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The antiplasmodial activity of norcantharidin analogs

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ABSTRACT

The antiplasmodial activities of sixty norcantharidin analogs were tested in vitro against a chloroquine sensitive (D6, Sierra Leone) and chloroquine resistant (W2) strains of *Plasmodium falciparum*. Forty analogs returned IC_{50} values <500 μM against at least one of the *P. falciparum* strains examined. The ring open compound **24** ((1*S*,4*R*)-3-(allylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid) is the most active aliphatic analog (D6 IC_{50} = 3.0 ± 0.0 and W2 IC_{50} = 3.0 ± 0.8 μM) with a 20-fold enhancement relative to norcantharidin. Surprisingly, seven norcantharimides also displayed good antiplasmodial activity with the most potent, **5** returning D6 = 8.9 ± 0.9 and W2 IC_{50} = 12.5 ± 2.2 μM, representing a five-fold enhancement over norcantharidin.

Malaria, the disease caused by the parasite *Plasmodium* spp., still contributes significantly to human mortality, especially in developing countries. Poverty and lack of proper medical care intensify the strong impact of this disease. Every year over one million people die because of an infection by this protozoan parasite.[1,2] This situation is complicated by *Plasmodium*'s complex life cycle with host and development stage changes and our limited knowledge of the molecular intricacies of these processes. Many researchers are currently examining antimalarial vaccines and drugs. However, there remains a lack of efficient vaccinations, and *Plasmodium* spp. easily evolve resistance to intensively used antimalarial drugs such as chloroquine and sulphadoxine-pyrimethamine.[3] Therefore, the discovery of new antimalarial leads with novel targets of action is an important goal.

In traditional Chinese medicine, dried beetle bodies have been used as a medicine for over 2000 years to cure hepatoma and esophageal carcinoma.[4,5] Cantharidin **1** is an active compound isolated from blister and oedemerid beetles.[6] It comprises a 7-oxabicyclo[2.2.1] frame fused with a 2,3- succinic anhydride moiety and two methyl groups. Cantharidin shows good anticancer activity; for example, recent reports indicated that this compound is an apoptosis inducer in multiple melanoma cells by interaction with proteins of the JAK/STAT pathway.[7] To date the high renal toxicity has precluded cantharidin's use in Western medicine.[5] This problem has promoted a search for less renal cytotoxic and more selective cantharidin analogs with a similar or improved anticancer profile. A number of analogs have been screened against various cancer cell lines such as breast, ovarian, lung, skin, prostate, leukemia, and colon.[8]

The most well-known analog of cantharidin is endothall, a dicarboxylic acid synthetic herbicide that had the best antiplasmodial activity among the commercial herbicides that we tested previously.[9] In addition to endothall, norcantharidin is the most well-known analog of cantharidin. This demethylated cantharidin derivative has lower nephrotoxicity than cantharidin,[10] and as such norcantharidin has been used as the lead compound in multiple investigations targeting the development of more potent and selective analogs.[8,11–14] Norcantharidin displays an about 10-fold activity reduction against cancer cell lines than cantharidin, but maintains cantharidin's favorable stimulation of white cell growth by bone marrow (in contrast to other anticancer drugs that readily induce myelosuppression). Most norcantharidin analogs can be classified as: (a) ring opened ester; (b) ring opened amides; or (c) ring closed imides, the norcantharimides.[8,10–14]

Cantharidin and its analogues have attracted significant interest, as they are the simplest members of the okadaic acid class of serine/threonine protein phosphatase (PPP) inhibitors. Cantharidin, norcantharidin and endothall are potent PPP inhibitors especially of mammalian and plant PP1 and PP2A (IC_{50} = 1.8 and 0.2 μM; 2.0 and 0.4 μM; 4 and 0.09 μM, respectively).[10–17] Except for the methyl groups, and limited synthetic variations associated with the anhydride moiety are permissible, all other structural elements have been shown to be crucial to the inhibitory activity of this compound. Removal of the 5,6-ethylbridge also renders

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cantharidin analogs inactive.[18] Bertini et al. noted that in a cantharidin-PP5 co-crystal, cantharidin sits in the catalytic site in its dicarboxylic acid form coordinating with one metal (either a Zn^{2+} or Mn^{2+}) via three oxygen atoms: one from each of the carboxylates, and the other from the 7-oxa moiety. This explains the crucial nature of the ethereal oxygen.[19] The two carboxylate moieties are in close proximity and expel all active site water molecules. Interestingly, in all instances a double conformation was observed (1:1 ratio) in which the bound structure only differed by the relative orientations of the carboxylate moieties. No double conformation was observed with norcantharidin, suggesting that the methyl groups, as suggested by Gauss et al., act as a conformational lock.[20]

Protein phosphatases and kinases complement one another by keeping equilibrium between dephosphorylated and phosphorylated forms of particular proteins. In this manner they regulate a substantial number of cellular processes such as gene expression, DNA replication, cellular proliferation and differentiation, apoptosis, etc.[21,22] Disturbance of PPP levels can lead to cell death in many cases.[23,24] Protein phosphatases have extremely sophisticated systems of their regulation of gene expression at both the transcription and translation levels. In spite of intense research, these regulation processes are still not well understood. Deregulation of protein phosphatase functioning contributes to a number of diseases such as tumor development, Alzheimer's disease and lupus erythematosus.[25] Examples of deregulating agents are viral proteins which act as modulators of both activity and specificity of protein phosphatases; that is, polyoma virus small T antigen and middle T antigen.[20]

The *Plasmodium falciparum* genome encodes six serine/threonine protein phosphatases that may be potential targets of cantharidin and/or norcantharidin, that is, PfPP1 (AAN36754), PfPP2A (CAB38970), PfPP2A-like (CAD51958), PfPPα (AAN37243), PfPPβ (CAD51935) and PfPP5 (CAD52675); their amino acid sequences contain a highly conservative PPP catalytic site GDXHG(X)$_n$GDXVDRG(X)$_n$RGNHE (Fig. 1).[26] Despite very high amino acid sequence identity of the catalytic site between species, some sequence differences occur that could affect substrate preference, for example, PfPPα sequence contains a 15 amino acid long insert between the GDXHG(X) and GDXVDRG sequences (Fig. 1). In the future, this fact can be applied towards the design of compounds specific only to *Plasmodium* serine/threonine protein phosphatases.

The intricate *Plasmodium* life cycle has hampered research on functions and roles of *Plasmodium* PPP. Recent studies have shown that the function of PP1 is essential for *P. falciparum* during release of mature merozoites from erythrocytes. Inhibition of PfPP1 caused hyperphosphorylation of PfSBP1 (*P. falciparum* skeleton binding protein 1) and subsequently reinforcement and stabilization of the RBC membrane.[27] PfSBP1 is involved in transport of the major malaria virulence antigen PfEMP1 on the surface of RBCs.[28]

Recently, extensive studies have been conducted on the synthesis of several novel norcantharidin analogs with the main goal of obtaining compounds with higher activity and specificity.[10–14,18,29] In the present investigation we were keen to examine the ability of a select range of norcantharidin analogs (60 analogs as shown in Tables 1–3) inhibit the D6 and W2 strains of *P. falciaparum*. The *P. falciparum* D6 and W2 strains represent one of the most common and virulent forms of *Plasmodium* spp.[30]

The norcantharidin analogs examined can be separated into two broad classes: (1) the human PP1 and PP2A (HsPP1 and HsPP2A) inactive norcantharimides (Table 1). The norcantharimides possess a N-substituted imide moiety and are often known as the ring closed variants. (2) The human PP1 and PP2A active ring opened acid-amide norcantharidins (Tables 2 and 3). These norcantharidin analogs are further subdivided into two groups, firstly the aliphatic (Table 2) which possess alkyl amides terminating in a range of alkyl functional groups. The second grouping is the aromatic substituted norcantharimides where the primary functional group is an anilide (or an alkyl aromatic) (Table 3). We have previously reported inhibitory activity of these compounds against human catalytic subunits of PP1, PP2A and also for anticancer activity (human cancer cell lines: HT29, SW480 (colon), MCF-7 (breast), A2780 (ovarian), H460 (lung), A431 (skin), DU145 (prostate), BE2-C (neuronal), and SJ-G2 (glioblastoma)) of these compounds.[8,10,11,16,29] We compared the human phosphatases data described earlier to our antiplasmodial results introduced in this paper. Facing a lack of data about norcantharidin analogs inhibitory activity against plasmodium phosphatases, the results of human-host PPP activity bring a potentially useful perspective.

Our lead compound in this evaluation, norcantharidin displayed IC_{50} values of 60 ± 0.0 and 50 ± 0.0 μM against the *P. falciparum* strains D6 and W2, respectively.[30] This compares reasonably favorably with the more toxic, and hence less viable, cantharidin with activities of 9.0 ± 0.8 and 9.0 ± 0.0 μM against the D6 and W2 strains, respectively (Table 1). As can be seen from the data presented in Table 1, seven norcantharimides **5**–**10** and **18** return IC_{50} values of <500 μM (Table 1). Analogs **5** and **18** deserve particular note, showing IC_{50} values of D6 8.9 ± 0.9, W2 12.5 ± 2.2 μM; and D6 24.0 ± 4.8, W2 25.0 ± 0.0 μM, representing a five-fold enhancement in activity relative to norcantharidin. The other active analogues provide crucial structure–activity data with **3**–**10** showing a significant correlation between alkyl chain length, their *c*Log *P* value and growth inhibition of *P. falciparum* (Table 1). Elongation of **5**'s butyl chain to hexyl (**6**) and octyl (**7**) reduced the antiplasmodial efficacy, although this may in part be due to reduced solubility and uptake in the assay media. Constraining the *N*-alkyl moiety in a cyclohexyl ring is less detrimental to activity (IC_{50} **10**

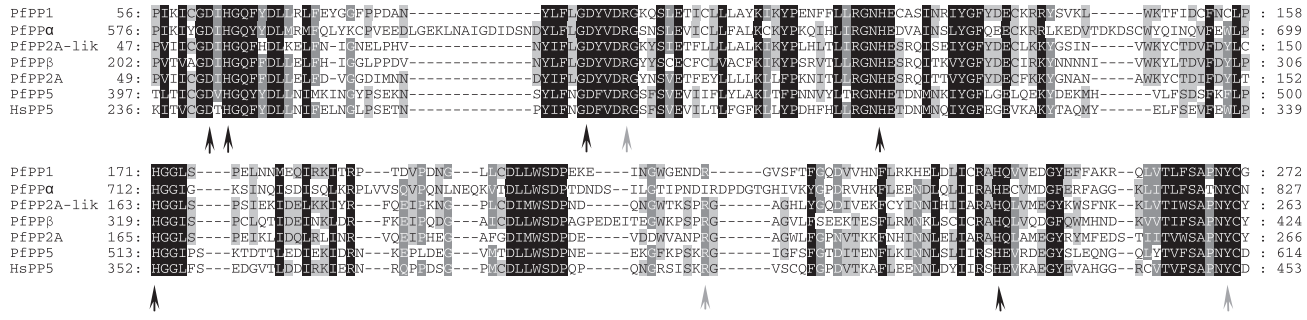

Figure 1. The alignment of *Plasmodium falciparum* serine/treonine protein phosphatases' and human PP5c[19] amino acid sequences. Fragments of catalytic sites of PPP with high similarity are marked. Black arrows pointing at amino acids responsible for coordination of metal ions located at active site Asp242, His 244, Asp 271, Asn 303, His 352 and His 427. Cantharidin and norcantharidin interact with two metal ions and Arg 275, Tyr 451 and Arg 400 (grey arrows).[19]