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Rhodanine-3-acetic acid derivatives as inhibitors of fungal protein mannosyl transferase 1 (PMT1)

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Abstract—The first inhibitors of fungal protein: mannosyl transferase 1 (PMT1) are described. They are based upon rhodanine-3-acetic acid and several compounds have been identified, for example, 5-[[3-(1-phenylethoxy)-4-(2-phenylethoxy)phenyl]methylene]-4-oxo-2-thioxo-3-thiazolidineacetic acid (**5a**), which inhibit *Candida albicans* PMT1 with IC_{50}s in the range 0.2–0.5 μM. Members of the series are effective in inducing changes in morphology of *C. albicans* in vitro that have previously been associated with loss of the transferase activity. These compounds could serve as useful tools for studying the effects of protein O-mannosylation and its relevance in the search for novel antifungal agents.

The incidence of life-threatening fungal infections has multiplied dramatically as the population of immunocompromised individuals has increased. Present therapeutic options are limited to three classes of compound: polyenes, azoles and the recently introduced candins.[1,2] However, the utility of polyenes is limited by nephrotoxicity and resistance is emerging to azoles. There is therefore a need for new anti-fungal compounds with novel modes of action for use in treating or preventing such fungal infections.

O-Linked mannoproteins constitute a significant component of the fungal cell wall of *Candida* spp. and other pathogenic fungi and are believed to confer the cell surface properties involved in adhesion and host interactions. The key step in the biosynthesis of these mannoproteins is catalysed by a family of protein: mannosyl transferases (PMTs).[3–6] Five family members of PMT have been identified in *C. albicans*[7–9] These enzymes are membrane proteins that are localised in the endoplasmic reticulum[10,11] and are responsible for the transfer of the initial mannose residue from dolichol-phospho-β-D-mannose onto serine/threonine residues with an inversion of the configuration to form an α-D-mannosyl bond.[12,13] There is accumulating evidence that PMTs may function as hetero or homodimers.[14] A family of mannosyl transferases, shown or postulated to be located in the Golgi, add subsequent mannose residues.[4,15,16]

Deletion of both copies of the PMT1 gene from *C. albicans* results in a strain that is no longer virulent in both immunocompetent[7] and immunocompromised animal models.[17] This strain shows a failure to form hyphae under conditions of nitrogen starvation and also displays increased sensitivity to agents associated with cell wall defects. The loss of virulence with the PMT-1 deletion mutant and the apparent absence of an equivalent enzymic activity in man have led us to pursue

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the *C. albicans* enzyme as a target for therapeutic intervention.

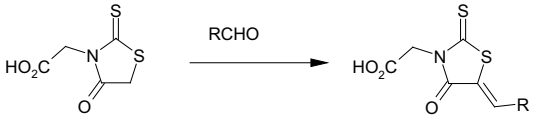
An assay suitable for testing large numbers of compounds for their inhibition of PMT1[18] has been established as well as a cellular assay, based upon the observed sensitivity of the PMT1$^{-/-}$ strain to aminoglycoside antibiotics, for evaluating the effects of such inhibitors on *C. albicans*[19] We herein describe the first series of inhibitors of this pivotal enzyme and their effects in vitro on *C. albicans*.

Screening a 50,000 corporate compound collection against PMT1 identified a series of weak inhibitors ($IC_{50} \sim 50\,\mu M$) of which the rhodanine-3-acetic acid derivative (**1a**) is representative. Confirmation of the suitability of **1a** as a chemical starting point for obtaining potent PMT1 inhibitors was facilitated by the straightforward preparative procedures, which were applied throughout this work (Scheme 1). The requisite benzaldehydes were either commercially available or could be synthesised via well-documented procedures.

Transposition of the benzyloxy group to the adjacent 3-position on the central aromatic ring (**1b**) had little effect on the PMT1 inhibitory activity. However, 3,4-bis-benzyloxy substitution (**1c**) resulted in a significant increase in activity, both against the enzyme and in the *C. albicans* assay (IC_{50}: 2.3 and 3.0 μM, respectively, Table 1). This result encouraged us to continue exploring this structural series for obtaining potent inhibitors of PMT1. What is noteworthy is that replacement of a phenyl group in **1c** by a 3- or 4-pyridyl ring, **1f** and **1g**, respectively, resulted in a marked loss of PMT1 inhibition and complete loss of cellular activity.

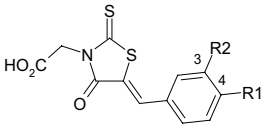
We next examined the effect of substitution on each of the phenyl rings of **1c** (Table 2). As a general observation, substitution in ring B was detrimental to PMT1 activity. Furthermore, little enhancement of the overall activity was achieved by substitution in ring A except for a modest effect in the case of the 4-Cl substitution (**2a**). By contrast, introducing a 4-Cl substituent into ring B (**2b**) led to a 7-fold fall in PMT1 inhibitory relative to **2a**. A similar trend was evident with the 3- and 4-CF_3 (**2e**–**h**) and 4-SO_2Me (**2c**,**d**) substitutions. In the case of 3-CF_3 and 4-CF_3 substitutions in ring B, **2f** and **2h**, activity in the *C. albicans* assay was abolished. Where examined, substitution in both ring A and ring B resulted in loss of activity in the PMT1 and *C. albicans* assays.

At this point we turned our attention to the length of the spacer between the ether oxygen and the pendant phenyl group at the 4-position (Table 1). Extending the spacer to generate a phenylethoxy (**1d**) or a phenylpropoxy (**1e**) group had only a minimal (favourable) effect on the PMT1 activity but in the case of phenylethoxy there was a significant improvement in potency in the *C. albicans*



Scheme 1. Reagents and conditions: NaOAc, AcOH, reflux 24 h or NH_4OAc, toluene, reflux 1 h or NH_4OAc, DMF, 80 °C 5 min.

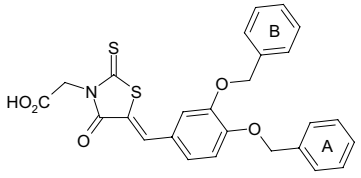
Table 1. In vitro inhibition of PMT1 and *C. albicans* proliferation by (**1a**–**g**)



Compound	R1	R2	PMT1 inhibition[a] IC_{50} μM	*C. albicans* G418-sensitive proliferation,[a] IC_{50} μM
1a	OCH_2Ph	H	55	50
1b	H	OCH_2Ph	50	NT
1c	OCH_2Ph	OCH_2Ph	2.3	3.0
1d	$O(CH_2)_2Ph$	OCH_2Ph	1.5	0.5
1e	$O(CH_2)_3Ph$	OCH_2Ph	1.4	2.4
1f	OCH_2Ph	$OCH_2$3-Py	43	>100
1g	OCH_2Ph	$OCH_2$4-Py	30	>100

[a] Mean of at least two determinations; NT: not tested.

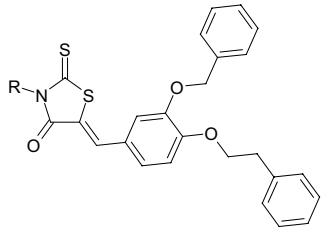
Table 2. Ring substitutions: in vitro inhibition of PMT1 and *C. albicans* proliferation by (**2a**–**u**)



Compound	A-ring substitution	B-ring substitution	PMT1 inhibition[a] IC_{50}, μM	*C. albicans* G418-sensitive proliferation,[a] IC_{50} μM
1c	H	H	2.3	3.0
2a	4-Cl	H	0.95	0.6
2b	H	4-Cl	7.0	NT
2c	4-SO_2Me	H	3.2	>100
2d	H	4-SO_2Me	20	>100
2e	3-CF_3	H	2.0	0.65
2f	H	3-CF_3	11	>100
2g	4-CF_3	H	1.9	4.0
2h	H	4-CF_3	11	>100
2i	2,4-Di-F	H	6.5	2.1
2j	H	2,4-Di-F	8.5	6.0
2k	3,4-Di-F	H	2.0	1.5
2l	H	3,4-Di-F	4.2	>100
2m	4-OMe	H	3.4	3.0
2n	4-OCF_3	H	2.0	0.5
2o	4-Me	H	2.7	0.6
2p	4-F	4-F	5.0	5.2
2q	2,4-Di-F	2,4-Di-F	5.0	>100
2r	2,6-Di-F	2,6-Di-F	20	>100
2s	H	3-OMe	7.5	>100
2t	H	3-Cl	4.0	4.0
2u	3-OMe	3-OMe	15	>100

[a] Mean of at least two determinations; NT: not tested.

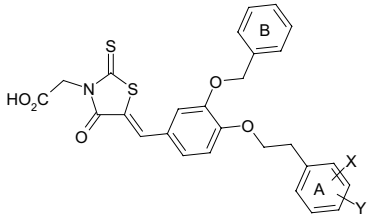
assay. Furthermore **1d** showed good selectivity over two other glycosyltransferases: no inhibition of dolichol-phosphomannose synthase and oligosaccharide transferase being evident at 70 μM. Before capitalising on these latter results we wished to confirm the necessity of having the acetic acid function on the rhodanine moiety. This was indeed established by the synthesis and evaluation of the analogues (**3a**–**d**), none of which displayed activity in the PMT1 and *C. albicans* assays.



(**3a**) R = H
(**3b**) R = CH_2CO_2Et
(**3c**) R = CH_2CONH_2
(**3d**) R = CH_2(5-tetrazolyl)

Substitution of ring A of **1d** was then undertaken (Table 3). Although this exercise did not identify compounds that had significantly greater activity against PMT1, it did reveal some, for example, **4a** and **4b**, which displayed potent activity (50 nM) in the *C. albicans* assays. By comparison, the ring A 4-Cl derivative (**2a**) is ~10-fold less active in the latter assay. The reason for the activity against the organism now outstripping that versus the isolated enzyme may be attributable to off-target activity (including inhibition of other PMT subtypes[20]) contributing to the anti-*Candida* effect or increased penetration to the endoplasmic reticulum membrane. However, where evaluated, the PMT1 inhibitors induce multiple phenotypic changes typical of *C. albicans* PMT1 mutants, for example, a decrease in formation of hyphae on spider medium and formation of cellular aggregates,[7] at concentrations in line with the inhibitory activity against the enzyme.

Table 3. Ring substitutions: in vitro inhibition of PMT1 and *C. albicans* proliferation by (**4a**–**f**)



Compound	Phenyl ring substitution: X, Y	PMT1 inhibition[a] IC_{50}, μM	*C. albicans* G418-sensitive proliferation,[a] IC_{50} μM
1d	H	1.5	0.5
4a	4-F	1.2	0.05
4b	4-Cl	1.6	0.05
4c	4-CF_3	4.0	0.18
4d	4-OCF_3	1.3	0.2
4e	2,4-Di-F	4.0	0.05
4f	3,5-Di-Me	0.85	0.11

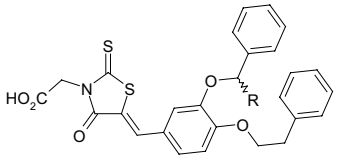
[a] Mean of at least two determinations; NT: not tested.

Introduction of sidechain substitutions in **1d** led to the most dramatic increase in potency, both against the enzyme and the organism (Table 4). Specifically, introduction of a methyl group (**5a**) or a hydroxymethyl group (**5d**) gave activities (as racemates) of 200 and 170 nM against PMT1 and 20 and 300 nM in the *C. albicans* assay, respectively. In this instance the excellent cellular activity of **5a** compared to **5d** may simply reflect the greater membrane penetration of the former compound relative to the more polar hydroxymethyl derivative. This is paralleled by the primary carboxamide (**5e**) which, although a potent PMT1 inhibitor showed only a weak effect in the *C. albicans* assay. Increasing the lipophilicity as in the case of the tertiary amide (**5f**) results in the re-gaining of potent activity against the organism. At this stage, relative to **1c**, an increase in activity of 10-fold against PMT1 and 150-fold in the *C. albicans* assay had been achieved without significant increase in molecular weight (Table 5).

The data on the racemic sidechain substituted analogues (**5a**–**d**) indicated that such modifications had a clear enhancing effect on compound profile and led us to undertake an evaluation of the effect of substituent type and absolute stereochemistry. The (*S*)-Me derivative (**6a**) was 5-fold more potent than the (*R*)-Me derivative (**6b**) against both the enzyme and the organism. However, this effect was muted when the 3-benzyloxy sidechain contained an (*R*)- or (*S*)-CF_3 group, for example, **6c/6d** and **6i/6j**.

In summary, starting from a 50 μM inhibitor of PMT1 identified from high throughput screening we have undertaken an optimisation programme, which has provided inhibitors of PMT1 in the mid-nanomolar range and displaying low nanomolar activity in the *C. albicans* assay. The reason(s) for the potency against the organism outstripping that versus the enzyme remain to be explored. They could partly relate to the somewhat artificial nature of the cellular assay as well as the

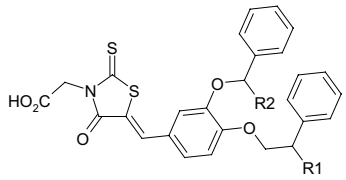
Table 4. Sidechain substitutions: in vitro inhibition of PMT1 and *C. albicans* proliferation by (**5a**–**g**)



Compound	R	PMT1 inhibition[a] IC_{50} μM	*C. albicans* G418-sensitive proliferation,[a] IC_{50} μM
5a	Me	0.2	0.02
5b	Et	0.95	0.02
5c	*i*-Pr	0.7	0.03
5d	CH_2OH	0.17	0.3
5e	$CONH_2$	0.35	7.0
5f	$CON(n\text{-}Pr)_2$	0.8	0.05
5g		1.0	43

[a] Mean of at least two determinations; NT: not tested.

Table 5. Sidechain substitutions: in vitro inhibition of PMT1 and *C. albicans* proliferation by (**6a–j**)



Compound	R1	R2	PMT1 inhibition[a] IC_{50} μM	*C. albicans* G418-sensitive proliferation,[a] IC_{50} μM
6a	····Me	H	1.8	0.07
6b	—Me	H	9.5	0.35
6c	····Me	—CF_3	0.4	0.015
6d	—Me	—CF_3	0.6	0.02
6e	·····Et	—CF_3	0.35	0.02
6f	—Et	—CF_3	0.5	0.02
6g	····Me	—Me	0.5	0.002
6h	····Me	····Me	1.8	0.03
6i	····Me	·····CF_3	1.8	0.2
6j	—Me	·····CF_3	3.5	0.55

[a] Mean of at least two determinations; NT: not tested.

reasons cited above. In any case, these inhibitors represent the first of their kind against this complex transferase and they could serve as useful tools for studying the cellular effects of protein O-mannosylation and its relevance in the search for novel antifungal agents.

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17. Unpublished data. Neutropenia was induced in DBA2 mice by single intraperitoneal injection of cyclophosphamide (150 mg/kg) two days before inoculation with *C. albicans*. A range of infective doses of *C. albicans* were injected into the tail veins and percent survival was followed over 21 days with body weight monitoring. The results were essentially identical to those with immunocompetent mice.
18. PMT1 assay method: In order to be able to screen a significant number of compounds against the PMT1 enzyme it was necessary to adapt the existing literature assay,[21] which involved a solvent extraction step. Dolicholphospho-^{3}[H]-mannose and phosphatidylcholine (100 mg/mL in chloroform) were dried down under vacuum and then resuspended in 1% TX100. The peptide substrate, Ac-DYATAV-NH_2 (final concentration 0.5 mM), was added in assay buffer (100 mM Tris/1 mM $MgCl_2$/0.04% 2-mercaptoethanol, pH 8.0) and test compounds in 10% DMSO, to give a final concentration of 1% DMSO. The reaction was started with the addition of a microsomal membrane suspension from a *Saccharomyces cerevisiae* strain, which had been engineered to over-express the *C. albicans* PMT1 protein. Although this assay cannot be demonstrated to be entirely specific for PMT1, the majority of the measured activity should be PMT1 associated. After 60 min at room temperature the reaction was stopped with cold methanol. The unreacted substrate was then removed by the addition of a suspension of C18 reverse phase column material in methanol. After mixing, the C18 was allowed to settle and a sample of supernatant was taken for scintillation counting.
19. *C. albicans* proliferation assay. Analysis of the *pmt1-/-Candida* strain reveals a number of striking phenotypic changes from the wild type, including failure to form hyphae, changes in adhesive properties and increased sensitivity to the aminoglycoside antibiotics Hygromycin B and G418, all of which reflect changes in the structure of the cell wall.[7] In order to assess inhibition of PMT1 activity in the intact organism we chose to follow the change in susceptibility to G418. A culture of early log-phase wild-type (CAF2-1) organism (OD_{600} 0.1 in YEPD broth) was added into 96-well plates in the presence of 100 μg/mL G418. Test compound was then added (to a final concentration of 1% DMSO) and after brief mixing the plate was incubated at 37 °C for 16 h. At the end of this period the OD_{600} was measured as an indicator of cell number. Inhibition of PMT1 was associated with a reduction in proliferation and individual IC_{50} values for the test compounds could be determined.
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