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Norcantharidin analogues with nematocidal activity in *Haemonchus contortus*

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ABSTRACT

With the major problems with resistance in parasitic nematodes of livestock to anthelmintic drugs, there is an urgent need to develop new nematocides. In the present study, we employed a targeted approach for the design of a series of norcantharidin analogues ($n = 54$) for activity testing against the barber's pole worm (*Haemonchus contortus*) of small ruminants in a larval development assay (LDA) and also for toxicity testing on nine distinct human cell lines. Although none of the 54 analogues synthesized were toxic to any of these cell lines, three of them (*N*-octyl-7-oxabicyclo(2.2.1)heptane-2,3-dicarboximide (**B2**), *N*-decyl-7-oxabicyclo(2.2.1)heptane-2,3-dicarboximide (**B3**) and 4-[(4-methyl)-3-ethyl-2-methyl-5-phenylfuran-10-oxa-4-azatricyclo[5.2.1]decane-3,5-dione (**B21**) reproducibly displayed 99–100% lethality to *H. contortus* in LDA, with LD_{50s} of 25–40 μM. The high 'hit rate' (5.6%) indicates that the approach taken here has advantages over conventional drug screening methods. A major advantage of norcantharidin analogues over some other currently available anthelmintics is that they can be produced in one to two steps in large amounts at low cost and high purity, and do not require any additional steps for the isolation of the active isomer. This positions them well for commercial development.

In spite of their massive impact, parasites of humans and other animals are seriously neglected in terms of funding for research and development (R&D) of drugs, vaccines and diagnostics. The current production losses caused by parasites to agriculture worldwide have a major adverse impact on farm profitability and exacerbate the global food shortage. For instance, nematodes of livestock cause major production losses to farmers due to poor productivity, failure to thrive and deaths.[1–5] In particular, strongylid nematodes are of paramount importance as pathogens of sheep, goats, cattle and pigs, causing gastrointestinal diseases and associated complications, often leading to death in severely affected animals.[5] Currently, these nematodes are controlled predominantly through the use of anthelmintics, but widespread resistance against a range of compounds (of three main classes) has compromised their efficacy.[6–11] Thus, there is an urgent need to work toward identifying new drug targets and developing new nematocides.

We have been pursuing the molecular characterization of a number of gender- and/or stage-enriched molecules in parasitic nematodes (Strongylida) using *C. elegans* as a reference organism, with a perspective on predicting novel drug targets.[12–30] Through a number of studies,[16,24,31] we have provided insights into genes encoding protein phosphatases (PPs) for selected strongylid nematodes. These studies have shown that selected serine–threonine phosphatases (STPs) are quite conserved between parasitic and free-living nematodes and have inferred that they play key roles in pathways required for the growth, development, survival and/or reproduction.[25] In addition, phylogenetic analysis has indicated that such STPs are specific to nematodes, clustering, with strong support, to the exclusion of related molecules in other invertebrates and vertebrates.[24]

Current literature indicates that inhibitors, such as cantharidin (1) (from the blister beetle, *Mylabris*)[32–36] and a number of analogues with the same pharmacophoric units, most notably some derived from norcantharidin (2), have no adverse toxic effects on well-defined, cultured human cells[34,35,37–39] but were considered to have unique potential for the development of nematocides (Fig. 1).[25] The former characteristic is important, as the focus should be on identifying compounds that have no adverse effect on mammalian cells (representing the host animal) but are lethal to parasitic nematodes or block their reproduction. Some norcantharidin analogues are known to display excellent STP (i.e., PP1 and PP2A) inhibitory activity.[34,35,40,41] Preliminary work conducted by us showed that some norcantharidin analogues, which had no toxic effect on human cell lines, killed larvae of the trichostrongylid nematodes *Trichostrongylus vitrinus* and/or *Haemonchus contortus*.[25] Homology modelling and in silico docking suggested that that

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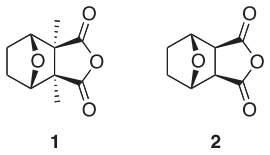


Figure 1. The chemical structures of cantharidin (**1**) and norcantharidin (**2**).

some prototype molecules designed bind specifically to active sites in selected STPs of these parasites.[25] These initial findings indicated an opportunity for the discovery of a novel class of nematocides and significant biotechnological outcomes. In the present study, we designed a series of norcantharidin analogues and then tested their nematocidal effect on *H. contortus* in a larval development assay (LDA).

Three families of norcantharidin analogues were synthesized: the ring-opened acid amides (library A, **A1**–**A24**), the ring-closed norcantharimides (library B, **B1**–**B22**) and the tetrahydroepoxyisoindole carboxamides (library C). These compounds were accessed through a series of robust in house-developed and generic approaches reported previously.[34,35,42–45] These approaches facilitated the rapid installation of a range of substituents for structure–activity relationship (SAR) studies.

All chemicals were tested for cytotoxicity in vitro against nine different human cancer cell lines: HT29 (colon), SW480 (colon), MCF-7 (breast), A2780 (ovarian), H460 (lung), A431 (skin), DU145 (prostate), BE2-C (neuronal) and SJ-G2 (brain), as previously described.[32,35,40,41] Following cytotoxicity testing, chemicals were tested in a larval development assay (LDA). *H. contortus* (Haecon 5 strain) was raised in helminth-free lambs (Merino crosses; 24 weeks of age), as described by Nikolaou et al.[46]

The present study employed a targeted approach for the design of a series of norcantharimide analogues for toxicity testing on nine different human cancer cell lines and for subsequent testing for nematocidal activity in LDA against *H. contortus*. A total of 54 analogues was synthesized (see Supplementary data). For both the ring-opened acid amide (library A) and ring-closed norcantharimide analogues (library B), the ease of synthesis typically related to the nucleophilicity of the amine used to develop each focused library member. The more nucleophilic amines favoured ring-closing to the corresponding norcantharimide, non-nucleophilic amines required prolonged heating to effect this transformation (path B in Scheme 1). Most notable was the reaction of *N*-methyl-1-(5-phenylfuran-3-yl)methanamine which, instead of affording the expected ring-opened analogue (path A in Scheme 1), underwent a very facile ring-closing to the quaternary ammonium norcantharimide (**B21**).

The synthesis of the tetrahydroepoxyisoindole carboxyamides was more challenging, but the products typically represented good yields (Scheme 2, library C).

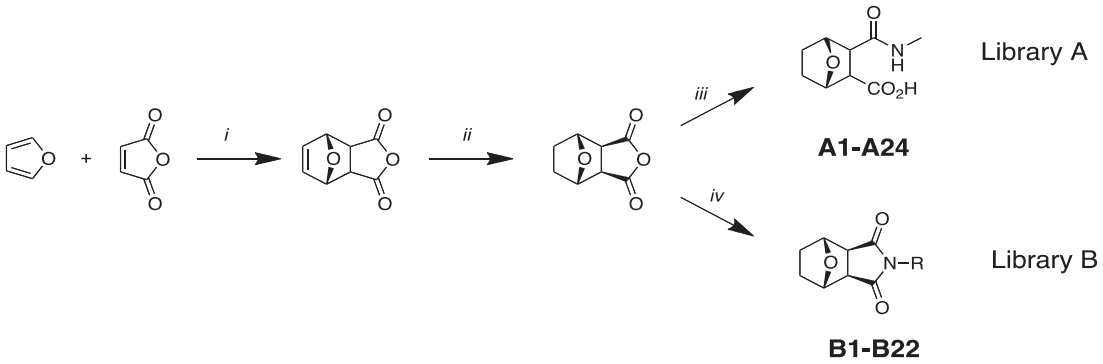
As a series of internal assay-validation standards were also synthesized, using a phase transfer catalyst approach, selected (*Z*)-2-phenyl-3-(1*H*-pyrrol-2-yl)acrylonitrile derivatives, which had been reported previously to be active against both *H. contortus* and the cat flea, *Ctenocephalides felis*.[47,48] The library compounds listed are those inhibitors that passed our toxicity pre-filter by displaying low levels, defined as a mean GI_{50} value >75 μM, of cell death in nine distinct human cancer cell lines (HT29, SW480, MCF-7, A2780, H460, A431, DU145, BE2-C and SJ-G2).[32,34,35,40,41] As reported previously,[34,35] the ring-opened acid amine analogues displayed higher levels of cytotoxicity (10–60 μM) than the corresponding ring-closed norcantharimides (>75 μM). All 54 analogues that passed the 'toxicity filter' were evaluated for nematocidal activity against *H. contortus* in LDA.

Based on our previous studies,[34–37,40,41] we anticipated our library A analogues to be the most potent (as we had shown protein phosphatase inhibition by some of these analogues). To our surprise, none of the analogues from library A returned notable parasite lethality. Analogues were tested over a drug concentration range of 12.5–100 μM.

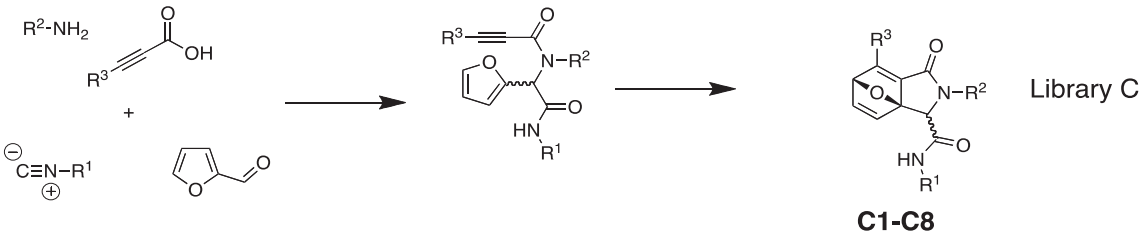
Subsequently, we examined the library B analogues representing the norcantharimide that we have shown previously to be devoid of protein phosphatase inhibition. To our surprise, three analogues (library B; **B2**, **B3** and **B21**; Fig. 2) from this library achieved 99–100% lethality of *H. contortus* in LDA (Table 1). All three compounds were retested on four separate occasions, achieving the same result. Progression to a full-dose response evaluation, at concentrations between 10 and 100 μM, revealed that each of these analogues had LD_{50} values in the range of 25–40 μM (Table 1). Interestingly, both **B2** and **B3** possess a long alkyl chain, which may enhance their bioavailability and assist their transport across the parasite cuticle and into tissues and cells, allowing access to the target in the parasite. Given our previous findings in relation to protein phosphatase inhibition by this class of norcantharidin analogue, we believe that it is unlikely that the ultimate target is a serine–threonine protein phosphatase. The highly hydrophobic nature of the phenylfuran moiety of **B21** most likely also assists transport through cell membranes. We had hoped that further elongation of the alkyl tail of **B2** and **B3** may improve uptake, but the dodecyl **B4**, tetradecyl **B5** and octadecyl **B6** were inactive. Presumably, this lack of activity is a consequence of poor water solubility.

Given the activity of **B2** and **B3**, we specifically tailored the synthesis of library C to include hydrophobic groups. However no compound from library C displayed any noteworthy nematocidal activity at the initial screening doses (12.5–100 μM).

Libraries A and B are related via a simple ring closing which effectively removes an acid and an amide moiety from the inactive pharmacophore. This, in turn, suggested that either the ultimate protein target disfavours the presence of these hydrogen bond



Scheme 1. Reagents and conditions: (i) Et_2O, rt, 48 h; (ii) acetone, 10% Pd–C, H_2 (g) 50 psi, 18 h; (iii) RNH_2, $PhCH_3$, Δ, 24–36 h.



Scheme 2. Reagents and conditions: (i) R^1NC, alkynoic acid, furan carbaldehyde, R^2NH_2, CH_3OH, rt 30 min; (ii) $PhCH_3$, sealed tube 200 °C, 36 h.

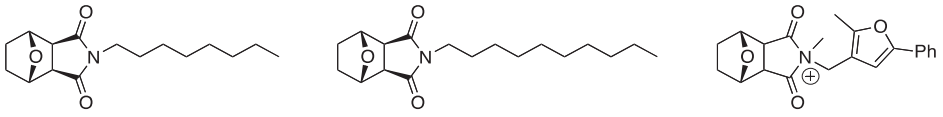
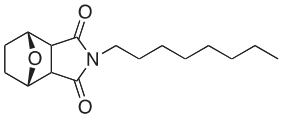
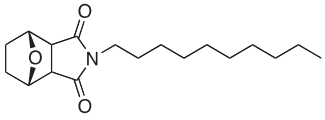
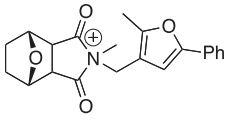
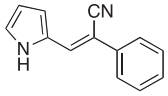
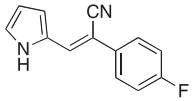
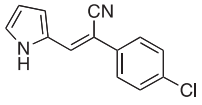
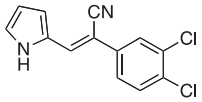


Figure 2. The three compounds (**B2**, **B3** and **B21**; ring closed norcantharimide analogues) from library B which consistently killed 99–100% of *Haemonchus contortus* in the larval development assay (LDA).

Table 1
Of 54 compounds synthesized (see Supplementary data, and Figs. 1 and 2) tested against *Haemonchus contortus* in a larval development assay (LDA), three norcantharimide analogues from library B reproducibly killed *H. contortus*. Included in the LDA were four control compounds (*Z*)-2-phenyl-3-(1*H*-pyrrol-2-yl)acrylonitrile, (*Z*)-2-(4-fluorophenyl)-3-(1*H*-pyrrol-2-yl)acrylonitrile, (*Z*)-2-(4-chlorophenyl)-3-(1*H*-pyrrol-2-yl)acrylonitrile, (*Z*)-2-(3,4-dichlorophenyl)-3-(1*H*-pyrrol-2-yl)acrylonitrile)[47,48]

Compounds	Concentration range (μM) tested	LD_{50} (μM)	LD_{99} (μM)
Test compounds with nematocidal activity:			
 **B2**	10–100	40	80
 **B3**	10–100	30	90
 **B21**	10–100	25	100
Control compounds:			
 (*Z*)-2-Phenyl-3-(1*H*-pyrrol-2-yl)acrylonitrile	6.25–100	6	100
 (*Z*)-2-(4-Fluorophenyl)-3-(1*H*-pyrrol-2-yl)acrylonitrile	6.25–100	10	50
 (*Z*)-2-(4-Chlorophenyl)-3-(1*H*-pyrrol-2-yl)acrylonitrile	6.25–100	10	25
 (*Z*)-2-(3,4-Dichlorophenyl)-3-(1*H*-pyrrol-2-yl)acrylonitrile	6.25–100	7	12.5

The LD_{50} and LD_{99} values for each compound are indicated.

donor–acceptor groups or that these groups prevent the penetration of the tissues or cuticle of the parasite, or that analogues with the library A pharmacophore are substrates for an active efflux or degradation mechanism. Which mechanism is operational is currently unknown. Library C differs in the relative spatial presentation of the key pharmacophoric moieties (relative to library B), suggesting that the position of these groups is crucial to eliciting the observed lethality of *H. contortus* for **B2**, **B3** and **B21**. Although an ovicidal effect has been reported for some commercially available anthelmintics, such as benzimidazoles,[49,50] there was no evidence of this effect for any of the analogues tested herein.

Until relatively recently, the search for novel drugs against parasites has usually been carried out using approaches which are decades old, such as the screening of many thousands of chemicals for inhibition or disruption of parasite growth and/or development in vitro. Today, genomic, proteomic, bioinformatic and/or chemoinformatic technologies are increasingly being used to assist the search for new compounds.[30,51–57] A major goal of current genomic and transcriptomic studies of parasites is the inference of novel candidate drug targets, guided by essentiality and genetic interaction studies.[16–23,27,28] However, the major challenge is not only to identify potential targets, but, importantly, to prioritize them, such that available resources can be focused on those most likely to lead to effective treatments. The length of time and the prohibitive costs associated with bringing a new drug to market, together with the knowledge that most lead-compounds fail at some stage in the development process, have deterred most pharmaceutical companies from investing in the discovery of entirely novel targets and classes of anthelmintics using integrated genomic–bioinformatic–chemoinformatic platforms. However, the recent success in developing monepantel through to a commercial product[58–64] provides fresh hope for the discovery of novel classes of anthelmintic compounds.[65]

In the present investigation, we were guided by a range of previous studies[16,24,25,31] showing that selected serine–threonine phosphatases (i.e., PP1 and PP2A) might represent suitable targets for strongylid nematodes, including *H. contortus*, because they are: (i) known to be essential for growth, development, survival and/or reproduction, (ii) conserved between these nematodes and *C. elegans* but (ii) divergent from related molecules in other invertebrates and vertebrates (including mammalian hosts).[25] That some norcantharidin derivatives display exquisite PP1 and PP2A inhibitor activity[34,35,40,41] suggested that a series of analogues, with no or limited toxicity to mammalian cell lines, could be designed and produced to specifically inhibit serine–threonine phosphatases of *H. contortus*. Three of the 54 analogues synthesized displayed almost complete lethality to *H. contortus* in LDA, achieving a 'hit rate' that exceeded (by at least five times) that reported previously for traditional screening methods.[66]

Although norcantharidin is known as a phosphatase inhibitor,[40,41,34–37,67] some of the novel analogues synthesized and tested herein (and which no longer closely resemble the original 'backbone molecule') might have molecular targets other than PP1s and/or PP2As. Currently, we are exploring new approaches to facilitate and determine the target(s) of these compounds. In addition, further work should also focus on improving the LD_{50} and bioavailability of the three compounds. A major commercial advantage of these chemicals over some other currently available anthelmintics is that they can be produced in one to two steps in large amounts at low cost and high purity, and do not require any additional steps for the isolation of the active isomer. By contrast, monepantel (an aminoacetonitrile derivative),[56] for example, needs to be synthesized in multi-step chemical reaction pathways, followed by the isolation of the active optical isomer. Given that the present norcantharimide analogues display a lack of toxicity to mammalian cells, they should now be tested directly in vivo (in sheep) against *H. contortus* and also for activity in vitro and in vivo against other parasitic nematodes. In future, compounds that are not toxic to mammalian cell lines and have failed screens on plant parasites and/or, for example, cancer cells should be screened for activity and lethality against parasitic nematodes of animals and humans.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.031.

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