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ScienceDirect
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 3392–3397

Heterocyclic substituted cantharidin and norcantharidin analogues—synthesis, protein phosphatase (1 and 2A) inhibition, and anti-cancer activity

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Received 10 February 2007; revised 21 March 2007; accepted 29 March 2007
Available online 2 April 2007

Abstract—Norcantharidin (**3**) is a potent PP1 (IC_{50} = 9.0 ± 1.4 μM) and PP2A (IC_{50} = 3.0 ± 0.4 μM) inhibitor with 3-fold PP2A selectivity and induces growth inhibition (GI_{50} ~45 μM) across a range of human cancer cell lines including those of colorectal (HT29, SW480), breast (MCF-7), ovarian (A2780), lung (H460), skin (A431), prostate (DU145), neuroblastoma (BE2-C), and glioblastoma (SJ-G2) origin. Until now limited modifications to the parent compound have been tolerated. Surprisingly, simple heterocyclic half-acid norcantharidin analogues are more active than the original lead compound, with the morphilino-substituted (**9**) being a more potent (IC_{50} = 2.8 ± 0.10 μM) and selective (4.6-fold) PP2A inhibitor with greater in vitro cytotoxicity (GI_{50} ~9.6 μM) relative to norcantharidin. The analogous thiomorpholine-substituted (**10**) displays increased PP1 inhibition (IC_{50} = 3.2 ± 0 μM) and reduced PP2A inhibition (IC_{50} = 5.1 ± 0.41 μM), to norcantharidin. Synthesis of the analogous cantharidin analogue (**19**) with incorporation of the amine nitrogen into the heterocycle further increases PP1 (IC_{50} = 5.9 ± 2.2 μM) and PP2A (IC_{50} = 0.79 ± 0.1 μM) inhibition and cell cytotoxicity (GI_{50} ~3.3 μM). These analogues represent the most potent cantharidin analogues thus reported.

Protein phosphorylation and dephosphorylation is a fundamental process for regulating cellular signaling pathways and is mediated by a delicate interplay between kinases and phosphatases. The protein phosphatases 1 (PP1) and 2A (PP2A) comprise ~1% of all cellular proteins and account for 90% of all serine/threonine protein phosphatase activity.[1–4] PP2A has a heterotrimeric architecture with the core PP2A enzyme consisting of a 65-kDa (A)-subunit and a 36-kDa catalytic (C) subunit. This AC core enzyme binds to at least 18 different regulatory subunits (B) which are implicated in regulating cellular localization, substrate specificity, and enzymatic activity.[5–9] Evidence is accumulating that PP2A is a pivotal mediator of many, if not all, cellular processes.[10–13]

There are numerous natural product inhibitors of PP1 and PP2A as exemplified by the okadaic acid class of compounds. Okadaic acid, tautomycin, calyculin A, and the microcystins are highly complex nanomolar potent inhibitors of both PP1 and PP2A, typically displaying modest levels of PP2A selectivity (~100-fold at best), with tautomycin the exception being ~4-fold PP1 selective.[3,4,14] Fostriecin (**1**), isolated from a bacterial broth, is the most selective PP2A (vs PP1) inhibitor yet reported (~40,000-fold PP2A selective).[15,16] Interestingly fostriecin has undergone considerable evaluation as a potential anti-cancer agent, and as yet no successful outcome has been reported, however most of these studies were compromised by poor compound supply, quality, and/or rapid in vivo deactivation. Cantharidin (**2**), the active component of Spanish Fly, is another toxin that inhibits both PP1 and PP2A albeit at higher concentrations.[3] Like fostriecin, cantharidin has been evaluated and indeed utilized as an anti-cancer agent. *Mylabris*, a natural source of cantharidin, was first reported as an anti-cancer agent in 1264.[3]

Keywords: Cantharidin; Norcantharidin; Small molecule protein phosphatase inhibitors; PP1; PP2A; Anti-cancer.
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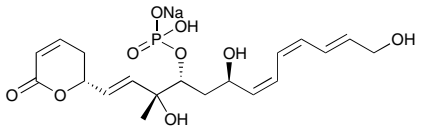
0960-894X/$ - see front matter
doi:10.1016/j.bmcl.2007.03.093



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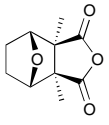

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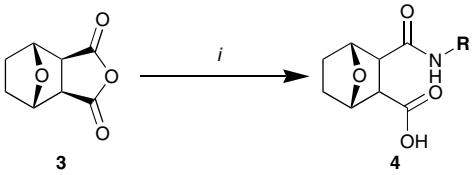
Structurally the simplest member of the okadaic acid class of compounds, cantharidin, has been the subject of multiple medicinal chemistry studies in efforts to improve its PP1 and PP2A potency and to induce either PP1 or PP2A selectivity.[3,4,17,18] Our group has developed many such analogues and explored the correlation between PP2A selectivity and in vitro anti-cancer activity. However we, as have others, have been frustrated by the apparent inability to improve potency beyond that of the native compound.[17,18] In this paper, we report on our recent efforts that have been somewhat more successful.

In keeping with our philosophy that medicinal chemistry should adopt elegantly simple synthetic approaches where possible, and in an extension of earlier work, our analogue development commenced with the readily accessible norcantharidin (**3**).[18a] Treatment, Scheme 1, of this active anhydride with a series of substituted heterocyclic amines in THF at room temperature rapidly furnished us with the family of analogues shown in Table 1. For comparative purposes cantharidin and norcantharidin were also included in all assays conducted.[18a]

Our synthesis and evaluation commenced with the cyclopentylamine analogue **5**.[18a] As shown in Table 1, **5** displayed modest levels of PP1 and PP2A inhibition, with no enzyme selectivity, which is contrary to the parent compounds which show modest levels of PP2A selectivity. Indeed, **5** displayed comparable activity to previously reported anilino derivatives from our group,[18a] suggesting that the aromatic ring at this juncture is not necessary for inhibition, but may play a role in imparting PP2A selectivity. A moderate level of broad spectrum cytotoxicity was also observed (GI_{50} ~36 μM).

Current literature consensus is that cantharidin and its analogues bind in the surface grooves of both PP1 and PP2A, in the same area as the larger toxins, and several groups including ours have made efforts to access these hydrophobic domains to improve potency and specificity.[3,4,17,18] This was the rationale for examining the chain extended analogues **6** and **7**. No activity, either phosphatase inhibition or growth inhibition, was observed (Table 1). There are two logical explanations for this unanticipated loss of activity: (1) the introduction of a second pyrrolidine (piperidine) nitrogen adversely affects interactions in the hydrophobic domain or (2) these analogues do not bind in the proximity of the hydrophobic domain. In previous work, we developed a series of analogues that seemed to suggest that accessing the hydrophobic domain was possible.[18c] However, a report by Shan on the evaluation of a family of norcantharidin analogues and enzyme kinetic experiments supplied tantalizing evidence that cantharidin may not bind in the same site as the okadaic acid class of toxins.[19] Our own enzymatic kinetic studies have been ambiguous. We are in the process of completing a series of synthesis and computer modeling studies in this area and will report our findings shortly.



3 4

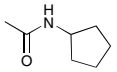
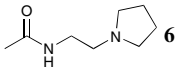
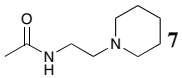
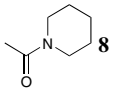
Scheme 1. Reagents and condition: (i) RNH_2, THF, rt.

Piperidine analogue **8** is by far the most intriguing entry in Table 1, with the removal of the basic nitrogen atom and drawing the hydrophobic entity closer to the amide linkage we observed highly potent PP inhibitor, more potent even than norcantharidin against PP1 and equipotent at PP2A, a first in our laboratory. In addition to a return to PP inhibition we also observed an increase in cytotoxicity across all cell lines evaluated, again at least comparable or better than that observed for norcantharidin.

Having established that the presence of the piperidine substituent conferred highly favorable PP inhibition and cytotoxicity, we extended our evaluations to include a number of similar small heterocyclic amines. These data are presented in Table 2.

Commencing with a series of simple bioisosteric replacements: **8** (4′-CH_2) ⇒ **9** (4′-O) ⇒ **10** (4′-S) ⇒ **11** (4′-NH) ⇒ **12** (4′-N-CH_3) it is immediately apparent that the 4′-heterocyclic ring substituent has a pronounced effect on PP inhibition and cytotoxicity. First the introduction of the morphilino oxygen is detrimental to PP1 inhibition whilst improving PP2A inhibition and hence PP2A specificity, with **9** now 4.6-fold PP2A selective. Gratifyingly, given our contention that PP2A specificity and potency correlates with cytotoxicity, we observed across the board improvement in GI_{50} values relative to **8** (**8** mean GI_{50} = 14.5 μM; **9** mean GI_{50} = 9.6 μM). Bioisosteric replacement O ⇒ S generates **10**, which adversely affects PP2A inhibition whilst positively affecting PP1 inhibition with **10** now PP1 selective (1.6-fold), and equipotent with **8**, i.e., the change from 4′-CH_2 to 4′-S has no real effect on PP inhibition, the effect on cytotoxicity is also negligible. Given the change in PP2A selectivity we had expected a decrease in cytotoxicity. Analogue **11** arises via installation of a 4′-NH and allows a return to PP2A specificity (3.4-fold), although at the expense of potency, and a modest level of cytotoxicity. Presumably the decrease in cytotoxicity is a combination of reduced PP inhibition and the bioavailability of **11**. The former effect could be due to a change in H-bonding character; the change in heterocycle polarity based on an O ⇒ N replacement, our data do not distinguish between these possibilities. N-methylation of **11** to **12** renders the analogue insoluble in assay media as such no data were obtained.

Table 1. Inhibition of PP1 and PP2A, and growth inhibition against a number of human cancer cell lines by **2**, **3**, and the acid–amide analogues **5**–**8**

R	IC_{50}[a] (μM)		GI_{50}[a] (μM)								
	PP1	PP2A	HT29[b]	SW480[b]	MCF-7[c]	A2780[d]	H460[e]	A431[f]	DU145[g]	BE2-C[h]	SJ-G2[i]
2	11 ± 2.0	1.2 ± 0.1	3.3 ± 0.1	4.6 ± 0.2	7.0 ± 0.2	5.8 ± 0.2	4.0 ± 0.1	3.4 ± 0.2	2.4 ± 0.1	4.9 ± 0.2	3.0 ± 0.1
3	9.0 ± 1.4	3.0 ± 0.4	48 ± 4.0	35 ± 1.1	64 ± 3.4	59 ± 0.0	45 ± 1.8	33 ± 0.8	30 ± 1.1	56 ± 1.8	35 ± 1.1
 **5**	18 ± 1.5	17 ± 1.0	44 ± 3.0	27 ± 4.5	48 ± 2.5	56 ± 1.0	29 ± 4.3	32 ± 2.0	33 ± 4.0	31 ± 2.0	31 ± 1.5
 **6**	(0)	(0)	(3.9 ± 3.0)	(2.4 ± 3.1)	(5.9 ± 6.6)	(8.4 ± 2.8)	(7.7 ± 4.8)	(6.3 ± 2.8)	(0)	(0)	(5.7 ± 3.3)
 **7**	(0)	(0)	(13 ± 4.7)	(5.1 ± 8.3)	(13 ± 1.8)	(11 ± 0.6)	(1.2 ± 7.7)	(4.8 ± 3.4)	(0)	(2.6 ± 1.4)	(0)
 **8**	3.0 ± 0.49	4.4 ± 0.18	14 ± 1.2	9.3 ± 0.40	14 ± 0.9	12 ± 1.0	19 ± 1.7	14 ± 0.2	16 ± 0.3	14 ± 0.3	18 ± 1.8

Values in parentheses represent percentage growth inhibition generated at 100 μM drug concentration with IC_{50} or GI_{50} values not determined.

[a] Means ± SEM of three experiments conducted in triplicate.

[b] Colorectal.

[c] Breast.

[d] Ovarian.

[e] Lung.

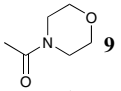
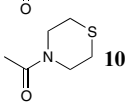
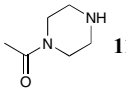
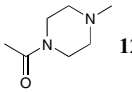
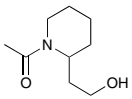
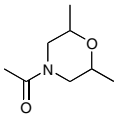
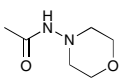
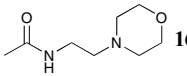
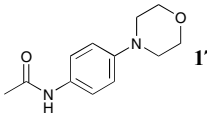
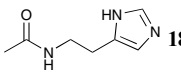
[f] Skin.

[g] Prostate.

[h] Neuroblastoma.

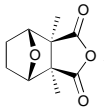
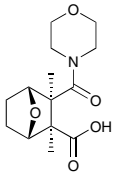
[i] Glioblastoma human cancer cell lines.

Table 2. Inhibition of PP1 and PP2A, and growth inhibition against a number of human cancer cell lines by acid–amide analogues **9**–**18**

R	IC_{50}[a] (μM)		GI_{50}[a] (μM)								
	PP1	PP2A	HT29[b]	SW480[b]	MCF-7[c]	A2780[d]	H460[e]	A431[f]	DU145[g]	BE2-C[h]	SJ-G2[i]
 **9**	13 ± 2	2.8 ± 0.1	7.4 ± 2.1	5.5 ± 0.8	7.7 ± 1.8	8.3 ± 0.4	18 ± 2.8	8.5 ± 1.3	14 ± 0.3	6.0 ± 0.6	11 ± 0.6
 **10**	3.2 ± 0.0	5.1 ± 0.4	7.7 ± 1.6	5.3 ± 0.6	7.0 ± 0.1	6.2 ± 0.1	13 ± 1.2	8.5 ± 0.7	11 ± 1.0	5.6 ± 0.2	12 ± 0.9
 **11**	32 ± 2.0	9.3 ± 0.8	17 ± 0.0	17 ± 0.9	20 ± 1.7	24 ± 2.5	21 ± 1.6	23 ± 3.0	29 ± 1.5	20 ± 0.7	13 ± 3.0
 **12**	Insol	Insol	Insol	Insol	Insol	Insol	Insol	Insol	Insol	Insol	Insol
 **13**	54 ± 13	13 ± 1.9	46 ± 4.9	76 ± 1.3	88 ± 10	(18 ± 0.9) > 100	(54 ± 2.9) > 100	96 ± 3.3	96 ± 3.7	57 ± 3.1	48 ± 4.0
 **14**	9.3 ± 1.2	4.4 ± 0.07	19 ± 0.9	15 ± 1.3	24 ± 2.7	24 ± 1.5	24 ± 0.9	21 ± 0.0	25 ± 0.0	18 ± 3.6	20 ± 0.7
 **15**	44 ± 6.3	18 ± 0.3	(29 ± 5.3)	(29 ± 1.0)	(12 ± 3.8)	(7.0 ± 1.8)	(18 ± 0.4)	(36 ± 4.9)	(3.1 ± 5.0)	(39 ± 8.1)	(30 ± 2.4)
 **16**	31 ± 2.9	43 ± 1.0	(37 ± 13)	(14 ± 1.6)	(7.5 ± 3.0)	(14 ± 1.2)	(10 ± 3.7)	(23 ± 5.6)	(17 ± 6.9)	(26 ± 20)	(19 ± 4.8)
 **17**	6.5 ± 2.4	7.9 ± 0.4	(23 ± 1.6)	(29 ± 0.7)	(20 ± 2.7)	(18 ± 3.8)	(19 ± 3.1)	(21 ± 3.9)	(17 ± 1.6)	(13 ± 4.1)	(21 ± 2.3)
 **18**	(35 ± 5.5)	(56 ± 8.7)	(21 ± 4.6)	(33 ± 5.3)	(17 ± 2.0)	(20 ± 1.9)	(25 ± 5.1)	(32 ± 2.5)	(14 ± 3.0)	(23 ± 2.3)	(27 ± 3.7)

Values in parentheses represent percentage enzyme or percentage growth inhibition generated at 100 μM drug concentration with IC_{50} or GI_{50} values not determined.
[a] Means ± SEM of three experiments conducted in triplicate.
[b] Colorectal.
[c] Breast.
[d] Ovarian.
[e] Lung.
[f] Skin.
[g] Prostate.
[h] Neuroblastoma.
[i] Glioblastoma human carcinoma cell lines.

Table 3. Inhibition of PP1 and PP2A, and growth inhibition against a number of human cancer cell lines by cantharidin (**2**) and the morpholino-substituted half-acid analogue **19**

Analogue	IC_{50}[a] (μM)		GI_{50}[a] (μM)								
	PP1	PP2A	HT29[b]	SW480[b]	MCF-7[c]	A2780[d]	H460[e]	A431[f]	DU145[g]	BE2-C[h]	SJ-G2[i]
 **2**	11.3 ± 1.97	1.2 ± 0.1	3.3 ± 0.1	4.6 ± 0.2	7.0 ± 0.2	5.8 ± 0.2	4.0 ± 0.1	3.4 ± 0.2	2.4 ± 0.1	4.9 ± 0.2	3.0 ± 0.1
 **19**	5.9 ± 2.2	0.79 ± 0.1	2.8 ± 0.0	3.9 ± 0.1	5.8 ± 0.2	4.9 ± 0.4	3.3 ± 0.1	2.9 ± 0.1	1.7 ± 0.0	2.5 ± 0.3	2.1 ± 0.1

[a] Means ± SEM of three experiments conducted in triplicate.
[b] Colorectal.
[c] Breast.
[d] Ovarian.
[e] Lung.
[f] Skin.
[g] Prostate.
[h] Neuroblastoma.
[i] Glioblastoma human cancer cell lines.

Introduction of additional substituents to the morpholine ring of **8** with a 2′-ethyl alcohol resulted in a decrease in both PP inhibition and cytotoxicity (Table 2, compound **13**), whilst the 3′,5′-dimethylmorphilino analogue **14** displayed a slight decrease in PP1 inhibition but no such adverse effect on either PP2A inhibition or cytotoxicity suggesting that this may be a site suitable for further modification. Insertion of an ethyl linker into analogue **9**, afforded analogue **16** which displayed very poor PP inhibition and cytotoxicity. This suggests that the proximity of the ring to the amide carbonyl is pivotal for activity with **9** as one of the most potent protein phosphatase norcantharidin analogues thus far reported. This effect is further confirmed with the position of the morpholine substituent as close to the amide carbonyl vital for high activity, with movement of even one bond length detrimental to activity (Table 2, compounds **15** and **16**). It is also likely that the type of nitrogen atoms present in these analogues impacts on the observed activity. The aniline-type nitrogen of **17** is less basic than the tertiary amine nitrogen of **16** and thus more favorable for a hydrophobic environment. Also, drop in potency of **16** compared to **9** may result from the increased polarity of the basic nitrogen of the morpholine ring in **16** (versus the amide nitrogen of **9**).

In this series the most potent and PP1 selective analogue is **10** and the most potent and selective PP2A analogue is **9**, and in both cases all the data generated indicate that both these analogues are more potent than the lead norcantharidin. The type of spacer (phenyl ring, **17**) has an effect on PP inhibition with the insertion of a phenyl ring restoring potency analogous to our previously reported anilino series, however analogue **18** possessing a simple imidazole terminated ethyl chain displays low levels of PP1/PP2A inhibition as well as very low levels of cytotoxicity.

Cantharidin has greater potency, selectivity, and cytotoxicity than norcantharidin, associated with the additional methyl groups. Thus, it was hypothesized that the synthesis of a cantharidin based variant of **9** would generate an analogue with improved PP inhibition and cytotoxicity. Hence, we applied our synthetic approach as shown in Scheme 1 and generated **19** and the results of our biological screening are given in Table 3.

The data in Table 3 clearly show that **19** is more potent in all aspects than the parent **2**. Given we observed no decrease in inhibition with the 3′,5′-dimethylmorphilino-substituted **14** we feel that this result has the potential to allow the rapid development of novel highly potent cantharidin analogues.

In this work, nucleophilic ring opening with a series of heterocyclic amines has for the first time allowed the generation of a series of novel (nor)cantharidin analogues that are more potent than the original lead compounds. Both PP inhibition and cytotoxicity are enhanced. In particular, further evaluation of substituted morphilino analogues should be investigated, and are currently underway in our laboratories and will be reported in due course. Interestingly, the data generated suggest that the consensus binding motif associated with analogues of this kind warrants further investigation and gives tentative support to the kinetic studies performed by Shan et al.[19]

Acknowledgment

We are grateful for financial support from the Biotechnology Innovation Fund (Australia).

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