

Biological evaluation of 2,3-dioxindolin-*N*-phenylacetamide derivatives as potent CDC25B and PTP1B phosphatase inhibitors

Qing-Hao Jin¹, Wen-Bo Chen^{1,2}, Ya-Nan Xia³, Bing-Yu Liu³, Li-Ping Guan^{3*}

¹Zhejiang Ocean University, DongHai Science and Technology College, Zhejiang, Zhoushan, P.R. China, ²Hailisheng Pharmaceutical Co Lid, Zhejiang, Zhoushan, P. R. China, ³Food and Pharmacy College, Zhejiang Ocean University, Zhejiang, Zhoushan, P. R. China

A series of 2,3-dioxindolin-*N*-phenylacetamide derivatives was evaluated for inhibitory activity against CDC25B and PTP1B enzymes. Most of the derivatives showed inhibitory activity against CDC25B (IC₅₀ = 3.2–23.2 µg/mL) and PTP1B (IC₅₀ = 2.9–21.4 µg/mL). Compound **2h** showed the most inhibitory activity *in vitro* with IC₅₀ values of 3.2 and 2.9 µg/mL against CDC25B and PTP1B, respectively, compared with the reference drugs Na₃VO₄ (IC₅₀ = 2.7 µg/mL) and oleanolic acid (IC₅₀ = 2.3 µg/mL). The results of selectivity experiments showed that the 2,3-dioxindolin-*N*-phenylacetamide derivatives were selective inhibitors against CDC25B and PTP1B. Enzyme kinetic experiments demonstrated that compound **2h** was a specific inhibitor with the typical characteristics of a mixed inhibitor. In cytotoxic activity assays compound **2h** had potent activity against A549, HeLa, and HCT116 cell lines. In addition, compound **2h** showed potent tumor inhibitory activity in a colo205 xenograft model *in vivo*.

Keywords: Dioxindolin-*N*-phenylacetamide. CDC25B. PTP1B. Phosphatase inhibitors. Enzyme kinetics.

INTRODUCTION

Cell division cycle 25 phosphatase (CDC25) enzymes are potential targets for the development of new cancer therapeutic agents (Contour-Galceran *et al.*, 2007). CDC25, a subfamily of dual-specificity protein tyrosine phosphatases, which includes CDC25A, B, and C homologues, plays a pivotal role in the regulation of the cell cycle (Rudolph, 2007). Current drug discovery efforts are being directed toward identifying novel CDC25 inhibitors that work *in vitro*, and compounds that may be active against human tumors *in vivo* have been reported. In particular, CDC25B isoforms are known to be overexpressed in primary tissue samples from various human cancers, and this overexpression is strongly associated with tumor aggressiveness and poor prognosis (Lavecchia, Di Giovanni, Novellino, 2011; Lavecchia *et al.*, 2008).

Protein tyrosine phosphatase 1B (PTP1B) is the most characterized of all the tyrosine phosphatases and acts as a critical negative and positive regulator of numerous

signaling cascades (Feldhammer *et al.*, 2013; Low, Chai, Yao, 2014). PTP1B is expressed in multiple tissues including the liver, adipose tissue, skeletal muscle, and the brain. PTP1B is involved in multiple signal transduction pathways (Julien *et al.*, 2007). PTP1B is also a key player in cancer regulation serving as both a tumor suppressor and tumor promoter depending on the cellular context.

Isatin (2,3-dioxindolin) is an endogenous compound in humans that possesses a wide range of biological activities. In recent years, various isatin derivatives have been identified that act as antibacterial, anticonvulsant, antifungal, antitubercular, antiviral, and anticancer agents (Pandeya *et al.*, 2005; Pandeya, Raja, 2002). It is well-documented that apoptosis, or programmed cell death, is the key mechanism by which chemo-therapeutic agents exert their cytotoxicity (Tripathi, Krishnamurthy, Ayyannan, 2016; Modi *et al.*, 2011). Recently, several isatin derivatives have been shown to display appreciable cytotoxicity (Figure 1). For example, Popp (Sukhramani, Desai, Suthar, 2011) synthesized 3-*o*-nitrophenyl hydrazones of isatin by condensation of isatin with *o*-nitrophenyl hydrazine. These compounds were found to be active intramuscularly against Walker carcinoma-256 and inactive against L-1210 lymphoid

*Corresponding: L.-P. Guan. Food and Pharmacy College, Zhejiang Ocean University, 316022 - Zhoushan, Zhejiang, P. R. China. E-mail: glp730@163.com

leukemia in animal models. A novel series of 5-(2-oxo-3-indolyl) thiazolidine-2,4-diones having substitutions prepared using various Mannich bases at positions 1 and 3 of the isatin and thiazolidine rings, respectively, has been synthesized by Eshba and Salama (Eshbha, Salama, 1985). Five compounds were evaluated for antileukemic activity against p388 lymphocytic leukemia in mice. The di-Mannich base with a dimethyl amino component exhibited the highest activity of the tested compounds. The introduction of bromine into the aromatic moiety of the isatin ring at position 5 increased the activity compared with the parent molecule to a small extent. Teitz *et al.* (1993) studied the selective repression of the V-abl coded protein (P120) on the oncogene product associated with tyrosine kinase activity using *N*-methylisatin-4',4'-diethyl thio-semicarbazone and *N*-allylisatin-4',4'-diallyl thio-semicarbazone. These compounds selectively suppressed the V-abl oncogene, as well as the Moloney murine leukemia virus. Broadbent, Thomas and Broadbent (1998) reviewed the chemistry and pharmacology of indole-3-carbinol and 3-methoxymethylindole; these compounds showed antimutagenic and anticarcinogenic properties against a variety of classes of carcinogens and acted as anticancer agents against certain common neoplasms.

In our previous work, we found that compound **I** [2-(6-bromo-2,3-dioxindolin-1-yl)-*N*-(2-bromophenyl) acetamide] displayed inhibitory activity against CDC25B and PTP1B ($IC_{50} = 3.87$ and 2.98 $\mu\text{mol/L}$, respectively), and also showed cytotoxic activity against three cancer cell lines (HeLa, A549, and HCT116). In addition,

compound **I** displayed potent tumor inhibitory activity in a colo205 xenograft model *in vivo* (Zhao *et al.*, 2015a). To further investigate the anticancer effects of CDC25B and PTP1B inhibitors, we synthesized a series of 2,3-dioxindolin-*N*-phenylacetamide derivatives and investigated the structure-activity relationships. The synthetic pathway for compounds **2a-2o** is illustrated in Scheme 1. Compound **2h** was selected for a kinetics study and selectivity analysis to determine whether these compounds are suitable for further development. The pharmacological results showed that all of the tested compounds significantly inhibited CDC25B and PTP1B *in vitro*. The mechanism of this inhibition was also studied.

MATERIAL AND METHODS

Reagents

2,3-Dioxindolin-1-yl-*N*-phenylacetamides (**2a-2o**) were synthesized as described previously (Xie *et al.*, 2014 a). All compounds were confirmed from the IR spectra (FT-IR1730, Bruker, Switzerland), ^1H -NMR and ^{13}C -NMR spectra (AV-300, Bruker, Switzerland), mass spectra (HP1100LC/MS, Agilent Technologies, USA), and elemental analyses (CHN) (Perkin Elmer 204Q CHN). 3-O-Methylfluorescein phosphate (OMFP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and A549, HeLa, and HCT116 cells were purchased from Sigma Aldrich (St Louis, MO, USA). All

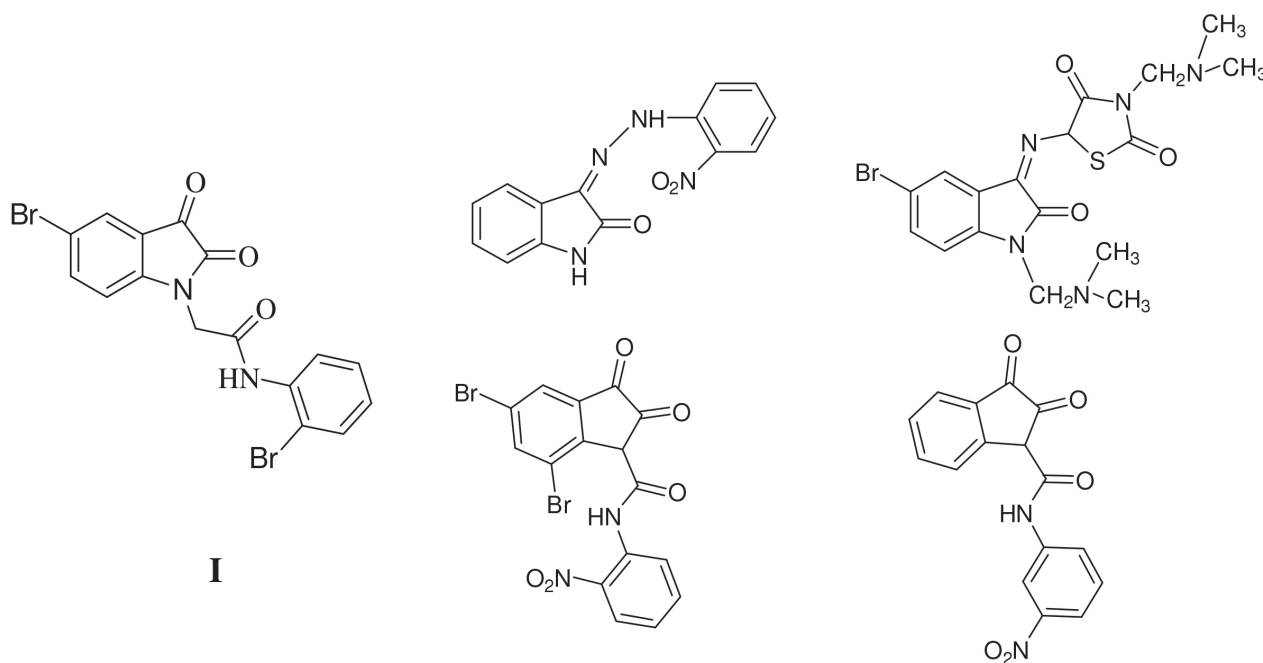
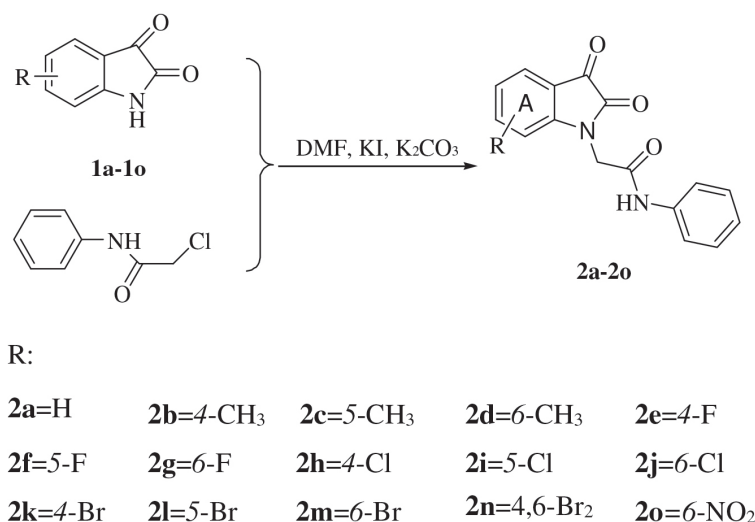


FIGURE 1 - Structures of isatin derivatives reported to be CDC25B and PTP1B inhibitors and **I**.

SCHEME 1 - The synthetic pathway for compounds **2a–2o**

other reagents and solvents used in the experiments were of analytical grade.

Biological activity assay for CDC25B

The enzymatic activity of the CDC25B catalytic domain was determined by monitoring the dephosphorylation of OMFP. The dephosphorylation of OMFP generates 3-O-Methylfluorescein (OMF), which was detected at 485 nm excitation/535 nm emission wavelengths. A typical 100- μ L assay mixture contained Tris-HCl (50 mmol/L), NaCl (50 mmol/L), OMFP (5 μ mol/L), recombinant CDC25B (20 nmol/L), 1% glycerin, and DTT (1 mmol/L), in the presence or absence, of 2 μ L of compounds **2a–2o** in dimethyl sulfoxide (DMSO); pH 8.0. The activity was continuously monitored and the initial rate of the dephosphorylation was determined using the early linear region of the enzymatic reaction kinetic curve. Continuous kinetic monitoring was performed in clear 96-well plates (Corning, Lowell, MA) (Cossy *et al.*, 2006; Zhao *et al.*, 2015b).

Biological activity assays for PTP1B

The enzymatic assays for PTP1B were performed as described elsewhere (Cossy *et al.*, 2006). Briefly, the enzymatic activity of the PTP1B catalytic domain was determined at 30 °C by monitoring the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP). Dephosphorylation of *p*NPP generates the product *p*-nitrophenyl (*p*NP), which was monitored at an absorbance of 405 nm using an EnVision multilabel plate reader (PerkinElmer Life Sciences, Boston, MA, USA). Assays were performed in a total volume of 100 μ L containing 50 mM

3-[*N*-morpholino]propanesulfonic acid (MOPS), 2 mM *p*NPP, and 20 nM recombinant PTP1B; pH 6.5, as well as varying concentrations of compounds **2a–2o**.

Enzyme kinetic analysis of compound 2h

Compound **2h** was tested in enzyme kinetic assays according to previous studies (McGovern *et al.*, 2003; Sun *et al.*, 2013a). The assays were performed in a total volume of 100 μ L including 30 nM PTP1B, 50 mM MOPS; pH 6.5, and *p*NPP in twofold dilutions from 80 mM, and different concentrations of **2h**. In the presence of a competitive inhibitor, the Michaelis-Menten equation is described by $1/v = (K_m/[V_{max}[S]])(1+[I]/K_i)+1/V_{max}$, where v = the initial rate, V_{max} = the maximum rate, and $[S]$ = the substrate concentration. The K_i value was determined from a linear replot of the apparent K_m/V_{max} slope from the primary reciprocal plot versus inhibitor concentration $[I]$ according to the equation $K_m/V_{max} = 1+[I]/K_i$.

Cell cultures

A549, HeLa, and HCT116 cells were kept at logarithmic growth in HG-DMEM, McCoy's 5A, and F12 mediums, respectively, at 5% CO₂ and 37 °C supplemented with 10% FBS and 100 units/mL each of penicillin G and streptomycin.

Cytotoxicity assays for compound 2h

Cytotoxicity assays were performed on human colon cancer (HCT116) and cervical carcinoma cell lines. Cells (6000–10000) in 100 μ L culture medium per well were seeded onto a 96-well microtest plate (Falcon, CA, USA). Cells were treated in triplicate; a gradient concentration of

tested compounds was added and the plate was incubated at 37 °C for 72 h. For the three cell lines, a MTT assay was performed to measure cytotoxic effects. The IC₅₀ values of the tested compounds for the tumor cells were determined from the dose-response curves.

Antitumor effect of compound 2h *in vivo*

The antitumor effect of compound **2h** was evaluated *in vivo* and compared with the reference drug IRT. BALB/C nude male mice (weight 18–20 g) were obtained from the Laboratory of Animal Research, College of Pharmacy, Zhejiang Academy of Medical Sciences. Colo205 cancer-cell suspensions were subcutaneously implanted into the right axilla region of the mice. Treatment began when the implanted tumor had reached a volume of 100–300 mm³ (after 17 days). The animals were randomized into treatment and control groups (10 animals per group), and BALB/C nude male mice administered by gavage once daily for 5 consecutive days from day 17 after implantation of the cells. Tumor volumes were monitored by caliper measurement of the length and width and calculated using the formula: TV = 1/2 × a × b², where a is the tumor length and b is the width. Tumor volumes and body weights were monitored every 4 days over the course of the treatments. Mice were sacrificed on day 35 after implantation of cells, and the tumors were removed and recorded for analysis. All procedures used were in accordance with the guide for the Care and Use of Laboratory Animals as adopted by the NIH, and the lab received ethical approval from the National Science and Technology Commission of China for the present study.

RESULTS AND DISCUSSION

Effects of derivatives 2a-2o on CDC25B and PTP1B *in vitro*

Human CDC25B is a central target and regulator of the G₂/M checkpoint mechanisms activated in response to DNA injury. The effect of these enzymes and their expression is finely regulated by multiple mechanisms, including interactions with regulatory partners, post-translational modifications, control of intracellular localization, and cell cycle-regulated degradation. CDC25B has been identified as a potential target for anticancer therapeutics (Zarling *et al.*, 2014; Aressy, Ducommun, 2008; Mak *et al.*, 2012).

The inhibitory effect of compounds **2a–2o** against CDC25B was tested using OMFP as a substrate. Na₃VO₄, a known CDC25B inhibitor, was used as a positive

control, and the results are summarized in Table I. The tested compounds dose-dependently inhibited CDC25B with IC₅₀ values ranging from 3.2 to 23.2 µg/mL. Among them, compound **2h** showed the most potent inhibitory activity for CDC25B (IC₅₀ = 3.2 µg/mL), and the inhibitory activity was close to that of the positive control Na₃VO₄ (IC₅₀ = 2.7 µg/mL) (Table 1).

TABLE I - Inhibitory activity of compounds **2a–2o** against CDC25B and PTP1B

Compounds	IC ₅₀ (µg/mL)	
	CDC25B	PTP1B
2a	16.7 ± 1.28	14.5 ± 2.12
2b	15.2 ± 1.61	11.3 ± 1.54
2c	21.5 ± 1.95	19.5 ± 2.70
2d	20.1 ± 2.74	21.4 ± 2.47
2e	10.3 ± 3.03	11.1 ± 3.44
2f	16.2 ± 3.92	15.1 ± 3.84
2g	13.2 ± 2.02	12.5 ± 1.96
2h	3.2 ± 0.98	2.9 ± 0.52
2i	14.7 ± 3.53	15.3 ± 1.36
2j	12.4 ± 2.37	13.9 ± 4.35
2k	4.7 ± 1.13	4.5 ± 1.88
2l	11.6 ± 1.89	12.7 ± 3.49
2m	15.8 ± 3.76	16.3 ± 2.77
2n	5.0 ± 1.80	6.4 ± 1.25
2o	23.2 ± 2.13	20.2 ± 2.43
Na ₃ VO ₄	2.7 ± 1.16	-
Oleanolic acid	-	2.3 ± 1.54

The structure activity relationships of compounds **2a–2o** were analyzed against CDC25B. Compounds **2b–2o** contained electron-withdrawing groups and electron-donating groups on the A ring. Three compounds **2b–2d** with electron-donor containing were designed and synthesized, containing *o*-CH₃, *m*-CH₃ and *p*-CH₃. Compound **2b** (*o*-CH₃) displayed the most potent inhibitory activity for CDC25B (IC₅₀ = 15.2 µg/mL). Compounds **2c** (*m*-CH₃) and **2d** (*p*-CH₃) exhibited slight activity inhibition effects with IC₅₀ > 20 µg/mL, with an activity order of was *o*-CH₃ > *p*-CH₃ > *m*-CH₃. Compound **2a** non-substituent (-H) on the A ring displayed good inhibition activity for CDC25B (IC₅₀ = 16.7 µg/mL). Except **2o** (IC₅₀ = 23.2 µg/mL), The halogen substituted compounds **2e–2n** with electron-withdrawing groups seemed to show better activity than compounds containing electron-donating groups on the whole level. These results

indicated that electron-withdrawing groups facilitated CDC25B inhibition. We also found that the position of the substituent on the A ring significantly influenced CDC25B inhibition effects, with an activity order of was $o\text{-F} > p\text{-F} > m\text{-F}$ for fluorinated compounds; $o\text{-Cl} > p\text{-Cl} > m\text{-Cl}$ for chlorinated compounds; $o\text{-Br} > 2,4\text{-Br}_2 > m\text{-Br} > p\text{-Br}$ for brominated compounds. Among them, compounds **2h** ($o\text{-Cl}$), **2k** ($o\text{-Br}$) and **2n** ($2,4\text{-Br}_2$) showed better inhibition activity against CDC25B with IC_{50} values of 3.2, 4.7 and 5.0 $\mu\text{g/mL}$, respectively. The electron-withdrawing group compound **2n** ($p\text{-NO}_2$) showed slightly inhibitory activity ($IC_{50} = 23.2 \mu\text{g/mL}$).

PTP1B is a ubiquitously expressed phosphatase that contains a catalytic domain and a C-terminal domain of mainly hydrophobic residues that are involved in targeting the enzyme to the cytoplasmic face of the endoplasmic reticulum. A range of biochemical, cellular and knockout mouse studies have revealed that PTP1B functions as a negative regulator of both the insulin and leptin receptor signaling pathways. Moreover, ablation of PTP1B confers resistance to obesity induced by a high-fat diet. PTP1B is also as key player in cancer serving as both tumor promoter and tumor suppressor depending on the cellular context. (Shi *et al.*, 2008; Barr, 2010; Feldhammer *et al.*, 2013; Malla, Kumar, Kumar, 2013; Reddy *et al.*, 2014).

The inhibitory activity of the synthesized compounds (**2a–2o**) against PTP1B was measured using *p*NPP as a substrate. Oleanolic acid, a known PTP1B inhibitor, was used as a positive control. The results are shown in Table I. All of the tested compounds showed inhibitory effects against PTP1B with IC_{50} values ranging from 2.9 to 21.4 $\mu\text{g/mL}$. Compound **2h** exhibited the most potent inhibitory activity for PTP1B ($IC_{50} = 2.9 \mu\text{g/mL}$), and the inhibitory activity was similar to the positive control oleanolic acid ($IC_{50} = 2.3 \mu\text{g/mL}$). In general, the halogen-substituted compounds **2e–2n** with electron-withdrawing groups displayed better activity than compounds containing electron-donating groups. The order of potency was $o\text{-F} > p\text{-F} > m\text{-F}$ for fluorinated compounds; $o\text{-Cl} > p\text{-Cl} > m\text{-Cl}$ for chlorinated compounds; and $o\text{-Br} > 2,4\text{-Br}_2 > m\text{-Br} > p\text{-Br}$ for brominated compounds. Compound **2o** with an electron-withdrawing group ($p\text{-NO}_2$) also exhibited potent inhibitory activity against PTP1B ($IC_{50} = 20.2 \mu\text{g/mL}$). For the three compounds **2b–d** with electron-donating groups, the order of activity was $o\text{-CH}_3 > m\text{-CH}_3 > p\text{-CH}_3$. The IC_{50} value for compound **2b** was 11.3 $\mu\text{g/mL}$. Compound **2a**, non-substituted (-H) on the A ring, displayed good inhibitory activity for PTP1B ($IC_{50} = 14.5 \mu\text{g/mL}$). CDC25B and PTP1B are potential targets for the development of new cancer therapeutic agents. Current drug discovery efforts are directed toward

identifying novel CDC25B and PTP1B inhibitors, and derivatives that may be active against human tumors have been reported (Li *et al.*, 2014; Xie *et al.*, 2014b). In the present study, all of the tested compounds significantly inhibited CDC25B and PTP1B, which indicates that these compounds may possess anticancer activity.

Enzyme kinetic assays of compound 2h

A kinetic study was used to clarify the inhibitory mechanism of compound **2h**. The assays were executed according to procedures described previously (Shi *et al.*, 2008; McGovern *et al.*, 2003; Sun *et al.*, 2012b). As illustrated in Figure 2A, compound **2h** showed time-independent inhibition of PTP1B and the results indicated that compound **2h** was a fast-binding inhibitor of PTP1B. The time-independent behavior of compound **2h** was also able to be eliminated with regard to PTP1B, so compound **2h** is a nonspecific inhibitor, because nonspecific inhibitors always exhibit time-dependent behavior and a steep inhibition curve. In further analysis of the mechanism of inhibition of PTP1B; compound **2h** displayed characteristics typical of a competitive inhibitor, with increased K_m values and reduced V_{max} values with increases in concentration (Figure 2B). Moreover, because all the plots for different concentrations of compound **2h** intersect on the y-axis, the Lineweaver-Burk plot suggested that compound **2h** is a competitive inhibitor for PTP1B (Figure 2C). The results indicated that compound **2h** binds to the catalytic pocket of PTP1B and competes with the physiological substrate. The K_i value, calculated from Figure 2D, was 0.502 μM .

Compound 2h inhibited cell proliferation

A549, HCT116, and HeLa cells were used to investigate the effect of compound **2h** on the proliferation of cancer cells. Cells were seeded at 2000 cells per well in a 96-well microplate. After 24 h, cells were treated with the indicated concentrations of compound **2h** for 72 h, using 1% DMSO as a negative control. The viability of cells was evaluated using the MTT method. As shown in Table II, compound **2h** markedly inhibited the proliferation of A549 cells ($IC_{50} = 2.86 \pm 0.19 \mu\text{mol/L}$), HCT116 cells ($IC_{50} = 3.59 \pm 0.27 \mu\text{mol/L}$), and HeLa cells ($IC_{50} = 2.78 \pm 0.11 \mu\text{mol/L}$).

Antitumor activity for compound 2h in vivo

On the basis of the favourable data for compound **2h in vitro**, its antitumor activity in the colo205 (colon

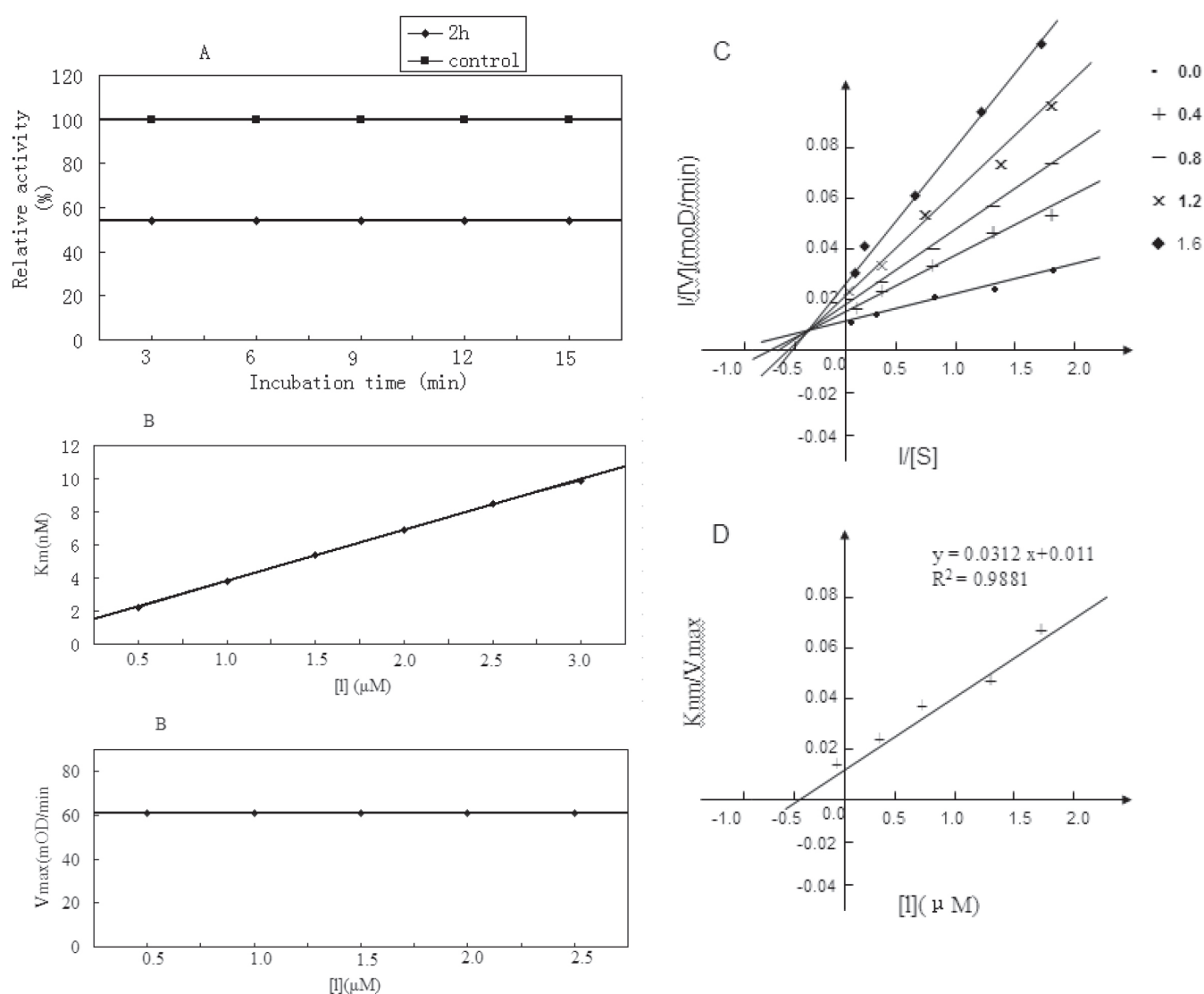


FIGURE 2 - Characterization of the inhibitory mechanism of compound **2h** toward PTP1B. (A) The time-independent inhibition of PTP1B by **2h**; (B) At various fixed concentrations of **2h** the initial velocity was determined with various concentrations of *p*NPP; (C) The mixed inhibition of **2h** shown by Lineweaver–Burk plot; (D) K_i determination of **2h**

TABLE II - The antitumor activity of compound **2h** *in vitro*^a

Compounds	IC_{50} (μ g/mL)		
	A549	HCT116	HeLa
IRT	1.65 ± 0.03	1.43 ± 0.06	1.62 ± 0.07
2h	2.86 ± 0.09	3.59 ± 0.07	2.78 ± 0.11

^aCancer cells: A549: lung cancer; HCT116: colon cancer; HeLa: cervical carcinoma cell.

carcinoma) human tumour xenograft model were evaluated (Figure 3). IRT, a clinical relevant camptothecin, was chosen as a reference drug. The tested compounds were formulated in carboxymethyl cellulose sodium and water were given the orally by gavage. In the colo205

xenograft model, compound **2h** produced a tumor volume inhibition of ~50% when administered once daily for five consecutive days. However, compound **2h** showed to be well tolerated at a dose of 10 mg/kg no lethal toxicity occurred.

CONCLUSION

In summary, 15 2,3-dioxindolin-1-yl-*N*-phenylacetamide compounds were synthesized and screened for inhibition of CDC25B and PTP1B *in vitro*. Compound **2h** showed the most inhibitory activity against both CDC25B and PTP1B. Enzyme kinetic experiments demonstrated that compound **2h** was a nonspecific inhibitor with the typical characteristics of a mixed

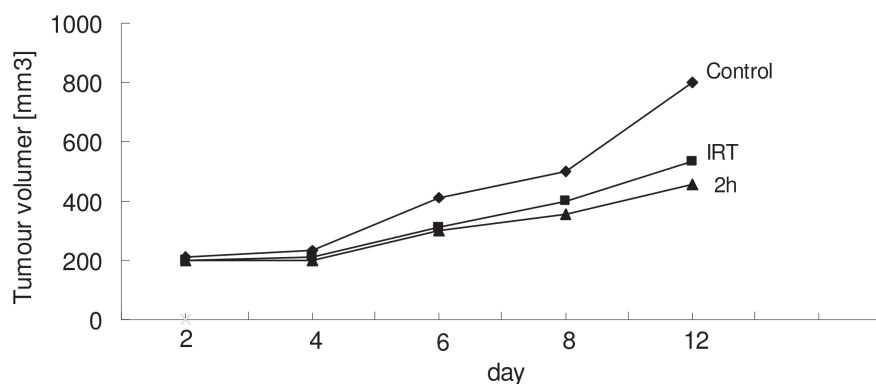


FIGURE 3 - Tumor growth inhibition of colo205 xenografts in nude mice by compound **2h** administered intragastrically.

inhibitor. Cytotoxic activity assays showed compound **2h** is the potent against A549, HeLa and HCT116. In addition, compound **2h** displayed potent tumor inhibitory activity in a colo205 xenograft model. These results indicate that compound **2h** is a promising lead compound for the development of a new group of cancer chemotherapeutics.

ACKNOWLEDGEMENTS

This work was supported by Zhejiang Province Public Technology Application Project (No. 2017C33131) and the Science and Technology Program Project of Zhoushan City of China (No. 2016C41005). We thank Victoria Muir, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

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Received for publication on 13th December 2016

Accepted for publication on 10th July 2018