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Novel tetramethylpiperidine-substituted phenazines are potent inhibitors of P-glycoprotein activity in a multidrug resistant cancer cell line

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The multidrug resistance (MDR)-neutralizing and cytotoxic properties of 16 novel tetramethylpiperidine (TMP)-substituted phenazines were compared with those of clofazimine and B669 using a P-glycoprotein (P-gp)-expressing undifferentiated, human leukemia cell line (K562/MMB). Unchlorinated TMP-substituted phenazine molecules were more cytotoxic than their chlorinated counterparts, while the halogenated molecules, especially those with chlorine atoms at position 3 on the aniline and phenyl rings, were less cytotoxic but more effective as chemosensitizing, P-gp-neutralizing agents. One of the TMP-substituted phenazines, B4121, increased the sensitivity of K562/MMB cells to vinblastine by 100-fold. TMP-substituted phenazines are a novel class of pharmacologic anti-cancer agents with both direct cytotoxic, as well as MDR-neutralizing anti-tumor properties.

Key words: Multidrug resistance, P-glycoprotein, riminophenazines.

Introduction

Acquired multidrug resistance (MDR) to chemotherapeutic agents is often associated with an increased expression of P-glycoprotein (P-gp), an energy dependent drug efflux pump,[1] and can be a major obstacle in the treatment of cancer. Although this pump can be inhibited by a variety of pharmacologic agents *in vitro*, clinical trials employing these drugs as chemosensitizers have invariably been disappointing, mainly due to the toxic side-effects of these agents at concentrations which neutralize P-gp.[2–5] The design of novel inhibitors of MDR that are active at low concentrations and free of serious side-effects is clearly a priority in cancer research.

We have recently reported that the riminophenazine compound clofazimine (B663), originally described in 1957 as an anti-tuberculosis agent[6] and which has since been used as a component of the recommended combination chemotherapy of leprosy,[7] as well as a more active derivative, B669, reverse MDR in a P-gp-positive small cell lung cancer cell line.[8] A new class of anti-mycobacterial riminophenazines, the tetramethylpiperidine (TMP)-substituted phenazines, has recently been described.[9–12] Unlike clofazimine, these agents do not crystallize inside cells, are less soluble in fat and lack direct toxicity in experimental animals.[9–12] In this study, the MDR-neutralizing properties and cytotoxic activities of 16 novel TMP-substituted phenazines, relative to those of clofazimine and B669, have been investigated using a P-gp-expressing cell line.

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Methods

Drugs

All the riminophenazine compounds were synthesized by Dr JF O'Sullivan (Department of Chemistry, University College Dublin, Republic of Ireland), and their molecular structures are shown in Figures 1 and 2. These agents were dissolved in ethanol containing 10 mM acetic acid, while vinblastine was dissolved in dimethyl sulfoxide (DMSO), both solutions giving stock concentrations of 2 mg/ml. Further dilutions were made in RPMI 1640 supplemented with 10% fetal calf serum (FCS). In addition to 16 TMP-substituted phenazines, clofazimine (B663) the prototype, chlorinated riminophenazine, its unchlorinated derivative B670, as well as B669 and its chlorinated analog B4175 were included for comparison.

MDR cell lines

An undifferentiated leukemia cell line (K562) and its P-

gp-expressing derivative (K562/MMB) were maintained as previously described.[13]

Cytotoxicity assay

This was performed using a metabolic assay based on the reactivity of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Sigma, St Louis, MO) with viable cells.[14] Cells were seeded at 1×10^4/well in 96-well microtiter plates in a final volume of 200 μl of FCS-supplemented RPMI 1640 in the presence of the riminophenazines at concentrations of 0.02–2 μg/ml with or without a fixed concentration (3 ng/ml) of the standard anti-neoplastic agent, vinblastine, which was non-toxic for K562/MMB cells. Appropriate solvent control systems were included. After incubation at 37°C for 7 days 20 μl of a 5 mg/ml solution in phosphate-buffered saline (PBS) of MTT was added to each well and the plates incubated for 4 h at 37°C.[8] The cells were then washed with PBS and the intracellular formazan solubilized with DMSO, and quantitated spectrophotometrically at a test wavelength of 540 nm and a reference wavelength of 620 nm.

Measurement of uptake of [^{3}H]vinblastine

The effects of B663, B3962 and five of the chlorinated derivatives of B3962 (B4100, B4112, B4121, B4128 and B4169) on the uptake of [^{3}H]vinblastine by K562 and K562/MMB cells were also investigated. Briefly, following a 30 min preincubation at 37°C with the riminophenazines at 0.5 μg/ml, the cells (1×10^6/ml) were treated with 25 ng/ml [^{3}H]vinblastine (1 μCi added, specific activity 11.4 Ci/mmol; Amersham, Buckinghamshire, UK) for 30 min. Uptake was terminated by the addition of ice-cold PBS and the radioactivity measured in a liquid scintillation spectrometer following washing of the cells and release of cell-associated [^{3}H]vinblastine with 1% Triton X-100/0.1 M NaOH.

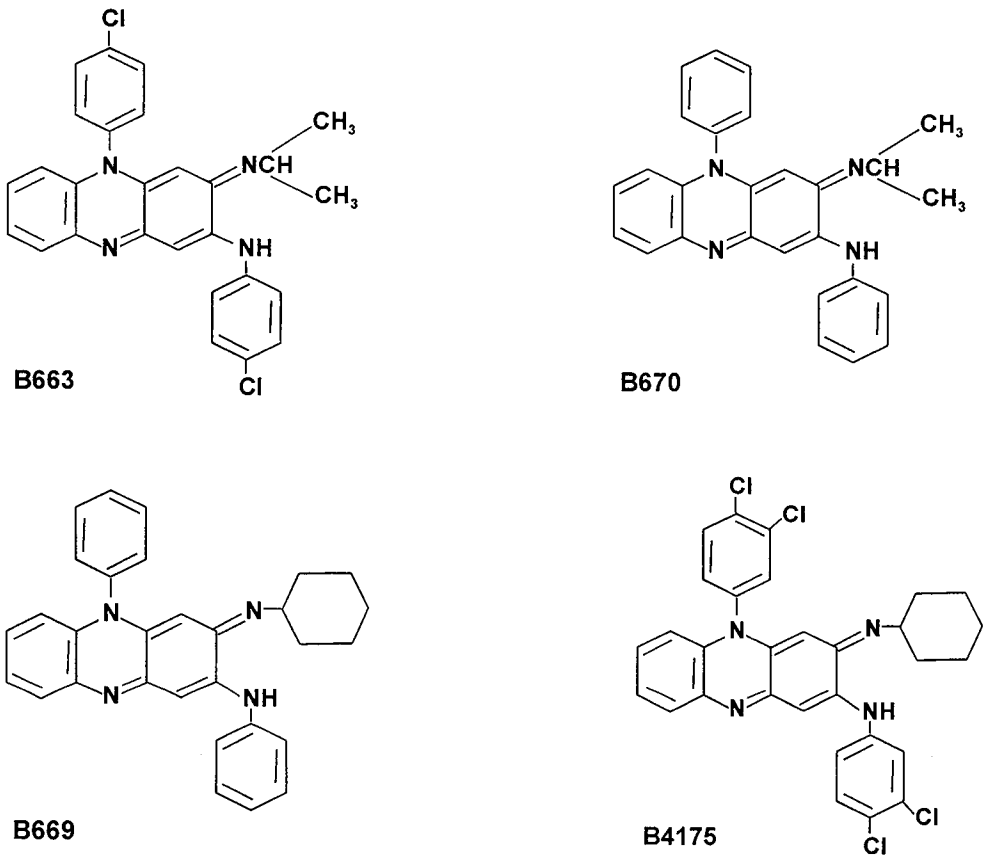


Figure 1. Chemical structures of B663, B670, B669 and B4175.

Statistical analysis

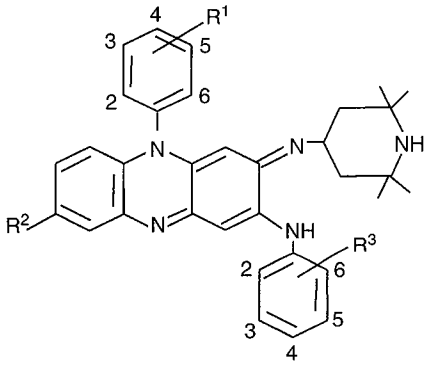
Results are expressed as the mean values ± SEM. Correlation between increased sensitivity in cytotoxicity assays and increased [^{3}H]vinblastine uptake was calculated using Spearman's correlation coefficient.

Results

Cytotoxic and MDR neutralizing activity of the riminophenazines

These results for all the test riminophenazines are shown in Table 1. The unchlorinated TMP derivatives were in general more cytotoxic than the corresponding chlorinated derivatives; however, this was not the case with those riminophenazines which contained alkylimino groups at position 2 on the phenazine nucleus. For example, the IC_{50} values for B3962 and B4169 were 0.285 and 2.861 μg/ml, respectively.

With respect to reversal of MDR using vinblastine (3 ng/ml) as the standard chemotherapeutic agent, the three most active chemosensitizing agents in increasing order of potency were B4121, B4169 and B4112. Full dose–response curves showing both the cytotoxic and MDR-neutralizing effects of B3962 and five of its chlorinated derivatives on K562/MMB cells are shown in Figure 3. The experimental compounds did not alter the level of sensitivity of the parent cell line (K562) to vinblastine (not shown). The parent cell line and the P-gp-positive cell line did not differ with respect to sensitivity to the direct cytotoxic effects of the riminophenazines (results not shown).



TMP-substituted phenazine

Compounds	R_1 and R_2	R_3
B3962	H	H
Chlorinated		
B4090	4-Cl	Cl
B4100	3,4-di-Cl	Cl
B4112	3-Cl	H
B4121	3,5-di-Cl	H
B4123	3-Cl	Cl
B4128	2,4-di-Cl	H
B4169	3,4,5-tri-Cl	H

*The substitutions in the phenyl- and analino-rings (R_1 and R_3) and at the R_2 position of the phenazine nucleus are indicated for each compound tested.

Figure 2. Chemical structures of the TMP-substituted phenazines tested.

Effects of the riminophenazines on cellular uptake of [^{3}H]vinblastine

All the compounds tested caused significant ($p<0.001$) enhancement of vinblastine uptake by K562/MMB cells (Table 2), with B4121 being the most active. A significant correlation was found between increased [^{3}H]vinblastine uptake and increased sensitivity of the cells to this agent ($r=0.8182$, $p<0.005$). Uptake of [^{3}H]vinblastine by the parent cell line (K562) was unaffected by the experimental compounds (results not shown). Total vinblastine uptake by K562 was 5.4 ± 0.1 ng/10^6 cells in the absence of the chemosensitizing agents.

Table 1. Effects of a 7 day exposure to different concentrations of various riminophenazine compounds on the sensitivity of the MDR leukemia cell line K562/MMB to vinblastine (3 ng/ml)

	IC_{50} (μg/ml)[a]	
	Alone (cytotoxicity)	Plus vinblastine (MDR-reversal)
Riminophenazine		
B670	0.566	0.136 (4.17)
B663	0.225	0.038 (5.92)
B669	0.139	0.023 (6.04)
B4175	>2.0	>2.0
TMP derivatives		
B3962	0.285	0.07 (4.07)
Chlorinated only		
B4090	0.197	0.027 (7.3)
B4100	0.335	0.095 (3.53)
B4112	0.156	0.008 (19.5)
B4121	2.250	0.023 (97.83)
B4123	0.333	0.049 (6.8)
B4128	0.383	0.059 (6.49)
B4169	2.861	0.116 (24.66)

[a]Data from two to four experiments are expressed as the mean drug concentration (μg/ml) causing 50% cell killing.
The values in parentheses represent the *n*-fold sensitivity compared with the riminophenazine compounds without the chemotherapeutic agent. The standard errors were 2–20%.

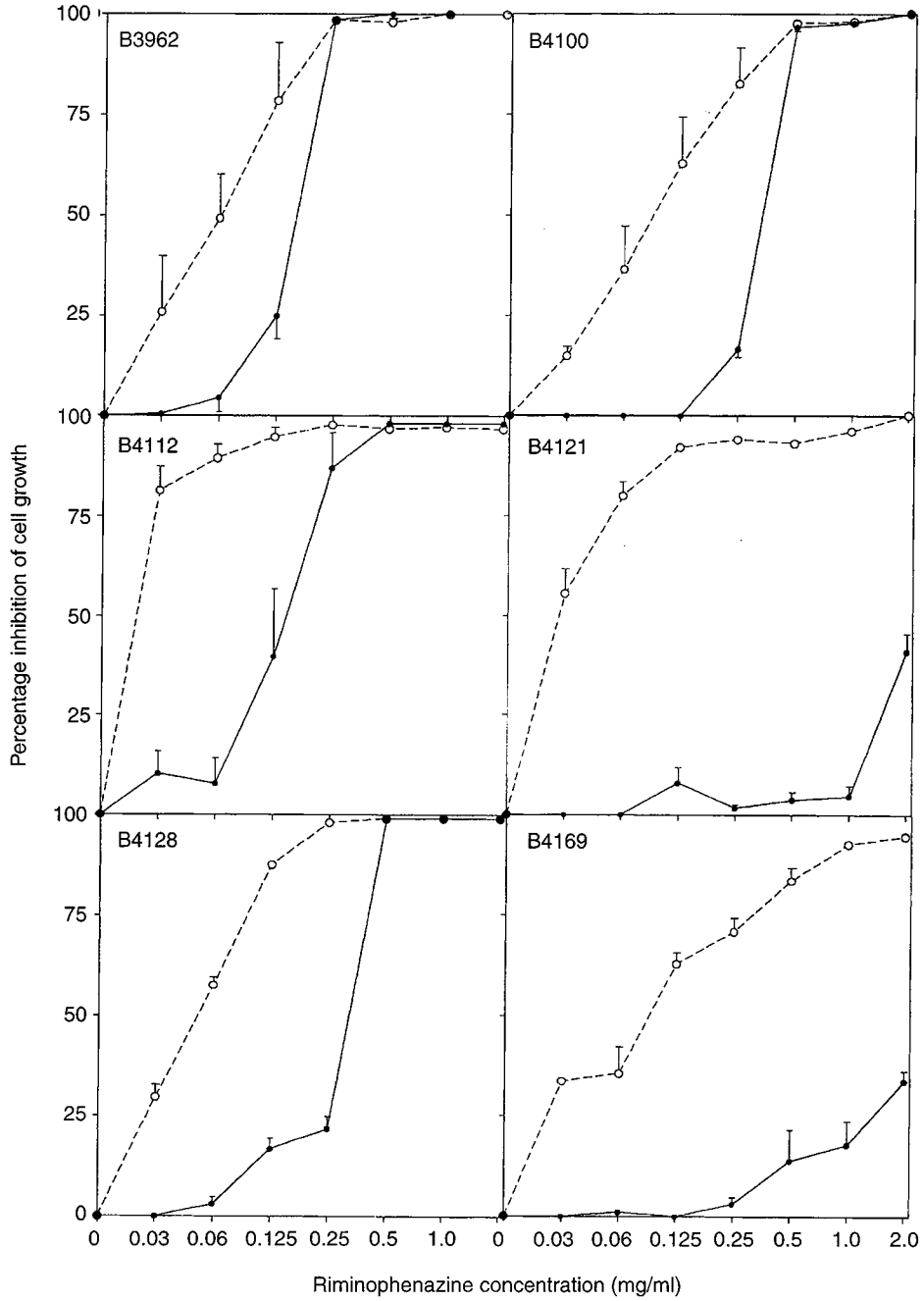


Figure 3. Effects of TMP-substituted phenazines on the growth of a P-gp-positive MDR cell line (K562/MMB) alone (filled circles) or in combination with a non-cytotoxic concentration of vinblastine (3 ng/ml) (open circles).

Discussion

The recognition of the association between chemosensitizing potency and common structural and physico-chemical properties of the various classes of MDR-reversing agents has enabled the development of novel chemosensitizers with improved P-gp-neutralizing activity in the setting of minimal cytotoxicity.[15] Our efforts in this area have been largely focused on the riminophenazines, of which clofazimine, primarily an anti-mycobacterial agent, is the prototype.[8] We have clearly demonstrated that the presence of an alkylimino group at position 2 on the phenazine nucleus is a critical requirement for biological activity of these agents.[16] In the search for derivatives with improved chemosensitization:cytotoxicity differentials

Table 2. Effects of a 60 min exposure of K562/MMB cells to various riminophenazine compounds (at 0.5 μg/ml) on [^{3}H]vinblastine (25 μg/ml) uptake

Treatment	[^{3}H]Vinblastine uptake (ng/10^6 cells)[a]
Control	1.174±0.298
B670	1.431±0.049
B663	2.595±0.236
B669	1.387±0.015
B4175	1.470±0.108
B3962	1.418±0.037
B4090	4.234±0.555
B4100	3.808±0.293
B4112	4.963±0.531
B4121	6.265±0.405
B4123	3.946±0.520
B4128	4.632±0.704
B4169	4.934±0.398

[a]Data from two to four experiments are expressed as the mean [^{3}H]vinblastine uptake by 10^6 cells±SEM.

(i.e. high chemosensitizing activity in the setting of low direct cytotoxicity), a recent major, molecular structure–function screening program conducted in our laboratories has identified the TMP-phenazines as a novel class of anti-tumor riminophenazines.[17] In the present study we have compared the chemosensitizing and direct anti-tumor properties of 16 novel TMP-substituted phenazines with those of clofazimine and B669. The TMP-phenazines varied with respect to their halogenation profiles.

All of the TMP-phenazines tested, with the exception of non-chlorinated B3962, outperformed clofazimine and B669 in assays of uptake of [^{3}H]vinblastine by P-gp-expressing K562/MMB cells, with B4121 being the most impressive. With respect to restoration of chemosensitivity in cytoxicity assays, only B4112 was superior to clofazimine in terms of chemosensitizing potency, with B4090, B4100, B4121, B4123, B4128 and B4165 being approximately equipotent. The apparent discrepancy between the results of the [^{3}H]vinblastine uptake and cytotoxicity assays may be related to the different incubation times used (30 min and 7 days, respectively). It may be that the chlorinated TMP-phenazines are accumulated more efficiently and/or are more rapidly acting than clofazimine, hence their superior performance in this short-term assay. These differences may be difficult to detect in the longer duration cytotoxicity assay.

Interestingly, the direct cytotoxic activity of two of the TMP-phenazines, B4121 and B4169, was about 10-fold less than that of clofazimine, resulting in markedly improved chemosensitization:cytotoxicity differentials (98 and 25, respectively, by comparison with 6 for clofazimine), a potentially important property of chemosensitizing agents. The importance of the TMP group is highlighted by the fact that B4112, a TMP-substituted compound chlorinated at the same positions as clofazimine, is a more active chemosensitizer than clofazimine.

In conclusion, B4121, a novel dichlorinated TMP-phenazine, is more impressive than clofazimine with respect to accelerated uptake of vinblastine into a P-gp-expressing leukemic cell line and has a vastly superior chemosensitization:cytotoxicity differential.

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