL) was dissolved in dry DCM (7 mL), and trimethylsilyl bromide (2.4 mL, 18.1 mmoL) was added dropwise at 0 °C. The reaction mixture was stirred at 0 °C for 12.0 hrs. When the reaction was completed, addition of methanol (2.0 mL) was performed. The reaction mixture was stirred for another 20 mins. The solvent was directly removed under diminished pressure to afford 1.2 g of **7** as a white powder.

Yield, 95.7%. ^{1}H NMR (500 MHz, DMSO) δ 10.33 (s, 1H), 8.49 (d, 1H, J = 9.0 Hz), 8.40 (d, 1H, J = 3.0 Hz), 8.29 (dd, 1H, J = 9.0, 3.0 Hz), 7.83 (d, 1H, J = 3.0 Hz), 7.68 (dd, 1H, J = 9.0, 3.0 Hz), 7.55 (d, 1H, J = 9.0 Hz,). ^{31}P NMR (200 MHz, DMSO) δ −6.11. ^{13}C NMR (125 MHz, DMSO) δ 162.6, 148.1, 143.4, 140.7, 132.7, 130.1, 128.3, 127.2, 124.8, 124.7, 123.3, 123.1, 123.0. TOF-MS, *m/z*: $[M-H]^-$, calcd for $C_{13}H_8Cl_2N_2O_7P^-$, 404.9452, Found, 404.9444.

4.1.7. 4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl phosphate (8)

To 20.0 mL of a stirred methanol solution of **7** (1.2 g, 2.96 mmoL) was added dropwise saturated sodium hydroxide. When the sediment appeared, the solution was filtered, and freeze-dried to afford 0.72 g of **8** as a white powder.

Yield, 54.0%. ^{1}H NMR (400 MHz, DMSO) δ 8.95 (d, 1H, J = 9.2 Hz), 8.43 (s, 1H), 8.27 (d, 1H, J = 2.8 Hz), 8.13 (dd, 1H, J = 9.2, 2.8 Hz), 7.67 (s, 1H), 7.05 (dd, 1H, J = 4.0, 4.0 Hz), 6.50 (t, 1H, J = 8.0 Hz).^{31}P NMR (160 MHz, DMSO) δ −4.72. ^{13}C NMR (100 MHz, DMSO) δ 168.4, 167.8, 147.1, 139.9, 132.2, 128.2, 124.7, 123.4, 122.6, 122.5, 120.6, 119.0, 114.4.

4.1.8. tertbutyl4-(((4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl) phenoxy) carbonyl)amino)piperidine-1-carboxylate (9)

A solution of tertbutyl 4-aminopiperidine-1-carboxylate hydrochloride (3.0 g, 12.7 mmoL) in DCM (40.0 mL) was fiercely mixed with saturated sodium bicarbonate solution (30.0 mL). The organic phase was dried over anhydrous $MgSO_4$, and then concentrated under reduced pressure to afford 2.3 g of 4-aminopiperidine-1-carboxylate as a yellow oil in a 91.0% yield. Next, a cooled solution (0 °C) of triphosgene (3.54 g, 11.96 mmoL) in dry DCM (10.0 mL) was introduced to a dry DCM solution (10.0 mL) of the above yellow oil (2.3 g, 11.5 mmoL) and *N,N*-diisopropylethylamine (3.8 mL, 23.0 mmoL) in a dropwise fashion. The mixture was allowed to warm to r. t. and stirred for 1.0 h, and then concentrated under diminished pressure to afford the residue without purification for next reaction. Finally, to a solution of niclosamide (3.76 g, 11.5 mmoL) dissolved in a mixture of dry DCM (10.0 mL) and dry pyridine (1.0 mL) were added dropwise the above residue and 0.20 g of 4-dimethylaminopyridine. The resulting solution was stirred at r.t. overnight. After the reaction was over, EtOAc (200.0 mL) was added. The organic layer was washed with diluted hydrochloric acid (1 M), water and brine in order, and dried by anhydrous Na_2SO_4, and then concentrated to afford a yellow solid, which was further purified by silica gel chromatography with a mixture of EtOAc and petroleum ether (V/V = 1/10) as an eluent to yield 1.33 g of **9** as an yellow solid.

Yield, 21.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (d, 1H, J = 2.4 Hz), 8.24 (dd, 1H, J = 8.0, 2.4 Hz), 8.03 (d, 1H, J = 2.4 Hz), 7.64 (dd, 1H, J = 8.0, 2.4 Hz), 7.50 (d, 1H, J = 8.0 Hz), 7.21 (d, 1H, J = 8.0 Hz), 3.96 (dt, 1H, J = 8.0, 4.0 Hz), 3.62–3.44 (m, 2H), 3.15–3.10 (m, 2H), 1.73–1.55 (m, 2H), 1.41 (s, 9H), 1.38–1.29 (m, 2H).

4.1.9. 4-(((4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenoxy) carbonyl) amino) piperidin-1-ium bromide (10)

9 (0.3 g, 0.54 mmoL) was dissolved in 33% hydrobromic acid contained in AcOH (3.0 mL) at 0 ℃, and the mixture was stirred at r.t. for 0.5 h. The solvent was directly concentrated under diminished pressure to yield 0.21 g of **10** as a white solid.

Yield, 72.0%. ^{1}H NMR (400 MHz, DMSO) δ 8.54 (d, 1H, J = 2.4 Hz), 8.39 (dd, 1H, J = 8.8, 2.4 Hz), 8.35 (s, 1H), 8.14 (s, 1H), 7.98–7.87 (m, 2H), 7.50 (d, 1H, J = 8.8 Hz), 4.08–4.01 (m, 1H), 3.02–2.88 (m, 4H), 1.89–1.75 (m, 2H), 1.54–1.40 (m, 2H).

4.1.10. (tert-butoxycarbonyl)glycine (11)

Glycine (3.0 g, 40.0 mmoL) was dissolved in a mixture of dioxane (15.0 mL) and water (15.0 mL), and then tri-ethylamine (5.54 mL, 40.0 mmoL) and *tert*-butyl dicarbonate (8.72 mL, 40.0 mL) were added in order. The resulting reaction mixture was stirred at r.t. overnight. After evaporation, the residue was re-dissolved in water, and extracted with diethyl ether (25.0 mL) to remove some unknown impurities. A resulting solution was adjusted to pH = 2.0 with diluted hydrochloric acid (2.0 M), and extracted with EtOAc (20.0 mL × 2). The combined organic layer was washed with water and brine in order, and dried over anhydrous Na_2SO_4, and then concentrated under diminished pressure to afford 6.7 g of **11** as a white solid. Yield, 94.5%.

^{1}H NMR (500 MHz, $CDCl_3$) δ 11.81 (d, 1H, J = 7.5 Hz), 5.40–5.14 (m,

1H), 3.91 (t, 1H, $J = 6.0$ Hz), 3.84 (t, 1H, $J = 6.0$ Hz), 1.41 (d, 9H, $J = 4.5$ Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.6, 157.5, 81.9, 43.4, 28.3, 28.2.

4.1.11. 4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl (tertbutoxycarbonyl) glycinate (12)

Niclosamide (1.86 g, 5.71 mmoL) and **11** (1.0 g, 5.71 mmoL) were dissolved in anhydrous DMF (20.0 mL), and then DMPA (60.0 mg, 0.57 mmoL) and *N,N'*-dicyclohexylcarbodiimide (1.76 g, 8.57 mmoL) were added. The reaction solution was stirred at r. t. for 12.0 hrs. After the reaction was finished, diluted hydrochloric acid (1.0 M) was added dropwise to neutralize the solution. The reaction mixture was extracted with EtOAc (200.0 mL), and the organic layer was concentrated under reduced pressure to afford the crude residue, which was purified by flash chromatography with an eluent containing a mixture of EtOAc and petroleum ether (V/V = 1/8) to yield 1.24 g of **12** as a white solid.

Yield, 45.1%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.83 (s, 1H), 8.77 (d, 1H, $J = 9.0$ Hz), 8.33 (d, 1H, $J = 2.5$ Hz), 8.20 (dd, 1H, $J = 9.0$, 2.5 Hz), 7.90 (d, 1H, $J = 2.5$ Hz), 7.55 (dd, 1H, $J = 9.0$, 2.5 Hz), 7.22 (d, 1H, $J = 9.0$ Hz), 5.09 (t, 1H, $J = 6.0$ Hz), 4.21 (d, 2H, $J = 6.0$ Hz), 1.41 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.8, 162.3, 155.8, 146.4, 143.6, 140.2, 133.4, 132.8, 130.3, 128.3, 125.2, 124.9, 123.8, 122.9, 121.0, 80.8, 43.0.

4.1.12. 4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl glycinate (13)

To a stirred solution of **12** (1.24 g, 2.57 mmoL) in DCM (10.0 mL) was added trifluoroacetic acid (6.0 mL). The mixture was stirred at r.t. until the white precipitate appeared. The solution was diluted with EtOAc (30.0 mL), and the organic layer was washed with saturated sodium bicarbonate solution (30.0 mL) and brine (15.0 mL) in order, and dried over anhydrous Na_2SO_4, and finally concentrated under reduced pressure to yield 0.95 g of **13** as a white solid with high purity.

Yield, 81.0%. ^{1}H NMR (500 MHz, CD_3OD) δ 8.41 (d, 1H, $J = 9.0$ Hz), 8.34 (d, 1H, $J = 2.5$ Hz), 8.17 (dd, 1H, $J = 9.0$, 2.5 Hz), 7.86 (d, 1H, $J = 2.5$ Hz), 7.37 (dd, 1H, $J = 9.0$, 2.5 Hz), 6.92 (d, 1H, $J = 9.0$ Hz), 4.33 (s, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 170.2, 169.7, 159.2, 145.2, 141.8, 134.7, 129.4, 126.0, 125.7, 125.2, 124.0, 123.9, 120.0, 118.6, 44.9. TOF-MS, *m/z*: [M + H$^+$], calcd for $C_{15}H_{12}Cl_2N_3O_5^+$, 384.0149, Found, 384.0133; [M + Na$^+$], Calcd. for $C_{15}H_{11}Cl_2N_3O_5Na^+$, 405.9968, Found, 405.9951.

4.1.13. 2-(4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenoxy)-2-oxoethan-1-aminium bromide (14)

0.3 g of **13** (0.78 mmoL) was dissolved in 33% hydrobromic acid contained in AcOH (3.0 mL) at 0 ℃, and the mixture was stirred at r. t. for 0.5 h. The solvent was directly concentrated under diminished pressure to yield 0.33 g of **14** as a white solid.

Yield, 91.7%. ^{1}H NMR (500 MHz, CD_3OD) δ 8.42 (d, 1H, $J = 2.5$ Hz), 8.30–8.23 (m, 2H), 7.94 (d, 1H, $J = 2.5$ Hz), 7.71 (dd, 1H, $J = 8.5$, 2.5 Hz), 7.40 (d, 1H, $J = 8.5$ Hz), 4.18 (s, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 167.4, 165.1, 147.9, 146.3, 141.6, 133.9, 133.5, 130.6, 130.2, 128.5, 126.5, 126.3, 126.1, 123.8, 41.4.

4.1.14. 4-chloro-2-((2-chloro-4-nitrophenyl)carbamoyl)phenyl sulfamate (15)

To vigorously stirred solution of chlorosulfonyl isocyanate (1.2 mL, 13.9 mmoL) was added dropwise formic acid (0.53 mL, 13.9 mmoL) at 0 °C. Five mins later, a white precipitate formed. The precipitate in the above reaction was re-dissolved in DCM (5.0 mL), and niclosamide (4.5 g, 13.9 mmoL) in a mixture of DCM (20.0 mL) and dry pyridine (2.0 mL) was added in order, and then the reaction mixture was stirred at 50 °C for 6.0 hrs. The resulting solution was adjusted with diluted hydrochloric acid (1.0 M), and extracted with EtOAc (100.0 mL). The organic layer was concentrated under reduced pressure to afford a yellow solid. The crude solid was further purified by silica gel-based chromatography with an eluent containing a mixture of EtOAc and petroleum ether (V/V = 1/6) to yield 4.25 g of **15** as a yellow solid.

Yield, 76.0%. ^{1}H NMR (500 MHz, CD_3OD) δ 8.42 (d, 1H, $J = 2.5$ Hz), 8.30–8.19 (m, 2H), 7.72 (dd, 2H, $J = 10.5$, 8.5 Hz), 7.33 (dd, $J = 1$H, 9.0, 1.5 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 153.8, 135.8, 135.0, 131.8, 131.2, 130.8, 128.7, 127.8, 126.2, 125.8, 124.4, 123.6, 121.6. TOF-MS, *m/z*: [M + H$^+$], calcd for $C_{13}H_{10}Cl_2N_3O_6S^+$, 405.9662, found, 405.9666.

4.1.15. (2R,3R,4S,5R,6R)-2-(acetoxymethyl)-6-bromotetrahydro-2H-pyran-3,4,5-triyltriacetate (16)

To a solution of penta-acetyl glucopyranose (0.76 g, 1.95 mmoL) in anhydrous DCM was added 33% hydrobromic acid contained in AcOH (3.0 mL) at 0 °C. The reaction mixture was allowed to heat to r.t. and stirred for 4.0 hrs. The whole progress was monitored by TLC plate. The reaction solution was quenched with a mixture of EtOAc (60.0 mL) and saturated sodium bicarbonate solution (20.0 mL). The organic layer was washed with saturated sodium bicarbonate solution (20.0 mL × 2), water (20.0 mL) and brine (20.0 mL) in order, and dried over anhydrous Na_2SO_4, and filtered. The filter was removed under reduced pressure to afford 0.73 g of **16** as a white solid.

Yield, 91.0%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.60 (d, 1H, $J = 4.0$ Hz), 5.55 (t, 1H, $J = 9.5$ Hz), 5.15 (t, 1H, $J = 9.5$ Hz), 4.83 (dd, 1H, $J = 9.5$, 4.0 Hz), 4.34–4.26 (m, 2H), 4.12 (dd, 1H, $J = 12.5$, 2.0 Hz), 2.09 (s, 6H), 2.04 (s, 3H), 2.03 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.6, 170.0, 169.9, 169.6, 86.7, 72.3, 70.7, 70.3, 67.3, 61.1, 20.9, 20.8, 20.7, 20.6.

4.1.16. (2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-chloro-2-((2-chloro-4-nitrophenyl) carbamoyl) phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (17)

Niclosamide (0.47 g, 1.45 mmoL), potassium carbonate (0.35 g, 2.56 mmoL), and tetrabutylammonium bromide (50 mg, 0.15 mmoL) were taken up in anhydrous DMF (5.0 mL) in presence of 4 Å molecular sieves, and a solution of **16** (0.3 g, 0.73 mmoL) in DCM (1.0 mL) was added dropwise at 0 °C. Subsequently, the mixture was rise up to r.t. and stirred overnight before the reaction mixture was quenched with diluted hydrochloric acid (1.0 M), and extracted with EtOAc (35.0 mL). The organic layer was concentrated under diminished pressure to produce the crude residue, which was purified by silica gel-based chromatography with a mixture of EtOAc and petroleum ether (V/V = 1/4) to afford 0.22 g of **17** as a white solid.

Yield, 45.0%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.70 (s, 1H), 8.78 (d, 1H, $J = 9.0$ Hz), 8.37 (d, 1H, $J = 2.5$ Hz), 8.20 (dd, 1H, $J = 9.0$, 2.5 Hz), 8.09 (d, 1H, $J = 2.5$ Hz), 7.50 (dd, 1H, $J = 9.0$, 2.5 Hz), 7.13 (d, 1H, $J = 9.0$ Hz), 5.39–5.26 (m, 2H), 5.22 (d, 1H, $J = 7.5$ Hz), 5.14 (dd, 1H, $J = 10.0$, 9.0 Hz), 4.25 (dd, 1H, $J = 12.5$, 5.0 Hz), 4.12 (dd, 1H, $J = 12.5$, 2.5 Hz), 3.85 (ddd, 1H, $J = 10.0$, 5.0, 2.5 Hz), 2.04 (s, 3H), 2.00 (s, 6H), 1.90 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.4, 170.2, 169.4, 169.1, 162.2, 153.0, 143.4, 140.8, 133.9, 132.2, 130.4, 125.8, 125.0, 123.8, 123.5, 121.1, 118.5, 101.1, 72.8, 72.4, 71.0, 67.9, 61.7, 20.7, 20.6, 20.6, 20.5.

4.1.17. 5-chloro-N-(2-chloro-4-nitrophenyl)-2-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzamide (18)

To a solution of **17** (0.22 g, 0.33 mmoL) dissolved in dry methanol (3.0 mL) was added a catalytic amount of sodium methoxide (2.0 mg). The mixture was stirred at r. t. for 0.5 h, and excessive Dowex 50 (H$^+$) resins were added to neutralize the resulting solution and shaken for another 10 mins. The solution was filtered and concentrated under diminished pressure to afford 0.16 g of **18** as a white solid.

Yield, 99.0%. ^{1}H NMR (400 MHz, CD_3OD) δ 8.68 (d, 1H, $J = 8.8$ Hz), 8.42 (d, 1H, $J = 2.4$ Hz), 8.26 (dd, $J = 8.8$, 2.4 Hz, 1H), 8.10 (d, 1H, $J = 2.4$ Hz), 7.61 (dd, 1H, $J = 8.8$, 2.4 Hz), 7.50 (d, 1H, $J = 9.0$ Hz), 5.20 (d, 1H, $J = 8.0$ Hz), 3.92 (d, 1H, $J = 2.4$ Hz), 3.78–3.67 (m, 2H), 3.53 (dt, 2H, $J = 14.4$, 5.6 Hz), 3.42 (t, $J = 8.8$ Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ 164.2, 155.8, 145.1, 142.1, 134.9, 132.2, 129.6, 126.0, 125.8, 125.3, 124.0, 123.9, 120.0, 104.0, 78.7, 78.1, 74.5, 71.1, 62.4. TOF-MS,

m/z: [M – H^+], calcd for $C_{19}H_{17}Cl_2N_2O_9$, 487.0317, Found, 487.0303.

4.2. Cytotoxicity assay

The *in vitro* anti-proliferative activities of carboplatin, niclosamide and its derivatives (**2**, **4**, **5**, **8**, **10**, **14**, **15**, **18**) were assessed with the standard MTT assay [46]. A549 cells or NCI-H446 cells or Jurkat cells or HBE cells were seeded into 96-well plates. After the addition of freshly prepared concentrations of the tested compounds, the incubation period was extended to 48.0 hrs. Five concentrations of the appropriate drugs with four replicates at each concentration were conducted, and each experiment was performed in triplicate. Removal of the supernatant and addition of 40 μL of MTT solution (5 mg/mL) was employed in each well. After re-incubation for another 4 hrs, 100 μL of DMSO was used to dissolve the formazan crystals formed in each well. Using a Multiskan MK3 microplate reader (BioTek Elx800, USA), The measurement of the percentage of cell viability was performed by the absorbance at a wavelength of 490 nm. Calculation of IC_{50} values of the tested compounds was carried out by nonlinear regression analysis with GraphPad Prism 5.0.

4.3. Water solubility assay

The determination of aqueous solubility was carried out according to the modified HPLC method [48]. HPLC analysis run on Agilent Technologies 1260 infinity II with the following conditions: isocratic elution was adopted, and a mixture of CH_3OH/H_2O (V/V = 15/85) containing 0.1% TFA or 0.1% triethylamine selected for an eluent depending on the tested compounds was used; Flow rate was set as 1.0 mg/mL; Column temperature was 35 °C; Detection wavelength was chosen as 330 nM. Agilent Eclipse Plus column (250 × 4.6 mm, 5 μm) was adopted as an analytic column. The calibration curves of **8**, **10**, **14**, **15, 2**, **1** and **18** were established as our previous method [48]. Finally, enough amounts of **8**, **10**, **14**, **15**, **18, 2** or **1** was dissolved in 0.2 mL of 0.1 M phosphate buffer (pH 7.4), and the solution was gently shaken at 25 °C for 24 hrs. After filtered with 450 nM filter membrane, 100 μL of the saturated filter was diluted to 1.5 mL solution, 10 μL of which was taken for HPLC analysis. The value of aqueous solubility was calculated by substituting the obtained peak into the corresponding calibration curve.

4.4. Western blotting assay

Immunoblotting assay was in accordance with our previous method [47]. Specifically speaking, 2×10^5 cells/well were seeded into six-well plates and then treated as required. When the treatment period was over, cells were harvested and washed with PBS. Cells were lysed on ice in RIPA buffer supplemented with competing amounts of 1.0 × protease-phosphatase inhibitor cocktail (Solarbio Institute of Biotechnology) for 30 mins, and then centrifuged at 12,000 g at 4 °C for 15 mins. Supernatants were extracted and the total protein concentrations were determined by BCA assay (Beyotime Institute of Biotechnology). Equal amounts of total protein (50 μg/lane) were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in Tris buffering solution containing 0.1% Tween 20 (TBS-T) for 1.0 h at r.t., and then incubated with the appropriate primary antibody overnight at 4 ℃. The primary antibody incubated was rabbit anti-human p53 monoclonal antibody (Abcam, dilution as 1:2000) or Cyt *c* (Abcam, dilution as 1:1000), or Hsp 90 (Abcam, dilution as 1:5000). *β*-actin (Abcam, dilution as 1:5000) was served as an internal reference. Following the primary incubation, goat anti-rabbit or mouse HRP-conjugated secondary antibodies (Santa Cruz Bio-technology, dilution as 1:4000,) was added and incubated for 1.0 h at r.t.. Proteins were determined with an enzyme-linked chemiluminescence detection kit. Densitometric analysis of each lane as well as *β*-actin was performed with Image J. Experiments were repeated three times, and data were shown as the ratio of the densitometric level with drug treatment to that without drug treatment after normalization to *β*-actin.

4.5. Xenograft model antitumor assay

Five- to six-week-old male BALB/c mice (Beijing Charles River Laboratories (CRL), China) were inoculated subcutaneously with 2×10^6 human small cell lung cancer NCI-H69 in the flank. When the grafted tumor volume reached the average volume of 100 mm^3, mice were randomly divided into five groups (n = 5). The treatment regimen was shown in Table 2. Compounds **1** and **2** dissolved in 0.5% hydroxypropyl methyl cellulose (HPMC E5, Dow chemical company, U.S.A) were dosing at 50 mg/kg once a day to the end. As a positive control group, carboplatin (Qilu Pharmaceutical, China) at 6 mg/kg was administered *i.g.* once a week to the end. On day 29, all the tested animals were executed with euthanasia, and the tumor was stripped off and weighted. The *in vivo* antitumor potency was evaluated in terms of tumor growth inhibition (TGI), which was defined as the following formula: TGI (%) = [(tumor weigh of control group-tumor weigh of treated group)/tumor weight of control group]*100%. Results were statistically evaluated by Student's *t*-test. This *in vivo* study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Guangzhou University of Chinese Medicine (Guangdong, China) and was performed according to the institutional guidelines.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.104574.

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