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In vitro and *in vivo* anti-*Trypanosoma brucei* activities of phenazinomycin and related compounds

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During the course of our screening program to discover new antitrypanosomal compounds, we have evaluated isolates from soil microorganisms as well as compounds from the antibiotic libraries of the Kitasato Institute for Life Sciences and Bioscience Associates. We have previously reported on various microbial metabolites exhibiting potent anti-*Trypanosoma brucei* properties, which are defined as antitrypanosomal properties.[1,2] We have recently evaluated the known phenazine antibiotics, phenazinomycin, griseoluteic acid, griseolutein B and related compounds, some of which have shown significant antitrypanosomal activities, both *in vitro* and *in vivo*. Here, we report the antitrypanosomal profiles of phenazinomycin and some related compounds (Figure 1) in comparison with those of some clinically used antitrypanosomal drugs, such as suramin and eflornithine. We also present our findings with respect to structure–activity relationships.

Griseoluteic acid, griseolutein B, phenazinomycin and 1,6-dimethoxyphenazine were obtained from the antibiotic libraries of the Kitasato Institute for Life Sciences and Bioscience Associates. Related compounds, namely clinically used clofazimine and phenazine, were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA), and phenazine 5-oxide was donated by Meiji Seika Kaisha (Yokohama, Japan).

In vitro antitrypanosomal activities against *Trypanosoma brucei brucei* strain GUTat 3.1 (low virulence strain against mice) and cytotoxicity against human diploid embryonic cell line MRC-5 were measured, as described previously.[1,3] *In vivo* antitrypanosomal activity for *T. b. brucei* strain S427 (high virulence strain against mice) was measured as described previously.[2] Test compounds were solubilized in an aqueous mixture of 10% DMSO-Tween 80 and EtOH (7:3) and administered i.p. to mice on the next day (day 1) following infection with parasites (day 0). Subsequently, the compounds were successively administered (i.p.) to the infected mice once a day for 3 days (days 2–4). Efficacies of the compounds were determined by parasitaemia levels and the mean of survival days (MSD), and compared with those of the untreated control mice.

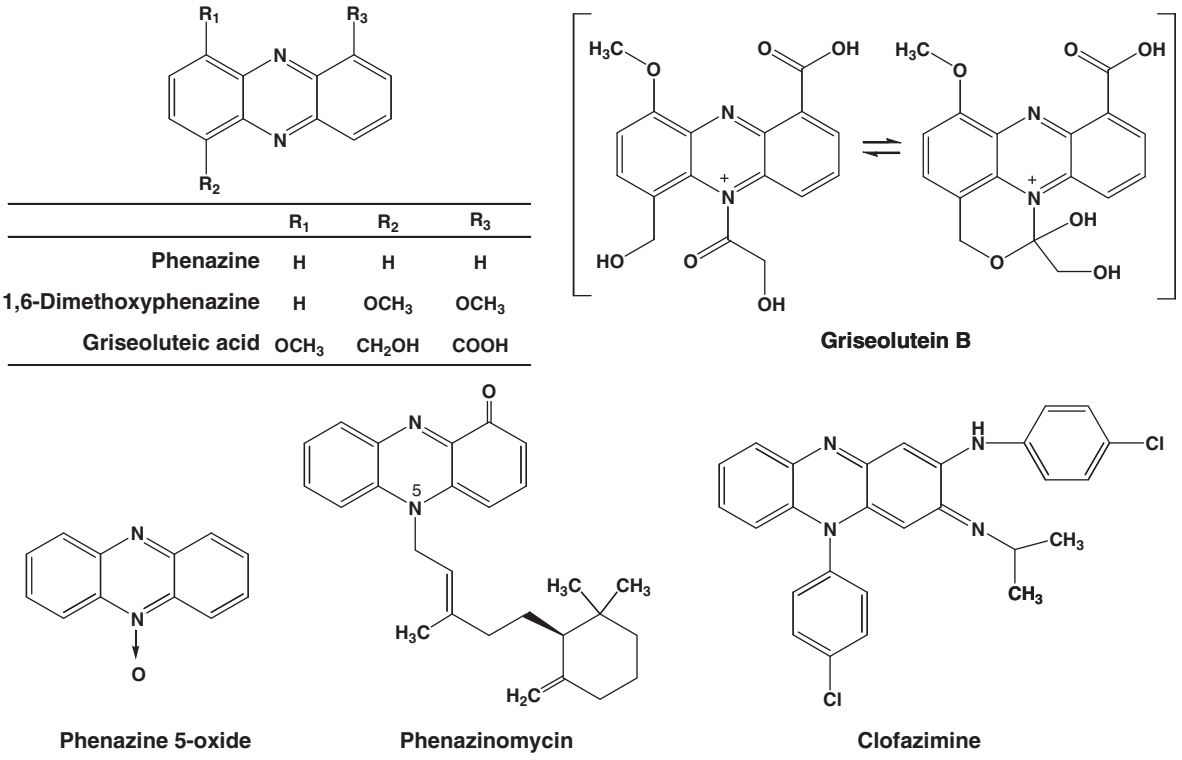
Table 1 shows the *in vitro* antitrypanosomal activities of phenazine compounds and selected standard antitrypanosomal drugs. Griseolutein B showed the highest antitrypanosomal activity, with an IC_{50} value of 1.4 ng ml^{-1}. The compound was 1100–1600-fold more potent than eflornithine and suramin. Phenazinomycin was 164-fold less active than griseolutein B, showing an IC_{50} value of 230 ng ml^{-1}. Griseoluteic acid and clofazimine showed moderate antitrypanosomal activity, with an IC_{50} value of approximately 2 μg ml^{-1}, which is comparable to those of both eflornithine and suramin, whereas 1,6-demethoxyphenazine, phenazine and phenazine 5-oxide showed no antitrypanosomal activity whatsoever.

The *in vitro* cytotoxicities of phenazine compounds and the antitrypanosomal drugs tested are also presented in Table 1. Griseolutein B showed the highest cytotoxicity against MRC-5 cells, with an IC_{50} value of 9 ng ml^{-1}. Griseoluteic acid and phenazinomycin were revealed to be slightly cytotoxic, demonstrating IC_{50} values of 5–6 μg ml^{-1}, while clofazimine had an IC_{50} value of >100 μg ml^{-1} and was shown to be not cytotoxic.

For appropriate evaluation of the combined antitrypanosomal activities and cytotoxicities of test compounds, we introduced selectivity indexes (SI; cytotoxicity (IC_{50} for the MRC-5 cells)/antitrypanosomal activity (IC_{50} for the GUTat 3.1 strain)) as presented in Table 1. Among the tested compounds, phenazinomycin and clofazimine showed a medium SI, with ratios of around 23 –> 52, less than or similar to those of eflornithine and suramin. Griseoluteic acid and griseolutein B showed a low SI, with ratios of around 3–6. Therefore, we selected phenazinomycin and clofazimine for *in vivo* efficacy tests.

The preliminary *in vivo* antitrypanosomal activities of phenazinomycin and clofazimine were measured in the *T. b. brucei* S427 acute mouse model. At a dose of 50 mg kg^{-1}, phenazinomycin did not achieve cure but did extend the MSD to 12.3 days, representing a 2.7-fold increase over control MSD (4.5 days). The same dose of clofazimine did not achieve cure but also extended the MSD to 6.0 days, similar to the control MSD (6.0 days). Under the same conditions, suramin showed a curative effect (MSD: >30 days) at a much lower dose of 1 mg/kg. The phenazinomycin data suggest that it might be a candidate compound for discovering new antitrypanosomal drugs. These efficacy tests, including *in vivo* antitrypanosomal activity, are ongoing.

1,6-Dimethoxyphenazine, phenazine and phenazine 5-oxide showed no antitrypanosomal activity. The most potent antitrypanosomal activity of griseolutein B and the moderate antitrypanosomal activity of griseoluteic acid and clofamizine, in comparison with phenazinomycin, provide very interesting information about structure–activity relationships. Griseoluteic acid lacks the substituent at N-5 and is 1450- and 9-fold less bioactive than griseolutein B and phenazinomycin, respectively. Furthermore, phenazinomycin possesses a substituent containing a sesquiterpene moiety at N-5, and has a medium SI compared with griseoluteic acid and griseolutein B. Our data therefore suggest



	R_1	R_2	R_3
Phenazine	H	H	H
1,6-Dimethoxyphenazine	H	OCH_3	OCH_3
Griseoluteic acid	OCH_3	CH_2OH	COOH

Figure 1 Structures of phenazine compounds.

that the substituent at N-5 present in phenazinomycin bestows significant antitrypanosomal activity and cytotoxicity.

Further studies are necessary for an extensive structure–*in vitro* antitrypanosomal activity evaluation of phenazinomycin-related compounds.

Griseoluteic acid (LL-14I352beta) is known to have antitumor and weak antibacterial activity, through its mode of action in inhibition of DNA synthesis.[4] Griseolutein B represents an equilibrium between the ketone form and the cyclic hemiacetal form (Figure 1),[5] and is reported to have antibacterial and antitumor activity.[6,7] We previously reported that phenazinomycin has antibacterial and antitumor activity,[8] although the mode of action is not clearly understood. However, a related antibiotic, lavanducyanin (WS-9659 A), containing a cyclohexenylmethyl moiety at N-5, is reported to have antitumor activity and to inhibit testosterone 5α-reductase in rat, dog and human prostates.[9,10] The mode of action of lavanducyanin is believed to be via disruption of signal transduction in cell growth.[11] Clofazimine, an anti-inflammatory and antileprosy drug, is a riminophenazine compound and demonstrates antituberculosis and anti-*Mycobacterium avium* complex activities, while also being used to treat several diseases, including leishmaniasis.[12] The modes of action of clofazimine are via inhibition of DNA synthesis and stimulation of phospholipase A_2.[12] The reported mechanisms of action of gliseoluteic acid, griseolutein B and clofazimine may be replicated against *Trypanosoma* too.

Table 1 ***In vitro*** **antitrypanosomal activity against** ***Trypanosoma brucei brucei*** **GUTat 3.1 and cytotoxicity in MRC-5 cells of phenazine compounds and some commonly used antitrypanosomal drugs**

	IC_{50} (ng ml^{-1})		
	Antitrypanosomal activity	*Cytotoxicity*	*Selectivity index (SI)*
Compound	*GUTat 3.1*	*MRC-5*	*MRC-5/GUTat 3.1*
Griseoluteic acid	2030	6110	3
Griseolutein B	1.4	9	6
Phenazinomycin	230	5190	23
1,6-Dimethoxyphenazine	>12 500	ND[a]	—
Clofazimine	1920	>100 000	>52
Phenazine	>12 500	ND[a]	—
Phenazine 5-oxide	>12 500	ND[a]	—
Suramin	1580	>100 000	>63
Eflornithine	2270	>100 000	>44

[a]Not determined.

Recently, phenazine compounds have been reviewed extensively, as they are producers of reactive oxygen species, and also with a view to identifying their biochemical communication systems in soil or in mammals with bacterial infections.[13] However, discovery of the antitrypanosomal activities of griseoluteic acid, griseolutein B, phenazinomycin and clofazimine is novel and our data constitute the first report of such properties.

The above results reveal that phenazinomycin is a candidate compound for development of novel antitrypanosomal drugs. Further study of the antitrypanosomal and other biological activities of phenazinomycin is in progress.

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