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Modified norcantharidins: synthesis, protein phosphatases 1 and 2A inhibition, and anticancer activity

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Abstract—Fourteen modified norcantharidin analogues have been synthesised and screened for their ability to inhibit the serine/threonine protein phosphatases 1 and 2A. The most potent compounds found were **10** (PP1 $IC_{50} = 13 \pm 5$ μM; PP2A $IC_{50} = 7 \pm 3$ μM) and **16** (PP1 $IC_{50} = 18 \pm 8$ μM; PP2A $IC_{50} = 3.2 \pm 0.4$ μM). Overall, only analogues possessing at least one acidic residue at the former anhydride warhead displayed any PP1 or PP2A inhibitory action. The ability of these analogues to inhibit PP1 and PP2A correlates well with their observed anti-cancer activity against a panel of five cancer cell lines: A2780 (human ovarian carcinoma), G401 (human kidney carcinoma), HT29 (human colorectal carcinoma), H460 (human lung carcinoma) and L1210 (murine leukemia).

The concept that protein kinases are the 'on-switch' while protein phosphatases are the 'off-switch' for signaling mechanisms is a naïve understanding of the action of both enzyme families. There are three major classes of protein phosphatases: tyrosine-specific, serine/threonine-specific, and dual-specificity phosphatases. However, we have focused on the serine-threonine family because of their ability to influence cell cycle control and growth. Traditionally, the serine-threonine, family has been classified into four subtypes based on their biological characteristics, sensitivities to specific inhibitors, and substrate specificity (PP1, PP2A, PP2B, and PP2C). Phosphorylation of structural and regulatory proteins is a major cell cycle control mechanism. Cell cycle abnormalities characterise malignancy. An intricate phosphorylation network involving interplay between kinases and phosphatases regulates the cell cycle.[1–3]

The role of protein phosphatases in the cell cycle is illustrated by the effect of gene mutations observed in yeast and drosophila.[1,4] Loss of function mutations in both PP1 and PP2A genes lead to a variety of defects in mitosis. In yeast PP1 mutants are unable to complete anaphase successfully, and are unable to instigate chromosome segregation, while PP1 over expression is lethal. In drosophila, PP1 mutants die at the larval-pupal boundary as a result of defective spindle organisation, abnormal sister chromatid segregation, hyperploidy and excessive chromosome condensation, as well as a delay in progression through mitosis. In yeast PP2A deficient mutants are not viable, however, mutants lacking one of the PP2A subunits display defects in cell septation and separation and the cells become multinucleated, while in drosophila it leads to abnormal anaphase resolution.

PP1 and PP2A exert their effects by modulating the activity of cyclin dependent kinases (cdk) and the retinoblastoma protein (pRb).[5,6] The activation of cdk/cyclin complexes requires the phosphorylation of a conserved threonine residue, as well as the removal of inhibitory phosphorylations. PP2A inhibits the activation of cdk/cyclin complexes by directly dephosphorylating cdks or indirectly by influencing upstream

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(CAK, Wee1, Myt) and downstream kinases/phosphatases (Cdc25). The pRb protein controls the movement of cells into the S-phase of the cell cycle and is regulated by PP1 and PP2A. Hyperphosphorylation of pRb releases the transcription factor E2F stimulating entry into the DNA synthesis phase of the cell cycle. PP1 and PP2A inhibit this entry by maintaining pRb in a dephosphorylated state. Other cell cycle events controlled by PP1 and PP2A include chromosome condensation, nuclear membrane disintegration, reorganisation of cytoplasmic microtubules, spindle formation, chromatid separation, nuclear membrane reassembly and cytokinesis. Abnormalities in these stages together with abnormal cell cycle movement and checkpoint abrogation have been described for various phosphatase inhibitors including cantharidin (blister beetles detailed below), fostriecin (streptomyces *pulveraceus*), okadaic acid (dinoflagellates), calyculin A (marine sponge *Discodermia calyx*), microcystin-LR (blue-green algae), and tautomycin (s*treptomyces spiroverticillatus*).[7–9] In particular, fostriecin and cantharidin have been shown to force cells prematurely through the cell cycle and into mitosis with multiple aberrant mitotic spindles subsequently inducing apoptotic cell death.[10,11]

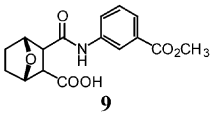
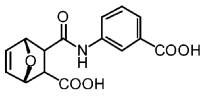
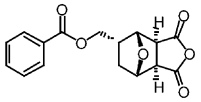
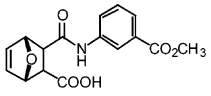
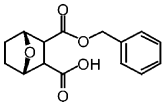
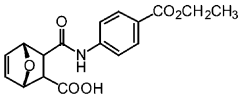
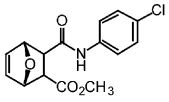
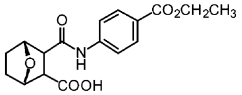
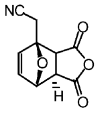
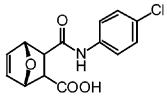
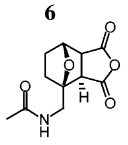
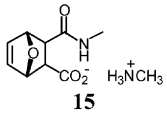
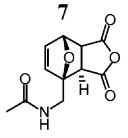
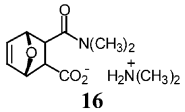
Norcantharidin, the demethylated analogue of cantharidin is also a PP2A and PP1 inhibitor and has been used in the treatment of primary hepatoma and upper gastrointestinal carcinomas, and it does not display the nephrotoxicity of cantharidin. We have shown the growth inhibition of norcantharidin (GI_{50} 13–47 μM) in the cancer cell lines listed in Table 2 to be slightly less than that of cantharidin.[12] Norcantharidin increased the mean survival time of 285 reported cases with primary hepatoma from 4.7–11.1 months, and the 1 year survival rate from 17–30%, as compared to 102 patients treated with conventional chemotherapy (5FU, hydroxycamptotherine, vincristine, thiophosphoramide and mitomycin).[13] As with cantharidin, norcantharidin not only failed to induce myelosuppression but also induced haemopoiesis via bone marrow stimulation.[14] This in vivo response was transient, lasting one week with the white blood count returning to normal following chronic administration. Interestingly, in mice, norcantharidin has been shown to block the leukopenia caused by cyclophosphamide, therefore, such an agent may antagonise myelosuppression induced by other chemotherapeutic agents.

There has been intense interest in developing potent and selective inhibitors of PP1 and PP2A in recent years. These attempts have included numerous analogues of cantharidin (so far with little success)[15] and the more complex toxins such as the Microcystin analogues, which resulted in moderate PP1 selectivity.[16] We too, have had an ongoing interest in cantharidin analogues, an interest that was further heightened with the discovery of fostriecin as a selective and potent PP2A inhibitor with powerful anticancer activity. We and previous researchers have shown that cantharidin brooks little in the way of modification whilst retaining activity against PP1 and PP2A.[15] Permissible modifications include removal of the methyl substituents; anhydride ring opening (as cantharidin binds as cantharidic acid); anhydride ring opening and mono-esterification. Modification of the bicyclo-skeleton is detrimental to PP1 and PP2A inhibition. Our more recent efforts have involved the synthesis of anhydride modified norcantharidin analogues: both ring opened ester analogues and the cantharimides, both of which maintained good levels of PP1 and PP2A inhibition. As a result we postulated that the inclusion of a single carboxylate and an ability to access the acidic groove of PP1 and PP2A were beneficial to inhibition. Accordingly our minimalist pharmacophore explores the effects of the amide NH of the cantharimides, and the single carboxylate associated with our ring opened analogues was also incorporated whilst maintaining other key features known to be crucial for activity. We thus developed a new series of cantharidin analogues and explored both their PP1 and PP2A inhibition and anticancer activities.

Each of the bicyclic norcantharidin analogues was prepared by minor modification of the published Diels–Alder conditions reported for the synthesis of **3**,[17] followed in some cases by catalytic hydrogenation of the resultant 5,6-dehydro adducts, and/or opening of the respective anhydride rings with amine or alcohol nucleophiles.[18] For example, the prototype compound, 5,6-dehydronorcantharidin, was prepared on a 25 g scale by the addition of furan (0.393 mol) to a stirred suspension of maleic anhydride (0.159 mol) in 100 mL of ethyl ether at ambient temperature; after 3 days the resultant white precipitate was collected by vacuum filtration, triturated briefly with more ether, and dried under vacuum to give 5,6-dehydronorcantharidin in 96% yield. All other cycloadditions were carried out similarly, with reaction times and yields varying depending on the furan substituents.[18] As with this representative example, the respective cycloadducts generally precipitated during the reaction, giving predominantly or exclusively the *exo* isomer as the isolated product. For cases in which small amounts of *endo* isomer or other impurities were detected in the proton NMR of the isolated products, one recrystallisation from ethyl ether provided pure material for testing. Catalytic hydrogenations of the 5,6-alkene bonds were carried out as previously described for similar analogues,[17] and the half-acid derivatives **4** and **9**–**16** were obtained by treating the corresponding anhydrides with the appropriate free amine or alcohol.[18]

The norcantharidin analogues, **3**–**16**, were screened for their ability to inhibit PP1 and PP2A.[12,19] Cantharidin (**1**) and norcantharidin (**2**) were included as internal standards. The results of the phosphatase inhibition study are shown in Table 1. We note that analogues **4**–**8** were inactive against either both PP1 and PP2A. This finding is in keeping with previous reports in this area and our more recent development of the cantharimides, which suggests that at least one free carboxylate is required in the absence of the anhydride group.[15e] For example, analogue **5** (–$COOCH_3$) IC_{50}s > 100 μM, but **14** (–COOH) displayed PP1 IC_{50} = 49μM and PP2A IC_{50} = 9.2 μM, a minimum of a 10-fold increase in

Table 1. Inhibition of protein phosphatases 1 and 2A by compounds **1**–**16**[a]

Compd	PP1 Inhibition	PP2A Inhibition	Compd	PP1 Inhibition	PP2A Inhibition
	IC_{50} (μM)			IC_{50} (μM)	
 **1**	0.43±0.10	0.6±0.12	 **9**	16±2	7.7±1.3
 **2**	0.57±0.18	0.96±0.33	 **10**	13±5	7.0±3.0
 **3**	25±6	13±3	 **11**	60±23	12±2
 **4**	>100	>100	 **12**	76±24	21±6
 **5**	>100	>100	 **13**	29±11	9.2±2.9
 **6**	>100	>100	 **14**	49±9	9.2±1.0
 **7**	>100	>100	 **15**	>100	>100
 **8**	>100	>100	 **16**	18±8	3.2±0.4

[a] Average of three experiments in duplicate.

potency. Closer scrutiny revealed that this may be an oversimplification of the enzyme requirements with **4** being inactive whilst displaying a free carboxylate. The SAR data presented herein also suggests amide NH is required. In our previous studies the free amine of the cantharimides fulfils this requirement.[15e] Of greater interest were analogues **9**–**14** and **16**, which displayed stronger PP1 and PP2A inhibition; note that in each of these analogues there is a minimum of one free carboxylate. The inhibition in this group of analogues varied, in the case of PP1, from 13–76 μM; and for PP2A from 3.2–21 μM. The lead compounds cantharidin (0.43 and 0.6 μM) and norcantharidin (0.57 and 0.96 μM) were somewhat more potent against PP1 and PP2A, respectively. Analogue **15** appears to be an anomaly, displaying neither PP1 nor PP2A inhibition, whist the dimethyl analogue, **16**, showed quite potent PP1 (18 μM) and PP2A (3.2 μM) inhibition (Pombo-Villar has previously observed that the dimethyl analogue is a 0.18 μM PP2A inhibitor[15b]). Taken together these data support a minimum hydrophobicity requirement for the amide in anhydride modified cantharidin analogues (greater than methyl).

Introduction of a second carboxylate, in the amide substituent, resulted in a modest improvement in potency; **9** PP1 $IC_{50}=16$ μM, PP2A $IC_{50}=7.7$ μM, **11** PP1 $IC_{50}=60$ μM, PP2A IC_{50} 12 μM) versus **10** PP1 $IC_{50}=13$ μM, PP2A $IC_{50}=7$ μM. The presence of the 5,6-double bond appeared to be detrimental to inhibition, more so with PP1 than PP2A, for example **12** PP1 $IC_{50}=76$ μM, PP2A $IC_{50}=21$ μM versus **13** PP1 $IC_{50}=29$ μM, PP2A $IC_{50}=9$ μM, and **9** PP1 $IC_{50}=16$ μM, PP2A $IC_{50}=7.7$ μM versus **11** PP1 $IC_{50}=60$ μM, PP2A $IC_{50}=12$ μM. This may be a result of the greater instability associated with the unsaturated species (a greater propensity to undergo a retro-Diels–Alder). Over all aromatic rings were well tolerated.

Table 2. Growth inhibition (GI_{50}, μM) of various tumour cell lines after 72 h continuous exposure to test compounds **1**–**16**

Compd	Tumour cell line[b]				
	A2780	G401	HT29 GI_{50} μM[a]	H460	L1210
1	10±2	3.5±0.3	6.4±0.7	4.3±0.9	15±2
2	39±5	35±2.3	33±7	50±4	13±0.3
3	30±8.0	53±7.4	57±7.7	43±1.5	49±5.8
4	81±1.0	>100	>100	>100	>100
5	98±2.5	>100	>100	>100	>100
6	85±5.0	>100	>100	>100	>100
7	95±5.0	>100	>100	>100	>100
8	98±2.5	>100	>100	>100	>100
9	90±0.1	>100	>100	>100	>100
10	82±10	>100	>100	>100	>100
11	97±3.3	>100	>100	>100	>100
12	83±8.8	>100	>100	>100	>100
13	80±5.8	>100	>100	>100	>100
14	87±7.3	90±5.8	>100	>100	>100
15	88±7.3	>100	89±0.8	>100	>100
16	40±10	25±1.5	49±15	41±9.2	48±1.5

[a] GI_{50} is the concentration that induces 50% growth inhibition compared with untreated control cells.
[b] A2780 (Human ovarian carcinoma), G401 (human kidney carcinoma), HT29 (human colorectal carcinoma), H460 (human lung carcinoma), L1210 (murine leukemia).

In view of the anticancer activity and clinical use of cantharidin, norcantharidin and fostriecin we also conducted cytotoxicity studies in a number of tumour cell lines, and the results from these studies are shown in Table 2.

Growth inhibition was evaluated using the MTT assay.[15] From Table 2 it was apparent that cantharidin (**1**) and norcantharidin (**2**) were potent growth inhibitory compounds in all cell lines tested.[2] Of the synthesized analogues, compounds **4**–**15** displayed minimal growth inhibition with GI_{50}s ranging from 80 μM to >100 μM. However, compounds **3** and **16** showed strong growth inhibition with GI_{50} values comparable to that of norcantharidin. Interestingly, compound **16** was the most potent PP2A inhibitor, suggesting that this was the mode of action leading to impaired cellular replication. Compound **3** on the other hand was less potent at inhibiting PP1/PP2A activity in vitro, however, the ester linkage in this compound is susceptible to intracellular enzymatic cleavage resulting in the potential production of a more potent intracellular PP1/PP2A inhibitor. Nonetheless, alternative modes of cell growth inhibition for this analogue can not be excluded. Similarly, variations in cellular uptake, membrane permeability, drug stability and drug metabolism may account for the lack of cell growth inhibition in those compounds that were moderate PP2A inhibitors such as compounds **9**, **10**, **13**, and **14**.

This study clearly illustrates the ability to produce modified cantharidin and norcantharidin analogues while maintaining protein phosphatase inhibitory activity and cell growth inhibition. This study provides the basis for further development of this class of protein phosphatase inhibitors for the treatment of malignancy.

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18. The Diels–Alder cycloadditions giving the respective 5,6-dehydronorcantharidin ring systems, and in some cases subsequent catalytic hydrogenations, were carried out essentially as described in ref 16. Half-acids were prepared by treating the appropriate anhydrides directly with alcohols or amines. Starting materials, reagents, reaction times, and yields for each analogue are summarised below: **3**: Prepared by the literature procedure, ref 17. **4**:

From **2**; benzyl alcohol, THF, DMAP, 60 °C, 31% yield. **5**: From **14**; $TMSCHN_2$, EtOAc/MeOH, 0 °C, 66% yield. **6**: Prepared by the general procedure described in the text, from 3-cyanomethylfuran and maleic anhydride; 19% yield. **7**: From **8**; H_2, Pd/C, THF, 20 °C, 100% yield. **8**: Prepared by the general procedure described in the text, from *N*-acetyl-2-(aminomethyl)-furan and maleic anhydride; 23% yield. **9**: From **2**; methyl *m*-aminobenzoate, THF, 20 °C, 88% yield. **10**: From **11**; LiOH in aqueous THF, −5 °C, 35% yield. **11**: From 5,6-dehydronorcantharidin; methyl *m*-aminobenzoate, THF, 20 °C, 24 h, 88% yield. **12**: From 5,6-dehydronorcantharidin; ethyl *p*-aminobenzoate, THF, 20 °C, 48 h, 83% yield. **13**: From **2**; ethyl *p*-aminobenzoate, THF, 20 °C, 48 h, 93% yield. **14**: From **2**; *p*-chloroaniline, CH_2Cl_2, Et_3N, 20 °C, 12 h, 80% yield. **15**: From 5,6-dehydronorcantharidin; 2 equiv methylamine, THF, 20 °C, 12 h, 100% yield. **16**: From 5,6-dehydronorcantharidin; 2 equiv dimethylamine, THF, 20 °C, 12 h, 100% yield.

19. **Protein phosphatase inhibition:** A non-radioactive in vitro assay detailed by Gupta was adopted to measure PP1 and PP2A enzyme activity in the presence of inhibitor drugs (Gupta, V.; Ogawa, A. K.; Du, X.; Houk, K. N.; Armstrong, R. W. *J. Med. Chem.* **1997**, *40*, 3199.). Serine/threonine protein phosphatase assay kit, purified PP1 (rabbit skeletal muscle), PP2A (human red blood cells) and a hexapeptide (Lys-Arg-pThr-Ile-Arg) substrate was purchased from Upstate Biotechnology (Lake Placid, NY). The concentration of PP2A, PP1 and substrate used in the assay was 0.3 mU/well, 30 mU/well, and 200 μM, respectively. The reactions were initiated by addition of substrate (5 μL) to a mixture containing enzyme (5 μL), reaction buffer (10 μL; 50 mM Tris–HCL, pH 7.0, 100 μM $CaCl_2$), and inhibitor (10 μL), producing a total reaction volume of 30 μL/well and incubated at room temperature for 60 min. Immediately prior to addition of substrate, the enzyme and inhibitor were preincubated for 10 min. Reactions were halted via addition of a malachite green solution (50 μL), and absorbance readings were taken at 650 nm after 10 min development time. Samples were blanked against wells containing enzyme (5 μL), and buffer (25 μL). Initial inhibitor dilutions were made in DMSO and subsequent dilutions were made in distilled deionised H_20. Enzyme dilutions were made with buffer containing 20 mM MOPS, pH 7.5, 0.15M NaCl, 60 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 2 mM EGTA, 0.1 mM $MnCl_2$, 1 mM DTT, 10% glycerol and 0.1 mg/mL serum albumin. A dose–response curve of percentage enzyme activity versus drug concentration was produced from which an IC_{50} value was calculated indicating the concentration of drug required to inhibit enzyme activity by 50%. Data represent the mean (±SEM) IC_{50} of three independent replicates. **Cell culture and stock solutions.** Stock solutions were prepared as follows and stored at −20 °C: Cantharidin (Biomol, USA) and cantharidin analogues as 5 mM solutions in DMSO. All cell lines were cultured at 37 °C, under 5% CO_2 in air. The A2780 (human ovarian carcinoma) and HT29 (human colon carcinoma) cell lines were maintained in DMEM (Trace Biosciences, Australia) supplemented with 10 mM sodium bicarbonate. L1210 (murine leukaemia) cells were maintained in RPMI 1640 (Trace Biosciences), while G401 (human kidney carcinoma), were maintained in McCoys (Trace Biociences). The H460 (human lung carcinoma) cell line was maintained in RPMI 1640 supplemented with glucose and pyruvate. All culture media was further supplemented with foetal bovine serum (10%) penicillin (100 IU/mL), streptomycin (100 μg/mL), and glutamine (4 mM). Cells were passaged every 3–7 days and all cell lines were routinely tested and found to be mycoplasma free. **Cytotoxicity assay**. Cells in logarithmic growth were transferred to 96-well plates. Cytotoxicity was determined by plating cells in triplicate in 100 μL medium at a density of 2,500–3,500 cells/well for all cell lines. On day 0, (24 h after plating) when the cells were in logarithmic growth, 100 μL medium with or without the test agent was added to each well. After drug exposure growth inhibitory effects were evaluated using the MTT (3-[4,5-dimethyltiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) assay and absorbance read at 540 nm The GI_{50} was the drug concentration at which cell growth is 50% inhibited based on the difference between the optical density values on the day 0 and those at the end of drug exposure (Sakoff, J. A.; Ackland, S. P. *Cancer Chem. Pharm.* **2000**, *46*, 477.)