Cytotoxicity of cantharidin analogues targeting protein phosphatase 2A

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Cantharidin is a natural toxin that possesses potent anti-tumor properties. Its clinical application, however, is limited due to severe side-effects. Its cytotoxicity is believed to be mediated by the inhibition of serine/threonine protein phosphatase 2A. In order to identify new compounds with potential clinical therapeutic use, a series of cantharidin analogues, including those with skeletal modifications at 1-C position (analogues 1–6) and those with anhydride modifications (analogues 7–13), were synthesized, and tested for their inhibitory effects on protein phosphatase 2A and their cytotoxicity to a panel of cancer cell lines. In addition, the mode of inhibition of cantharidin and analogue 13 on protein phosphatase 2A was determined by enzymatic kinetics assay. The data indicated that analogue 13 exhibited potent cytotoxicity to all cancer cell lines, and analogues 9, 11 and 12 showed relatively weak cytotoxicity to one or more cell lines, while other analogues showed little cytotoxicity. Accordingly, analogue 13 exhibited potent inhibitory activity on protein phosphatase 2A, and analogues 9, 11 and 12 showed weak inhibitory activity, while other analogues did not show any inhibitory activity. The findings indicate that the cytotoxicity of synthetic cantharidin analogues is likely to be associated with their protein phosphatase 2A inhibitory activity. The mode of inhibition of cantharidin and analogue 13 on protein phosphatase 2A is identified as noncompetitive inhibition by the Lineweaver–Burk plot. *Anti-Cancer Drugs* 17:905–911 © 2006 Lippincott Williams & Wilkins.

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Introduction

The reversible phosphorylation of intracellular proteins containing serine (Ser), threonine (Thr) and tyrosine (Tyr) residues plays a major role in the regulation of diverse cellular processes, such as metabolism, cell motility, membrane transport, transcription, RNA splicing, translation, DNA replication and cell cycle progression. The actual phosphorylation level of these proteins is achieved as the result of a delicate balance between protein kinases and phosphatases. Recent studies of the signaling events leading to cell proliferation have shown the important role of phosphatases in these pathways. Inhibition of protein phosphatases can break the balance of reversible phosphorylation, and influence cell cycle control and growth, which can subsequently induce apoptotic cell death [1,2].

Ser/Thr phosphatase has been classified into four subtypes (i.e. PP1, PP2A, PP2B and PP2C) on the basis of their respective biological characteristics, sensitivity to different specific inhibitors and substrate specificity. Protein phosphatase 2A (PP2A) mainly exists as a trimeric complex in the cytoplasm, and accounts for a major portion of Ser/Thr phosphatase activity in most eukaryotic tissues and cells [3]. It dephosphorylates a myriad of substrates and regulates multiple cell signaling pathways, and is thought to be the target for exogenous toxins [4–6]. Therefore, PP2A is considered a promising target of anti-tumor drugs.

Cantharidin (exo, exo-2,3-dimethyl-7-oxobicyclo[2.2.1] heptane-2,3-dicarboxylic acid anhydride) is a natural toxin in over 1500 different species of Chinese blister beetles (*Mylabris phalerata* or *Mylabris cichorii*) and Spanish flies (*Cantharis vesicatoria*) [7,8]. The basic structure of cantharidin is composed of a 7-oxobicycloheptane and a dicarboxylic acid anhydride (Fig. 1). The use of the dried bodies of the Chinese blister beetles as an anti-cancer agent can be traced back to over 2000 years in traditional Chinese medicine [8]. Although cantharidin has been proven to be lethal to cancer cells, its clinical application in oncology has been limited due to its severe side-effects

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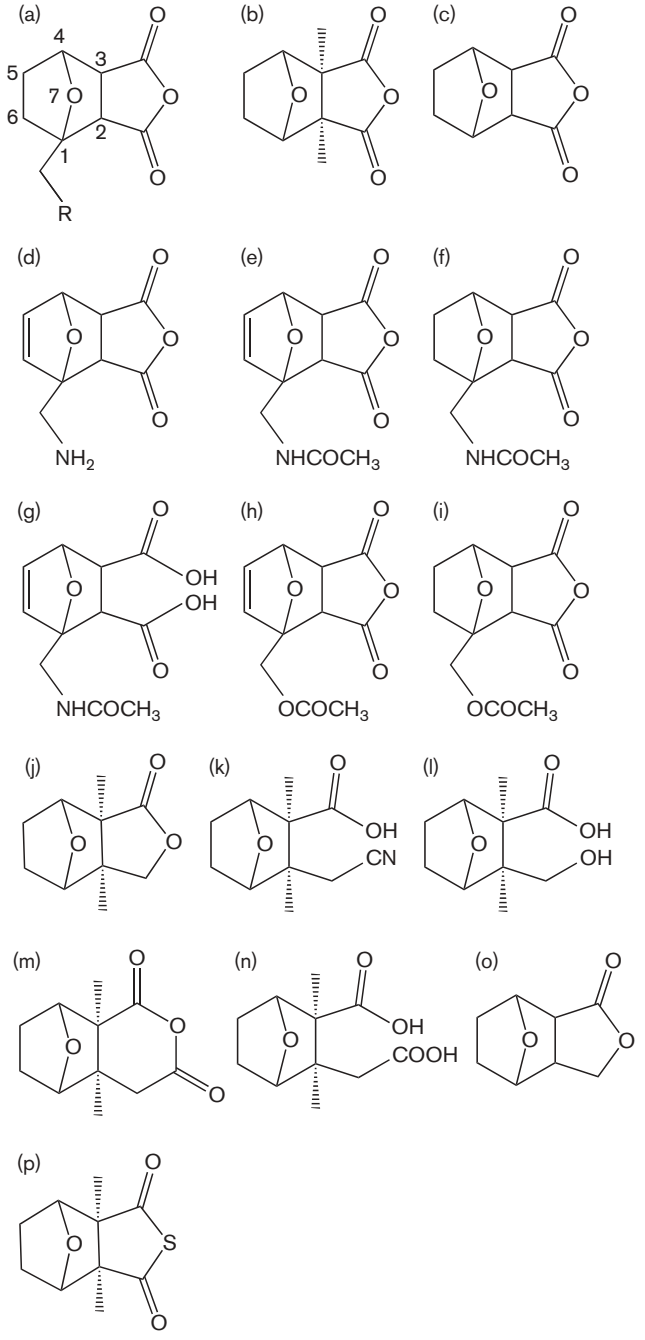
Fig. 1



Structural formulas of cantharidin and its analogues. (a) General formula and positions of skeletal atoms of cantharidin analogues, (b) cantharidin, (c) norcantharidin and (d–p) analogues **1–13**.

such as dysphagia, dysuria, hematemesis, liver congestion and renal toxicity. Since the discovery of cantharidin as a PP2A inhibitor in 1992 [9], efforts have been made to modify cantharidin that focus on PP2A inhibition in order to identify novel candidates for anti-cancer drugs. Limited success, however, has been found. Norcantharidin (Fig. 1) is the demethylated analogue of cantharidin and the only successful anti-cancer drug so far [10–12]. Numerous other skeletally modified cantharidin analogues or anhydride-modified cantharidin analogues have been synthesized. Although some of these analogues demonstrate PP2A inhibition or cytotoxicity, they are far from ideal for therapeutic use [13–17].

In the present study, we synthesized a series of skeletally modified analogues, those with anhydride modifications and their ring-opened derivatives. We determined their cytotoxicity to a panel of cancer cell lines and their inhibitory effect on PP2A. The mode of inhibition of cantharidin and analogue **13** on PP2A was determined by enzymatic kinetics assay.

Materials and methods

Cantharidin analogues

The synthetic cantharidin analogues were classified into two categories: those with additive side-chains to the 7-oxobicycloheptane framework at the 1-C position and those with modifications of the anhydride bridge, including atom or group substitution and ring opening. The former included analogues **1–6** and the latter included analogues **7–13** (Fig. 1).

Stock solutions

All analogues were supplied as dry powder. Stock solutions for cantharidin and analogues (10 mg/ml) were prepared in dimethyl sulfoxide at 25°C and stored at 4°C. They were diluted with phosphate-free distilled water for in-vitro assays for the inhibition of protein phosphatase.

Cell culture

All cancer cell lines were supplied by the Cancer Center, Sun Yat-sen University, Guangzhou, and cultured with 5% CO_2 at 37°C. The cell lines, including MGC803 (human gastric adenocarcinoma), HepG2 (human hepatoma), KB-3-1 (human oral cancer), HL-60 (human promyelocytic leukemia) and Glc82 (lung cancer), were maintained in RPMI 1640 (Trace Biosciences, Sydney, Australia) supplemented with 10% newborn calf serum (Gibco/BRL, Grand Island, New York, USA) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin). All cell lines were cultured on 10 cm cell culture dishes.

Detection of cytotoxicity by the MTT assay

Cells in logarithmic growth were diluted to 4–5 $\times 10^4$ cells/ml and transferred, in triplicate, into wells of 96-well plates, with a density of 4000–5000 cells/well. On day 0, 100 µl medium with or without analogues was added to the corresponding wells. After 72 h of drug exposure, cytotoxic effects were evaluated using the MTT (3-[4,5-dimethyltiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) assay. The absorbance was read at 570 nm in a microtiter plate reader. The 50% inhibitory

concentration (IC_{50}) values, which represent the drug concentration at which cell growth is inhibited by 50% at the end of drug exposure [18], were calculated by means of Bliss's algorithm.

Inhibition of protein phosphatase *in vitro*

Malachite green assay was used to measure Ser/Thr protein phosphatase activity. In principle, the malachite green molybdate dye/additive mixture can be bound with free phosphate in solution and then the color of the dye solution changes from light yellow to green with an absorption peak at 630 nm. The optical density (OD) value at 630 nm is linearly related to the concentration of free phosphate in solution. Therefore, it is possible to measure the effect of inhibitors on the Ser/Thr protein phosphatase enzyme activity [19].

Purified PP1 (from rabbit skeletal muscle) and PP2A (from human red blood cells), which were stored at −20°C, were diluted with enzyme dilution buffer (20 mmol/l MOPS, pH 7.5, 150 mmol/l NaCl, 60 mmol/l 2-mercaptoethanol, 1 mmol/l $MgCl_2$, 1 mmol/l ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.1 mmol/l $MnCl_2$, 1 mmol/l dithiothreitol, 10% glycerol and 0.1 mg/ml serum albumin). Malachite green (solution A), malachite green additive (solution B), hexapeptide (K-R-pT-I-R-R) substrate (1 mmol/l) and Ser/Thr assay buffer (a vial containing 20 ml of 50 mmol/l Tris–HCl, pH 7.0, 100 mmol/l $CaCl_2$) were stored at 4°C. All reagents were purchased from Upstate Biotechnology (Lake Placid, New York, USA). The assay was performed on 96-well microtiter plates (i.e. 1/2 volume flat-bottom plates).

A malachite green phosphate detection solution was prepared freshly by adding 10 µl of solution B to 1 ml of solution A. The concentration of PP2A or PP1 used in the assay was 0.05 U/well and the concentration of hexapeptide substrate was 250 µmol/l. The reactions were initiated by adding the substrate (5 µl/well) to a mixture containing an enzyme (0.05 U/well), the Ser/Thr assay buffer and an inhibitor (5 µl/well), which produced a total reaction volume of 20 µl/well. This was then incubated at 37°C for 60 min. Before the addition of the substrate, the enzyme and inhibitor were preincubated for 5 min. Reactions were halted by the addition of the malachite green phosphate detection solution (80 µl/well) and the absorbance was read at 630 nm. The blank control wells contained distilled water (10 µl) and buffer (10 µl).

An IC_{50} value that indicates the concentration of the drug required to inhibit enzyme activity by 50% was calculated. Data represent the means of IC_{50} of three independent replicates. PP2A selectivity is represented by the ratio of PP1 inhibition (IC_{50}) to PP2A inhibition (IC_{50}) [5].

Determination of the activity of intracellular protein phosphatase 2A

The PP2A immunoprecipitation phosphatase assay kit was purchased from Upstate Biotechnology. This kit contained anti-PP2A IgG (200 µg of IgG in 200 µl of 0.1 mol/l Tris–glycine, pH 7.4, 0.15 mol/l NaCl, containing 0.05% sodium azide, stored at −20°C) and protein A agarose (provided as 50% gel slurry for a final volume of 1 ml, stored at 4°C).

MGC803 cells were cultured under the above-mentioned conditions. Logarithmically growing cells were plated on 10-cm cell culture dishes (about 5×10^6 cells/dish). The potent analogues **9**, **11** and **13** as well as cantharidin were added and the solvent was used as the control. After the cells were incubated for consecutive 24 h, the cells were scraped off the culture dishes and transferred into 1 ml ice-cold TBS buffer (50 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl), washed three times with TBS, suspended in phosphatase buffer (50 mmol/l Tris–HCl, pH 7.0, 0.1 mmol/l ethylenediamine tetraacetic acid with 10 µg/ml aprotinin leupeptin and 1 mmol/l phenylmethylsulfonyl fluoride) and shock-frozen in liquid nitrogen until further use.

Cellular lysate was prepared by shock-freezing the cells in liquid nitrogen and thawing at 37°C three times, and then centrifuged at 14 000 r.p.m. at 4°C for 10 min. The supernatant was collected, and the total protein concentration was measured by ultraviolet spectrophotometry at 280 nm.

To measure the inhibition of PP2A in MGC803 cells, the total protein (100 µg) was immunoprecipitated with 4 µg anti-PP2A IgG and 30 µl protein A agarose slurry. The reaction volume per sample was added to 500 µl with Ser/Thr assay buffer. The mixture was incubated at 4°C for 2 h with constant rocking. PP2A in the lysate was immunoprecipitated by anti-PP2A IgG and kept in protein A agarose beads slurry. Then, these beads were washed three times with 700 µl TBS, followed by one wash with 500 µl Ser/Thr assay buffer to remove the remaining free phosphate in the slurry.

The enzyme activity reaction was initiated by adding 40 µl of diluted phosphopeptide (final reaction concentration was 500 µmol/l) and 40 µl of Ser/Thr assay buffer. The mixture was incubated at 37°C for 30 min and then centrifuged. The supernatant (25 µl) was transferred into each well of the 1/2 volume microtiter plate and 100 µl of malachite green phosphate detection solution was added into each well. The plate was placed at room temperature for 5 min and the OD was read at 630 nm. The blank wells contained distilled water, buffer and the substrate. The percentage of inhibition of PP2A was determined by comparing the results of the treated groups with those of

the controls, which were the cells cultured without cantharidin analogues [19,20], i.e. inhibition of PP2A (%) = (OD of controls – OD of treated cells)/(OD of controls – blank OD) × 100%.

Determination of the content of intracellular protein phosphatase 2Ac (catalytic subunit)

Treated cells were washed three times with ice-cold phosphate-buffered saline buffer, lysed in a lysis buffer (50 mmol/l Tris–HCl, pH7.5, 150 mmol/l NaCl, 1% NP-40, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin leupeptin) for 20 min and then centrifuged at 12 000 rpm for 10 min at 4°C. Supernatants were stored at −80°C before use.

Total proteins (20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts, USA). Nonstimulated A431 lysate (20 μg) was loaded as a positive antigen control (Upstate Biotechnology). The content of intracellular PP2Ac was detected by immunoblotting with primary antibodies (purified mouse anti-PP2Ac antibodies; Biosciences Pharmigen, USA). Hsp70 was detected by immunoblotting with anti-Hsp70 (Sigma-Aldrich, Shanghai, China) as an internal control. After the polyvinylidene difluoride membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG; Cell Biolabs, San Diego, California, USA), the proteins were visualized by an enhanced chemiluminescence detection kit (Cell Biolabs).

Protein phosphatase 2A enzymatic kinetics assay for the mode of inhibition of cantharidin and 2,3-dimethyl endothall thioanhydrid (analogue 13)

The standard phosphate solution (solution C; Upstate Biotechnology) was diluted with distilled water to make a series of concentrations of phosphate from 200 to 2000 pmol per 25 μl. OD was read at 630 nm in a microtiter plate reader. Subsequently, the OD value was plotted against the concentration of free phosphate for the standard curve, and thus the concentration of liberated phosphate and the velocity of the dephosphorylation reaction could be calculated.

The velocity of enzymatic dephosphorylation was measured by the malachite green assay in the presence of inhibitors as previously described. Four concentrations of each inhibitor were used: 0, 0.0625, 0.125 and 0.25 μmol/l for cantharidin, and 0, 0.625, 1.25 and 2.5 μmol/l for analogue **13**. The reaction speed was examined when the concentration of hexapeptide (K-R-pT-I-R-R) substrate was 100, 150, 225, 325 and 450 μmol/l, respectively. The reactions were initiated by adding the substrate to a mixture containing the enzyme (0.03 U/well), the Ser/Thr assay buffer and one of the inhibitors. Data were represented by the means of four independent replicates and Lineweaver–Burk plots were then applied according to the Michaelis–Menten equation. The reciprocal of velocity ($1/V$) was plotted as the ordinate with the reciprocal of the concentration of substrate (1/[S]) as the abscissa. The characteristics of inhibition can be recognized on a Lineweaver–Burk plot, as the noncompetitive inhibition alters the intercept on the ordinate (i.e. V_{max} is decreased), while leaving the intercept on the abscissa unchanged, and vice versa [21].

Results

In-vitro cytotoxicity assay

The structural modifications of these agents influenced their cytotoxicity (Table 1). Like cantharidin, analogue **13** exhibited strong cytotoxicity to all cancer cell lines studied. Like norcantharidin, analogues **9** and **11** showed relatively weak cytotoxicity to KB-3-1, MGC803 and HepG2 cell lines. Analogue **12** showed slight cytotoxicity to the KB-3-1 cell line only. Other analogues showed very poor cytotoxicity ($IC_{50} > 200$ μmol/l). Regarding the sensitivity of the cancer cell lines, KB-3-1 (human oral cancer) was the most sensitive to the cantharidin analogues. HepG2 (human hepatoma), however, did not show particular sensitivity to these analogues, although norcantharidin is clinically used to treat hepatoma. The sensitivity of MGC803 (human gastric adenocarcinoma) was below the average level.

Inhibition of protein phosphatase *in vitro*

Cantharidin and norcantharidin were potent PP2A inhibitors as expected (Table 2). Analogues with the modifications of dicarboxylic acid anhydride that showed cytotoxicity also showed inhibitory activity to protein phosphatase. Analogue **13** exhibited potent inhibitory activity. Analogues **9**, **11** and **12** showed weak inhibition to phosphatases, while analogues **7**, **8** and **10** did not show any inhibitory activity (Table 2). It appeared that

Table 1 Cytotoxicity of cantharidin analogues to a panel of cancer cell lines after 72 h of continuous exposure

	Cytotoxicity (IC_{50}, μmol/l) to different cancer cell lines				
Agents	KB-3-1	MGC803	HepG2	HL-60	Glc82
Cantharidin	2.7	2.8 ± 0.6	19.1	2.7	1.2
Norcantharidin	69.5 ± 8.5	161.9 ± 56.9	212.9 ± 26.2	15.7	5.3
1	>200	>200	>200	>200	>200
2	>200	>200	>200	>200	>200
3	>200	>200	>200	>200	>200
4	>200	>200	>200	>200	>200
5	>200	>200	>200	>200	>200
6	>200	>200	>200	>200	>200
7	>200	>200	>200	>200	>200
8	>200	>200	>200	>200	>200
9	79.2	123.6	218.5	103.2	92.1
10	>200	>200	>200	>200	>200
11	56.2	94.9	155.8	>200	>200
12	112.0 ± 5.5	>200	>200	>200	>200
13	1.8 ± 0.2	1.3 ± 0.1	14.7 ± 7.0	1.1	2.0

IC_{50} is the drug concentration effectively inhibiting cell growth by 50% as measured by the MTT assay after 72 h of drug exposure.

Table 2 **Inhibition of protein phosphatases 1 (PP1) and 2A (PP2A) *in vitro* by modified cantharidin analogues**

Analogues	Inhibition of PP2A (IC_{50}, μmol/l)	Inhibition of PP1 (IC_{50}, μmol/l)	PP2A selectivity[a]
Cantharidin	0.28 ± 0.05	2.24 ± 0.42	8.0
Norcantharidin	0.79 ± 0.20	2.59 ± 0.68	3.3
1	> 200	> 200	–
2	> 200	> 200	–
3	> 200	> 200	–
4	> 200	> 200	–
5	> 200	> 200	–
6	> 200	> 200	–
7	> 200	> 200	–
8	> 200	> 200	–
9	23.4 ± 5.4	39.4 ± 4.2	1.7
10	> 200	> 200	–
11	34.4 ± 4.3	96.4 ± 11.3	2.8
12	62.2 ± 10.1	99.9 ± 8.0	1.6
13	2.11 ± 0.26	3.05 ± 1.05	1.4

IC_{50} is the drug concentration effectively inhibiting serine/threonine protein phosphatase activity by 50% as measured by the malachite green assay.
[a]PP2A selectivity is represented by the ratio of inhibition of PP1 (IC_{50}) over inhibition of PP2A (IC_{50}).

analogues with additive side-chain at the 1-C position (i.e. analogues **1–6**) showed no inhibition to either PP2A or PP1 (all $IC_{50} > 200$ μmol/l). In conclusion, the cytotoxicity of the analogues to the cells was probably associated with their inhibitory activity on protein phosphatase. Unlike cantharidin, however, none of the candidate analogues demonstrated particular selectivity to PP2A (Table 2).

Influence of cantharidin analogues on intracellular protein phosphatase 2A activity

To determine the influence of the potent analogues on the activity of intracellular PP2A, the activity of PP2A in the cytoplasm of MGC803 cells was examined after these cells were treated with cantharidin and analogues **9**, **11** and **13** for 24 h. As shown in Fig. 2, cantharidin and analogues **9**, **11** and **13** inhibited intracellular PP2A activity in a dose-dependent manner.

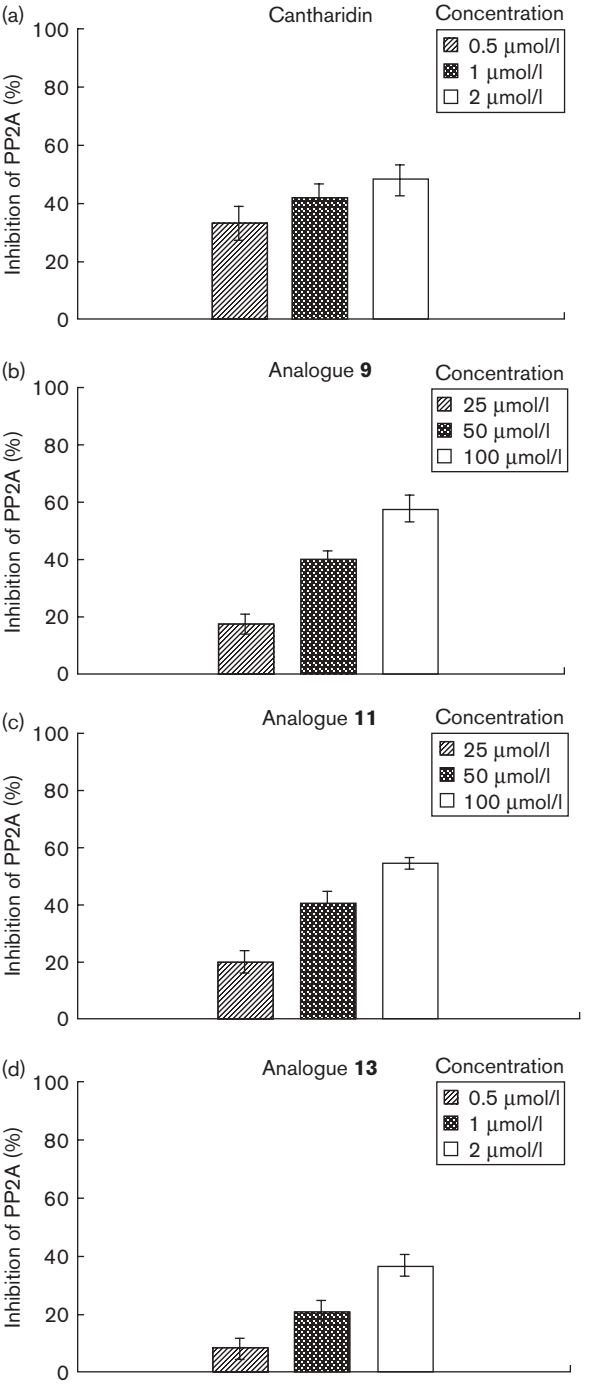
Influence of cantharidin analogues on the expression of intracellular protein phosphatase 2Ac (catalytic subunit)

After treatment with cantharidin and analogue **13** for 24 h, MGC803 cells did not show any visible changes in the expression of PP2Ac (catalytic subunit) (Fig. 3).

Mode of inhibition of cantharidin and analogue 13 on protein phosphatase 2A

On the Lineweaver–Burk plot, the mode of inhibition was identified as noncompetitive. This was demonstrated by the result that the slope of the experimental line was increased and the intercept on the ordinate was altered (i.e. V_{max} was decreased), but the intercept on the abscissa remained unchanged (i.e. K_m remained constant) (Fig. 4).

Fig. 2

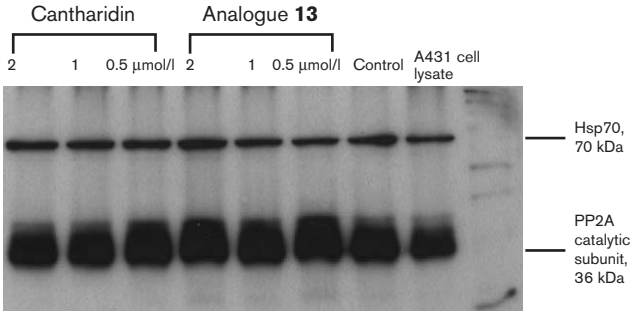


MGC803 cells were treated with various concentrations of cantharidin (a) and analogues **9** (b), **11** (c) and **13** (d). After cells were cultured for 24 h, intracellular PP2A was inhibited in a dose-dependent manner. Inhibition of PP2A (%) = (OD of controls – OD of treated cells)/(OD of controls – blank OD) × 100%. Data are mean ± SD of three determinations. PP2A, protein phosphatase 2A; OD, optical density.

Discussion

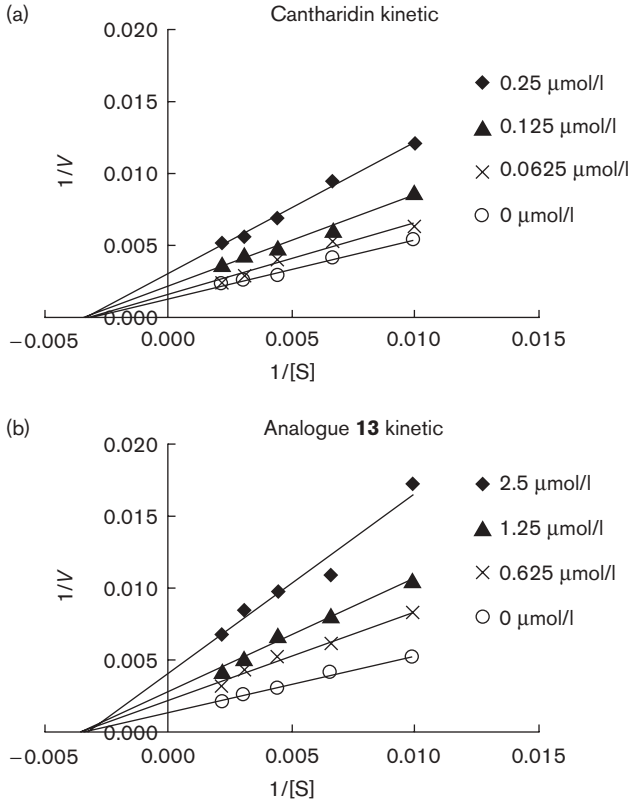
Although cantharidin possesses anti-tumor activity, it is too toxic to be used clinically as an internal medicine. Its analogues, however, may be potential candidates for anti-cancer drugs with fewer and milder side-effects. It has been reported that the toxicity of cantharidin is

Fig. 3



MGC803 cells were incubated with cantharidin or analogue **13** for 24 h. Total proteins (20 μg of) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Nonstimulated A431 lysate (20 μg) was loaded as a positive antigen control. Immunoblotting was conducted by purified mouse anti-PP2Ac antibodies (1:200). Hsp70 was detected by immunoblotting with anti-Hsp70 antibodies (1:20 000) as an internal control and horseradish peroxidase-conjugated secondary antibodies (1:2000). PP2A, protein phosphatase 2A.

Fig. 4



The mode of inhibition of cantharidin (a) and analogue **13** (b) on PP2A. The lines of the reciprocal of reaction velocity (pmol/h) against the reciprocal of concentration (μmol/l) of substrate were plotted, with the reciprocal of velocity (1/*V*) as the ordinate and the reciprocal of the concentration of substrate (1/[S]) as the abscissa. The synthetic phosphorylated hexapeptide (K-R-pT-I-R-R) was used as the substrate. PP2A, protein phosphatase 2A.

attributable to its binding affinity to PP2A, as PP2A is the key enzyme to reverse phosphorylation; the inhibition of PP2A will break the balance of reversible phosphorylation and will thus affect cell growth [9]. The structural modifications of cantharidin focusing on its inhibitory activities for PP2A have been explored, but there has been limited success so far [13–17].

In the present study, it was observed that subtle structural modifications of cantharidin brought significant alterations in its cytotoxicity against a panel of cancer cell lines. It was also observed that the cytotoxicity of cantharidin and some analogues appeared to be associated with their protein phosphatase inhibitory activities. Moreover, cantharidin and these analogues (i.e. analogues **9**, **11** and **13**) inhibited intracellular PP2A activity in a dose-dependent manner. These findings indicate that the cytotoxicity of these analogues, like that of cantharidin, is likely to be mediated by PP2A inhibition. It was found that the inhibitory activity of analogue **13** on intracellular PP2A was slightly weaker than that of cantharidin, although the two agents showed similar cytotoxicity. Therefore, it is possible that several factors, including the intracellular bio-modification, degradation and/or other intracellular targets, contribute to cytotoxicity. Previous studies have shown that multiple mechanisms are involved in cantharidin or norcantharidin-induced cytotoxicity [22,23]. Thus, it indicates that we cannot preclude the possibility that analogue **13** interacts with other intracellular targets.

The present study showed that a series of skeletally modified cantharidin analogues (**1–6**) with an additive side-chain at the 1-C position lost their cytotoxicity and the PP2A inhibitory activity. It has been demonstrated by molecular modeling analysis that 7-O of cantharidin can approach PP2A and form H-binding with PP2A [15], which indicates that substitution at the 1-C position may prevent cantharidin analogues from binding with PP2A. Modifications of the anhydride structure, however, might maintain the activity of cantharidin. Results demonstrated that analogues **9**, **11** and **13** exhibited PP2A inhibitory activity and effective cytotoxicity ($IC_{50} < 200$ μmol/l) to a panel of cancer cell lines.

A previous modeling analysis showed that the ring-opened dicarboxylic acid form of cantharidin could interact with PP2A and predicted that the anhydride ring opening is crucial for the inhibition of PP2A [15]. As demonstrated in the present study, analogues **9** and **11**, which have at least one free carboxylate, showed inhibitory activity on PP2A. On the contrary, analogue **10**, which has a ring-closing structure, showed little PP2A inhibitory activity. These observations indicate that ring-opened forms may contribute to the PP2A inhibitory activity of anhydride-modified analogues.

Analogue **8** is the leading compound of analogue **9**. It has a cyano instead of a hydroxy. Compared with analogue **9**, analogue **8** completely loses cytotoxicity and the inhibitory activity on PP2A. Therefore, the presence of only the ring opening and a carboxylate does not guarantee the activity of a cantharidin analogue, and the inhibitory activity can be influenced by other adjacent substitutions.

Analogue **12** showed weak phosphatase inhibitory activity and weak cytotoxicity to the KB-3-1 cell line. Interestingly, analogue **12** has a ring-closing monoester structure. We do not know, however, whether this ring is open under our assay conditions, as at least one free carboxylate is also required for the activity. Analogue **13** has been shown to be a highly potent inhibitor of PP2A which has strong cytotoxicity. In analogue **13**, the oxygen atom linking the two acid residues of anhydride is replaced by sulfur (Fig. 1), which is an active atom that is likely to bind to PP2A like a free carboxylate.

PP2Ac (catalytic subunit) can exert dephosphorylation activity alone [24] and thus the content of intracellular PP2Ac influences intracellular PP2A activity. In the present study, it was found that the treatment with cantharidin and analogue **13** for 24 h did not affect the content of intracellular PP2Ac, although they inhibited the activity of PP2A in the cytoplasm, indicating that these PP2A inhibitors do not interfere with the expression of intracellular PP2Ac. Further studies, however, are required to confirm these observations.

In addition, we determined the PP2A enzymatic kinetics of cantharidin and analogue **13** to determine their mode of inhibition on PP2A, which will help one understand how cantharidin and analogue **13** bind to PP2A. We showed that analogue **13** is a noncompetitive inhibitor of PP2A like cantharidin.

In conclusion, anhydride modifications of cantharidin may maintain its cytotoxicity and its inhibitory activity on PP2A, while skeletal modifications of cantharidin at the 1-C position abolish its cytotoxicity and PP2A inhibitory activity. PP2A inhibitory activity of synthetic cantharidin analogues is correlated with their cytotoxicity. Cantharidin and the potent analogue **13** inhibit PP2A via a noncompetitive mechanism. Further experiments are required to determine specificity of the cytotoxicity of analogue **13,** i.e. whether analogue **13** is toxic only to cancer cells and not to normal cells.

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