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Preliminary communication

Correlation of calculated molecular orbital energies of some phenothiazine compounds with MDR reversal properties

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Abstract

Molecular orbital energies of energetically minimized series of extended aromatic and aminoalkyl side chain substituted phenothiazine compounds have been considered with respect to charge transfer (CT) binding properties to P-glycoprotein (P-gp) amino acids of the first P-gp loop. A dependency of decreasing energies of lowest unoccupied orbitals (E_{lumo}) with reduced CT binding properties to an increasing P-gp mediated multidrug resistance (MDR) has been found for the extended aromatic compounds.

Keywords: Phenothiazines; MDR modulators; Molecular orbital energies

1. Introduction

Phenothiazines and related tricyclic compounds belong to one of the eldest classes of modulators of multidrug resistance (MDR) in cancer cells. MDR has become a main problem in actual cancer treatment mediated by membrane transport efflux pumps like P-glycoprotein (P-gp) [1,2]. Efflux pumps transport various structurally different anticancer drugs out of the cells partly before they reach the cytosol after membrane passage [3,4].

Although phenothiazines are an almost classical class of MDR modulators their mode of molecular mechanism is still unclear. Early studies with flupentixol, a thioxanthene derivative, suggested interactions with membrane phospholipids beside P-gp binding properties for the more active *trans* compound compared to the less active *cis* isomer [5,6]. Correlations of such drug–membrane activities with MDR reversal activities have been reported for one phenothiazine series with *N*-aminoalkyl side chains [7]. However, with only varying the alkyl side chain length no correlations of both activities were found [8], so that one may conclude that possible drug–membrane activities actually do not correlate with the observed P-gp inhibitory activities. The observed significant difference in P-gp inhibitory activities for the stereoisomers of flupentixol are only explicable by a different binding of the compounds to a stereochemically sensitive potential P-gp binding site. As a P-gp binding of P-gp substrates to such a binding site is known to precede conformational changes in the α-helical P-gp transmembrane subunits which then result in the efflux of the P-gp substrates, a competition with P-gp inhibiting compounds at such a binding site will result in lowered MDR reversal activities of the P-gp substrate [9,10]. Such P-gp binding interactions each depend on the molecular characteristics of the inhibiting molecules and have been suggested in literature with transmembrane segments TM 6 and TM 12 involving hydrophobic as well as H-bonding interactions [11].

It will be of great importance to identify and characterize such a potential binding site to improve the P-gp inhibiting properties of MDR-modulators.

Recent competition experiments with tomato lectin proved phenothiazine interactions with the first transmembrane loop overspanning TM 1 and TM 2 by influencing tomato lectine interactions with this loop [12]. Beside binding of both glycosylated parts of tomato lectine and the first loop certain interactions with neighboring amino acids will be involved interfer-

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ing with the phenothiazine compounds so that those neighboring amino acids have to be considered as a potential P-gp binding site for the interacting phenothiazine compounds.

Most of the investigated phenothiazines discussed below have a quinone partial structure which is known to undergo certain charge transfer (CT) interactions with aromatic compounds. With respect to the prevailing aromatic amino acids Phe and Tyr within this first loop we investigated probable CT interactions between the phenothiazines and these aromatic amino acids by calculating energies of the lowest unoccupied molecular orbital (LUMO) and highest occupied molecular orbital (HOMO) of the phenothiazines which both decisively contribute to such CT interactions. These energies then have been compared to the MDR reversal properties of the phenothiazines to estimate whether there may be a correlation proving the first loop to serve as a P-gp binding region.

2. Chemistry

Chlorpromazine **4**, 6,9-dioxochlorpromazine **7**, 5-oxo-5*H*-benzo[a]phenothiazine **1** and 6-hydroxy-5-oxo-5*H*-benzo[a] phenothiazine **3** have been synthesized as described in [13]. Formation of 7,8-dioxochlorpromazine **6** and 6,9-dihydroxychlorpromazine **5** followed recent protocols [14]. Verapamil, promethazine **8** and trifluoperazine **9** have been obtained from EGIS Works (Budapest, Hungary).

3. Pharmacology

MDR reversal activities have been measured in mouse T-lymphoma cell lines in comparison of parental cell line L5178Y and P-gp expressing subline each in comparison to untreated control by fluorescence uptake assay using fluorescent P-gp substrate rhodamine 123. Fluorescence activity ratios (*R*) have been calculated as described with *R* values > 1.1 corresponding to active P-gp inhibiting compounds. Verapamil has been used as reference compound at a concentration of 22 μM and a resulting *R* value of 15.58.

4. Results and discussion

As for phenothiazine compounds divers structural differences in functional patterns may cause differing P-gp binding modes which result in different MDR reversal properties, we considered among all investigated compounds **1**–**9** (Fig. 1) two structurally different series: one of the benzo-annelated phenothiazines with derivatives **1**–**3** and the other one of the aminoalkyl substituted phenothiazine skeletons **4**–**9**.

With poor basic properties of the nitrogen in the extended aromatic system and the quinone-imine partial structure compounds **1**–**3** serve as preferred electron acceptors in CT complexes with aromatic amino acids within the first P-gp loop (73–119), i.e. Phe 99, 103, 104 and Tyr 114, 116–118, which act as electron donors in a CT complex [15].

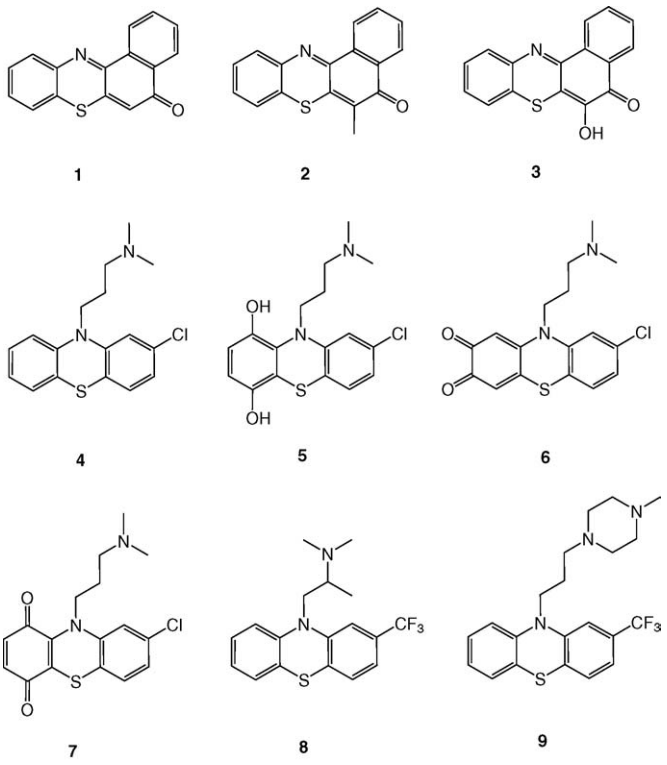

Fig. 1. Phenothiazine compounds series.

Table 1
Molecular orbital energies and MDR reversal activities (*R*-values) of compounds **1**–**9**

	Energies (eV)		*R*-values	
Compounds	E_{lumo}	E_{homo}	8 μM	80 μM
1	–1.577	–8.347	0.85	1.40
2	–1.523	–8.248	3.44	2.92
3	–1.594	–8.248	1.20	1.10
4	–0.307	–7.565	3.88	–[a]
5	–0.451	–8.053	1.14	–[a]
6	–1.684	–8.520	1.48	–[a]
7	–1.752	–8.120	1.40	–[a]
8	0.097	–7.420	1.75	5.29
9	–0.644	–7.788	30.14	–[a]

[a] Not determined because of observed cytotoxic effects.

Comparing calculated LUMO energies (E_{lumo}) (Table 1) for compounds **1**–**3** which just differ in the 6-substituents the hydroxy compound **3** has the highest negative value and the 6-methyl compound **2** the relatively lowest one. Compounds of relatively low E_{lumo} values form less stable CT-complexes with electron donors as the differences of energy values of each donor and acceptor determine the CT-energies and thus the strength of intermolecular bonding [16,17].

In comparison of the biological activities as MDR reversers compound **1** proved to be inactive at the low concentration, while the 6-methyl derivative **2** was found most active with an *R* value of 3.44. At the higher concentration compound **1** showed higher activity than the 6-hydroxy derivative **3**. Non observed increase in activity for the substituted compounds **2** and **3** may result from saturation effects of P-gp as have been previously reported for different classes of MDR reversers at such high concentrations [18].

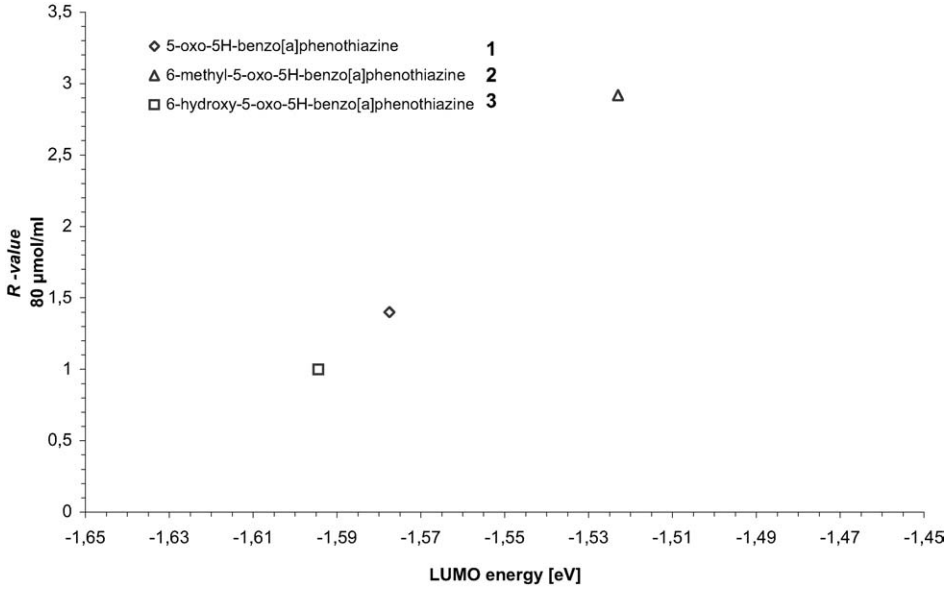

Fig. 2. Plotting of LUMO energies of extended aromatic phenothiazines **1**–**3** against MDR reversal activities at 80 μM.

Plotting the calculated LUMO energies against biological data (Fig. 2) an increase of MDR reversal activity is found with reduced LUMO energies suggesting a dependency of both compound properties which has to be proven by an increased set of extended aromatic phenothiazine compounds, so that the observed trend may give a preliminary hint.

In this case, published suggested CT interactions with aromatic amino acids of the P-gp loop [12] do not correlate with biological activity data in the way that better CT-binding properties lead to increased biological activity. The fact that poorer CT binding properties seem to correlate with increasing biological activity suggests additional P-gp binding properties of the phenothiazines which are supposed to bind to the loop but interfere with P-gp in a different way to cause the observed P-gp inhibitory effects. May be that the extent of possible loop binding reduces concentrations for P-gp inhibitory actions with another P-gp binding site.

From the series of aminoalkyl side chain substituted phenothiazines only chlorpromethazine **4**, promethazine **8** and trifluoperazine **9** showed MDR reversal activities at the low concentration in mouse T-lymphoma cells. With the side chain amino functionality and its lonely electronic pair, respectively, these compounds serve as electron donors in CT-complexes with phenylalanine amino acids of the P-gp loop. With lowest calculated HOMO energy values of all compounds the resulting CT-energies for complex formation with an electron acceptor are most favorable resulting in highest binding affinities to this P-gp binding region. However, these HOMO energies which lie within reported ranges of MDR modulators [19] do not correlate with the observed biological activities. Similar differences to biological data have also been found for the LUMO energies. The dihydroxy- as well as the dioxochlorpromazines **5**–**7** showed MDR reversal activities in P-gp expressing brain capillary endothelian cells. But with differing energy values for each LUMO as well as HOMO and similar activity data any influence of possible CT-binding properties to the P-gp loop on the MDR reversal properties is unlikely, so that interactions are possible and will undergo close investigations by further modeling of the peptide sequence of the P-gp loop but the mode of phenothiazine action leading to MDR reversal effects will be different from binding to this binding region as has been demonstrated.

5. Conclusions

Extended aromatic phenothiazine compounds with substituted quinone-imine partial structures structurally serve as electron pair acceptors in CT-complexes with amino acids phenylalanine and tyrosine, respectively, of the first P-gp loop thus explaining phenothiazine interactions with this loop. Compounds with calculated higher LUMO energies facilitating such CT complex formations showed lower activity as MDR reversers, while those of comparably higher energies show increased activities. So binding properties to amino acids of the first loop correlate with decreased biological activities as is suggested from these preliminary results. Phenothiazines with aminoalkyl side chain show HOMO energies within the range of reported MDR reversers thus suggesting electron donor properties in CT complexes. Neither HOMO nor LUMO energies correlate with biological data thus reflecting different phenothiazine interactions of these compounds with P-gp. Consequently, the observed tomato lectin MDR reversal interactions with phenothiazines do not correlate with the CT interactions so that this first loop is unlikely to serve as a P-gp binding site leading to the described changes in MDR reversal properties.

From the given results it may be concluded that tomato lectin as a wide spanning macromolecule like cyclosporine interacts with multiple P-gp binding sites [20,21] and thus may interfere with the phenothiazine P-gp binding site which will be different from that of the first loop.

6. Experimental protocols

6.1. Energy calculations

LUMO energies corresponding to the electron affinity of a molecule as well as HOMO energies corresponding to its ionization potential [22] have been calculated with the HyperChem 6.06 version program running on an AMD Athlon standard PC after performing geometry optimization of constructed starting structures using the semiempirical AM1-method as implemented in HyperChem. Resulting minima for each molecule have been given with a 0.001 kcal mol^{-1} $Å^{-1}$ energy gradient convergence criterion.

6.2. Cell culture

L5178Y mouse T-lymphoma cell line which was a gift from the National Cancer Institute (NCI) was infected with the PHA *mdr-1*/A retrovirus as described in [23]. P-gp expressing cells were selected by culturing the infected cells in 60 ng ml^{-1} colchicine. The L5178Y *mdr* subline and the L5178 parental cell lines were grown in McCoys 5A medium with 10% heat inactivated horse serum, L-glutamine (2 mM) and antibiotics.

6.3. In vitro fluorescence uptake assay of MDR reversal

The cultured mouse T-lymphoma cells were each adjusted to a concentration of 2×10^6 ml^{-1}, resuspended in serum free McCoys 5A medium and distributed into 0.5 ml aliquots in Eppendorf centrifuge tubes. Test compounds were added from stock solutions (1.0 mg ml^{-1}) and the samples were incubated for 10 min at room temperature. Then 10 μl of rhodamin 123 (5.2 μM as final concentration) were added to the samples and the cells were incubated for further 20 min at 37 °C, then washed twice and resuspended in 0.5 ml phosphate-buffered saline (PBS) for analysis. Fluorescence of 1×10^4 cells was measured by flow cytometry using a Beckton Dickinson FACScan instrument. Fluorescence activity ratios (R) have been calculated from fluorescence uptake relations of treated and untreated control cell lines.

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