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Research article

THE IMMUNOSUPPRESSIVE ACTIVITIES OF NEWLY SYNTHESIZED AZAPHENOTHIAZINES IN HUMAN AND MOUSE MODELS

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Abstract: In this study, we evaluated the activities of new types of azaphenothiazines in the following immunological assays: the proliferative response of human peripheral blood mononuclear cells induced by phytohemagglutinin A or anti-CD3 antibodies; lipopolysaccharide-induced cytokine production by human PBMC; the secondary, humoral immune response in mice to sheep erythrocytes (*in vitro*); and delayed-type hypersensitivity in mice to ovalbumin (*in vivo*). In some tests, chlorpromazine served as a reference drug. The compounds exhibited differential inhibitory activities in the proliferation tests, with 10H-2,7-diazaphenothiazine (compound **1**) and 6-(3-dimethylaminopropyl)diquinothiazine (compound **8**) being most suppressive. Compound **1** was selected for further studies, and was found to be

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Abbreviations used: AFC – antibody-forming cells; CNS – central nervous system; DMF – dimethylformamide; DMSO – dimethyl sulfoxide; DTH – delayed-type hypersensitivity; FCS – fetal calf serum; GI_{50} – inhibition of cell growth (the concentration needed to reduce the growth of treated cells to half that of untreated cells); IFN-γ – interferon gamma; IL – interleukin; i.p. – intraperitoneally; LPS – lipopolysaccharide; MDR – multidrug resistance; MTT – (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NS – not significant; OVA – ovalbumin; PBMC – peripheral blood mononuclear cells; PHA – phytohemagglutinin; RPMI-1640 – Rosewell Park Memorial Institute Medium; s.c. – subcutenaously; SRBC – sheep red blood cells; TNF-α – tumor necrosis factor alpha

strongly suppressive in the humoral immune response even at low concentrations (1 μg/ml). Compound **1** also inhibited the delayed-type hypersensitivity lipopolysaccharide-induced production of tumor necrosis factor and interleukin-6 in cultures of human blood cells. As there were only two subjects in this study, the effects of these compounds on human blood cells need to be confirmed. In this paper, we also discuss the structure-activity relationships of selected compounds.

Key words: Azaphenothiazines, Immune response, PBMC, Proliferation, Cytokines

INTRODUCTION

Phenothiazine-like drugs exhibit significant pharmacological effects, including antipsychotic, antihistamine, antitussive and antiemetic activities, and they possess interesting chemical properties [1]. Recent reports also describe anticancer [2-4], antiplasmid [5] and antibacterial activities [6, 7], and reversal of multidrug resistance (MDR) [8, 9]. Furthermore, there are potential applications for both classical and newly synthesized phenothiazines in the treatment of Alzheimer's disease [10], Creutzfeldt-Jakob disease [6] and AIDS [11]. Neuroleptic phenothiazines are potent modulators of immune functions. Numerous papers have described the inconsistent effects of phenothiazines, in particular chlorpromazine, on induced cytokine production in human and animal models. However, several articles [12-16] reported that this compound inhibits the release of tumor necrosis factor-alpha (TNF-α), which is induced by various stimuli. Although chlorpromazine was shown to reduce the endotoxin-induced production of interleukin TNF-α in a mouse model, it did not inhibit IL-1 or IL-6 production, and IL-10 production even increased [16]. In cultures of human peripheral blood mononuclear cells (PBMC), chlorpromazine had no effect on endotoxin-induced cytokine production, but some studies showed its suppressive effects on TNF-α, IL-2 and IFN-γ expression or release by human immunocompetent cells [15-18].

New phenothiazines were obtained by modifying the parent phenothiazine structure mainly by introducing a new substituent at the thiazine nitrogen atom at position 10 and by substituting one or two benzene rings with homoaromatic and heteroaromatic rings. In the next step of the search for pharmacoactive pyridine and quinoline derivatives, we modified the phenothiazine structure with pyridine and quinoline rings to form new types of heterocyclic compounds: dipyrido-1,4-thiazines (2,7-diazaphenothiazines), and the linear and angular fused diquino-1,4-thiazines (pentacyclic dibenzo-1,9-diazaphenothiazines and dibenzo-3,7-diazaphenothiazines) [19-23]. Some of these azaphenothiazines exhibit promising anticancer activities against human tumor cell lines deriving

from the colon, breast, kidneys, ovaries, prostate and CNS, and against melanoma and leukemia cell lines [19, 23, Pluta *et al.*[1]].

To the best of our knowledge, there has been no report on the immunomodulatory action of any azaphenothiazines. In this paper, we describe and discuss the immunomodulatory activities of the recently synthesized azaphenothiazines **1-10** – five 10H- or 10-substituted 2,7-diazaphenothiazines (**1-5**), and five 6-substituted dibenzo-1,9-diazaphenothiazines (**6-10**) – in selected immunological assays using human and mouse models. These compounds possess different types of substituents at the thiazine nitrogen atom (hydrogen, alkyl, aryl, dialkiloaminoalkyl, acetylaminoalkyl and sulfonylaminoalkyl). Of note, compound **9** possesses the nitrogen half-mustard unit (the 2-chloroethylamino group), and compound **6** has the ethylene unit forming the additional dihydro-1,3-diazole ring (Fig. 1).

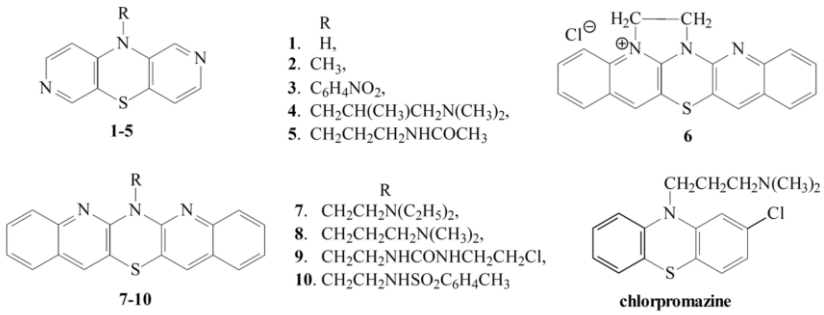

Fig. 1. The structures of azaphenothiazines **1-10** and chlorpromazine.

MATERIALS AND METHODS

Reagents

Sheep red blood cells (SRBC) were provided by the Wrocław University of Environmental Sciences (Wrocław, Poland). They were kept in Alsever's solution at 4ºC until needed. MTT, RPMI-1640 medium, DMSO (dimethyl sulfoxide), LPS (lipopolysaccharide; *E. coli* serotype O111:B4; 3×106 E.U./mg), PHA (phytohemagglutinin), ovalbumin (OVA) and chlorpromazine (2-chloro-10-(3-dimethylaminopropyl)phenothiazine) hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was supplied by Gibco (Paisley, UK). The complete and incomplete Freund's adjuvants were from Difco Laboratories (Detroit, Michigan, USA). Lymphoprep was from Pasching (Austria) and anti-CD3 antibodies were from R&D (Minneapolis, USA). All the tissue culture plates and flasks were purchased from Nunc (Denmark).

[1] Pluta, K., Morak-Młodawska, B. and Jeleń, M. National Cancer Institute Developmental Therapeutics Program, In-Vitro Testing Results, Bethesda, USA (unpublished data)

Mice

CBA mice of both sexes, 1-12 weeks old, were purchased from the Ilkowice Breeding Facility, Poland. The mice were fed a commercial pelleted food and filtered tap water *ad libitum*. The local ethics committee approved the study.

Blood donors

Blood from a non-smoking 36-year old woman and a non-smoking 60-year old man was used for the study. Written consent was obtained from both donors.

Syntheses

Azaphenothiazines **1-10** were obtained according to recently described procedures. 10*H*-2,7-diazaphenothiazine **1** was obtained from 3-amino-3'-nitro-4,4'-dipyridinyl sulfide in DMF [19]. Compounds **2-4** (10-methyl-, 10-(p-nitrophenyl)- and 10-(3-dimethylamino-2-methylpropyl)-2,7-diazaphenothiazine) were obtained from compound **1** by *N*-alkylation and *N*-arylation with methyl iodide and with 4-fluoronitrobenzene in DMF in the presence of sodium hydride, and with 3-dimethylamino-2-methylpropyl chloride in dioxane in the presence of sodium hydroxide [19]. Compound **5** (10-(3-acetylaminopropyl)-2,7-diazaphenothiazine) was obtained by *N*-alkylation of compound **1** with N-(3-bromopropyl) phthalimide in toluene in the presence of sodium hydride followed by hydrolysis with hydrazine and acetylation with acetic anhydride [24]. Compound **6** (5,6-ethylenediquinothiazinium chloride) was synthesized in the annulation reaction of 2,2'-dichloro-3,3'-diquinolinyl sulfide with 2-chloroethylamine in a methyl ether of diethylene glycol in an autoclave [22]. Compounds **7** and **8** (6-(2-diethylaminoethyl)diquinothiazine and 6-(3-dimethylaminopropyl)diquinothiazine) were synthesized from 6*H*-diquinothiazine in a reaction with 2-diethylaminoethyl and 3-dimethylaminopropyl chlorides in dioxane in the presence of sodium hydroxide. Compounds **9** and **10** (6-(2-chloroethylureido)ethyldiquinothiazine and 6-(2-*p*-toluenesulfonylamino) ethyldiquinothiazine) were synthesized from 2,2'-dichloro-3,3'-diquinolinyl chloride in a reaction of 1,2-diaminoethane followed by a reaction with 2-chloroethyl isocyanate and *p*-toluenenesulfonyl chloride [23].

The proliferation test

The venous blood from the two healthy donors was taken into heparinized tubes and diluted twice with phosphate buffered saline (PBS), applied onto Lymphoprep (density 1.077 g/ml) and centrifuged for 20 min at 800×g. The cells from the interphase were collected, washed twice with Hanks' medium and re-suspended in a culture medium consisting of RPMI-1640 supplemented with L-glutamine, sodium pyruvate, 2-mercaptoethanol and 10% FCS (referred to hereafter as the culture medium). The cells were distributed into 96-well flat-bottom plates (2×10^5/100 µl/well). PHA was added at a dose of 5 µg/ml, and the studied compounds were added at a concentration of 1, 10 and 100 µg/ml. The cells were cultured for 3 days in a cell culture incubator. After the incubation, the proliferation rate was measured using a colorimetric MTT method [25].

The results were presented as the mean optical density values from quadruplicate wells.

The toxicity test

Mouse splenocytes (pooled from three mice) were distributed in the culture medium into 96-well flat-bottom plates at a concentration of 2×105/100 µl/well. The studied compounds were added at a final concentration range of 50.0-1.56 µg/ml. After overnight incubation, the cells were treated with 0.25 µl of MTT [25], and the cell viability was measured as the optical density at 550/630 nm. The results were presented as the mean optical density values from quadruplicate wells.

The secondary, humoral immune response to sheep erythrocytes *in vitro*

Mice were sensitized intraperitoneally (i.p.) with 0.2 ml of a 5% sheep red blood cell (SRBC) suspension. After 4 days, the spleens from 5 mice were isolated, and a single cell suspension was prepared in the culture medium. The cells were distributed into 24-well plates at 5×10^6 cells/ml/well, and 0.1 ml of 0.005% SRBC was added. The studied compound was added at a concentration of 1 and 10 µg/ml at the beginning of a 4-day culture in a cell culture incubator. The number of antibody-forming cells (AFC) was determined by a method of local hemolysis in agar gel [26]. The results were presented as mean AFC numbers from 4 wells per 10^6 splenocytes.

Delayed hypersensitivity to ovalbumin

Mice were sensitized subcutaneously (s.c.) in the tail base with 5 µg of ovalbumin (OVA) emulsified in complete Freund's adjuvant. After 4 days, the delayed-type hypersensitivity (DTH) reaction was elicited by a s.c. injection of 50 µg OVA emulsified in incomplete Freund's adjuvant into the hind foot pads. The studied compound was administered i.p. 1 h before the sensitizing dose of antigen. The control mice were non-sensitized animals given only the eliciting dose of the antigen. The foot pad edema was measured using a caliper 24 h after the s.c. injection. The antigen-specific foot pad edema was calculated by subtracting the background control response, and the results were expressed in DTH units [27].

Induction of cytokines in peritoneal exudate cell cultures

Peritoneal exudate cells were obtained from mice by washing the peritoneal cavities with 5 ml of Hanks' medium containing 5 units of heparin. After the wash in Hanks' medium, the cells were re-suspended in the culture medium and placed at a density of 10^6/ml/well in 24-well culture plates. The cells were stimulated with 5 µg/ml of *E. coli* LPS for 24 h in a cell culture incubator. The compound was added at 1, 10 and 100 µg/ml concentrations with DMSO at the appropriate dilutions. The activities of TNF-α and IL-6 were respectively determined in the supernatants via bioassays using WEHI 164.13 [28] and 7TD1 cell lines [29].

Induction of cytokines in human blood cell cultures

Venous blood was diluted 5× with RPMI-1640 (0.5×10^6 cells/ml). The cell suspension was placed in 24-well culture plates (1 ml/well) and stimulated with 1 μg/ml of LPS. The compound was used at concentrations of 0.1, 1 and 10 μg/ml. After a 24-h culture, the activities of TNF-α and IL-6 were measured in the supernatants using bioassays [28, 29].

Statistics

The differences across groups were determined by analysis of variance after testing the homogeneity of variance using Levene's test. Individual grades were then compared using Tukey's test for multiple comparisons. The data is expressed as means ± SE (standard error). The effects of the compounds were compared with the appropriate dilutions of DMSO (the solvent). Differences were considered significant when P was less than 0.05. The statistical analysis was performed using STATISTICA 6.0 for Windows.

RESULTS

The effects of the compounds on phytohemagglutinin A and anti-CD3 antibody-induced proliferation of human peripheral blood mononuclear cells

The effects of the compounds on the proliferative response of human PBMC to PHA (assessed on one representative blood donor) are shown in Figs 2 and 3. Chlorpromazine served as a reference drug. Compounds **6-10** (Fig. 3) were distinctly inhibitory at concentrations as low as 10 μg/ml, and compounds **6** and **8** even exhibited strong activity at 1 μg/ml. Compounds **9** and **10** were much less active, although compound **9** did show a strong suppressive activity at 100 μg/ml. Compounds **1** and **2** were distinctly inhibitory at 10 μg/ml, but **3-5** showed a little activity (Fig. 2). Compound **1** was exceptionally suppressive at 100 μg/ml.

We also tested the effects of compounds **1** and **8**, at 10 and 100 μg/ml concentrations, on the proliferative response of human PBMC stimulated with anti-CD3 antibodies in parallel with stimulation with PHA (Fig. 4). It appeared that cell proliferation was blocked by both concentrations of compound **8** and chlorpromazine, but by compound **1** only at 100 μg/ml.

Toxicity of the compounds

Since the inhibitory effects of the compounds could have been caused by high cytotoxicity, the compounds were tested for their effects on mouse splenocyte viability in an overnight culture. The compounds were used at a wide concentration range (50 μg/ml-1.56 μg/ml). The reference compound was chlorpromazine. The results, presented in Fig. 5A and B, revealed that at the concentrations tested, compounds **1-5** were virtually devoid of toxicity, whereas some of compounds **6-10** appeared to be toxic (**6**, **7** and particularly **8**, which exhibited high toxicity even at their lowest concentrations). High toxicity was found with chlorpromazine (50 μg/ml-12.5 μg/ml).

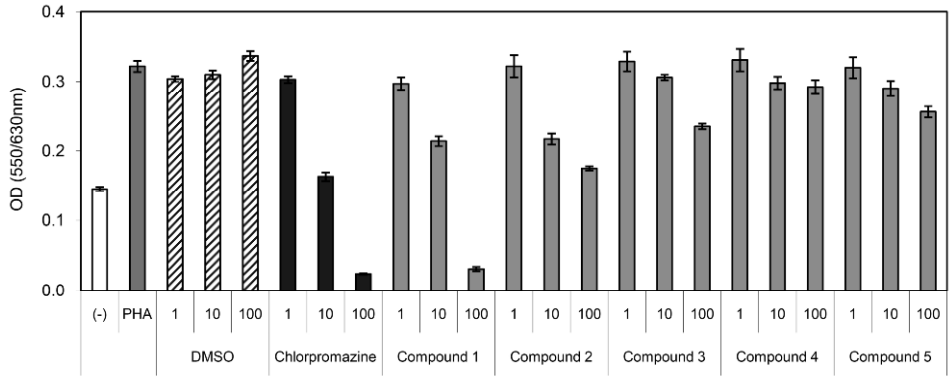

Fig. 2. The effects of compounds **1-5** on the proliferative response of human mononuclear blood cells to phytohemagglutinin A. The compounds were added to PHA-stimulated PBMC cultures at concentrations of 1, 10 and 100 μg/ml. After a 3-day incubation, the rate of cell proliferation was determined using the MTT colorimetric method. (-) – no additions. Statistics: 1 μg/ml: all comparisons NS; 10 μg/ml: DMSO vs Chlorpromazine, P = 0.0001; DMSO vs Compound **1**, P = 0.0001; DMSO vs Compound **2**, P = 0.0001; 100 μg/ml: DMSO vs Chlorpromazine, P = 0.0001; DMSO vs Compound **1**, P = 0.0001; DMSO vs Compound **2**, P = 0.0001; DMSO vs Compound **3**, P = 0.0001; DMSO vs Compound **4**, P = 0.0021; DMSO vs Compound **5**, P = 0.0001 (ANOVA).

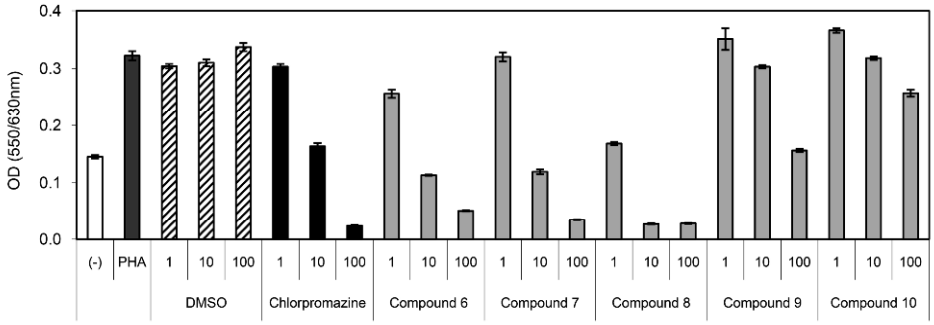

Fig. 3. The effects of compounds **6-10** on the proliferative response of human mononuclear blood cells to phytohemagglutinin A. The experiment was performed as described under Fig. 2. The compounds were used at concentrations of 1, 10 and 100 μg/ml. (-) – no additions. Statistics: 1 μg/ml: DMSO vs Compound **6**, P = 0.0025; DMSO vs Compound **8**, P = 0.0001; 10 μg/ml: DMSO vs Chlorpromazine, P = 0.0001; DMSO vs Compound **6**, P = 0.0001; DMSO vs Compound **7**, P = 0.0001; DMSO vs Compound **8**, P = 0.0001; 100 μg/ml: DMSO vs Chlorpromazine, P = 0.0001; DMSO vs Compound **6**, P = 0.0001; DMSO vs Compound **7**, P = 0.0001; DMSO vs Compound **8**, P = 0.0001; DMSO vs Compound **9**, P = 0.0001; DMSO vs Compound **10**, P = 0.0001 (ANOVA).

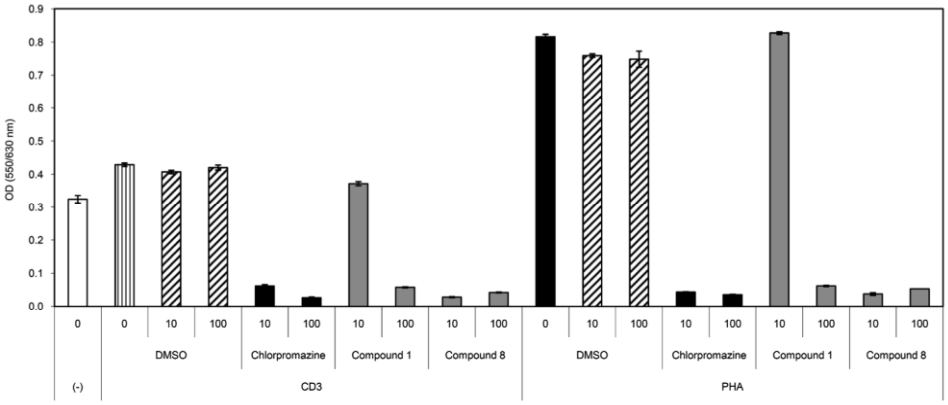

Fig. 4. The effects of compounds **1** and **8** on the proliferative response of human mononuclear blood cells to PHA and anti-CD3 antibodies. The compounds were added to the cultures at concentrations of 10 and 100 μg/ml. Statistics: CD3 – Control (-) vs CD3, P = 0.0047; DMSO (10-100) vs Chlorpromazine (10-100), P = 0.0002; DMSO (10) vs Compound **8** (10), P = 0.0002; DMSO (100) vs Compound **1** (100), P = 0.0002; DMSO (100) vs Compound **8** (100), P = 0.0002; PHA – Control (-) vs PHA, P = 0.0002; DMSO (10-100) vs Chlorpromazine (10-100), P = 0.0002; DMSO (10) vs Compound **8** (10), P = 0.0002; DMSO (100) vs Compound **1** (100), P = 0.0002; DMSO (100) vs Compound **8** (100), P = 0.0002 (ANOVA).

The effect of compound 1 on the secondary humoral immune response of mouse splenocytes to SRBC *in vitro*

Because of its low toxicity and high suppressive activity, compound **1** was selected to evaluate effects on the immune response. Fig. 6 demonstrates that the compound is very strongly suppressive with regard to the secondary humoral immune response *in vitro*, measured as the number of antibody-forming cells to SRBC. The actions of both concentrations (1 and 10 μg/ml were comparable (72.3 and 82.6% suppression, respectively).

The effect of compound 1 on the delayed-type hypersensitivity to OVA *in vivo* in mice

Compound **1** was also tested for its potential ability to suppress the induction phase of the delayed type hypersensitivity to ovalbumin *in vivo*. The compound, given before the sensitizing dose of the antigen, inhibited the DTH response in a dose-dependent manner (both inhibitory effects were statistically significant; Fig. 7).

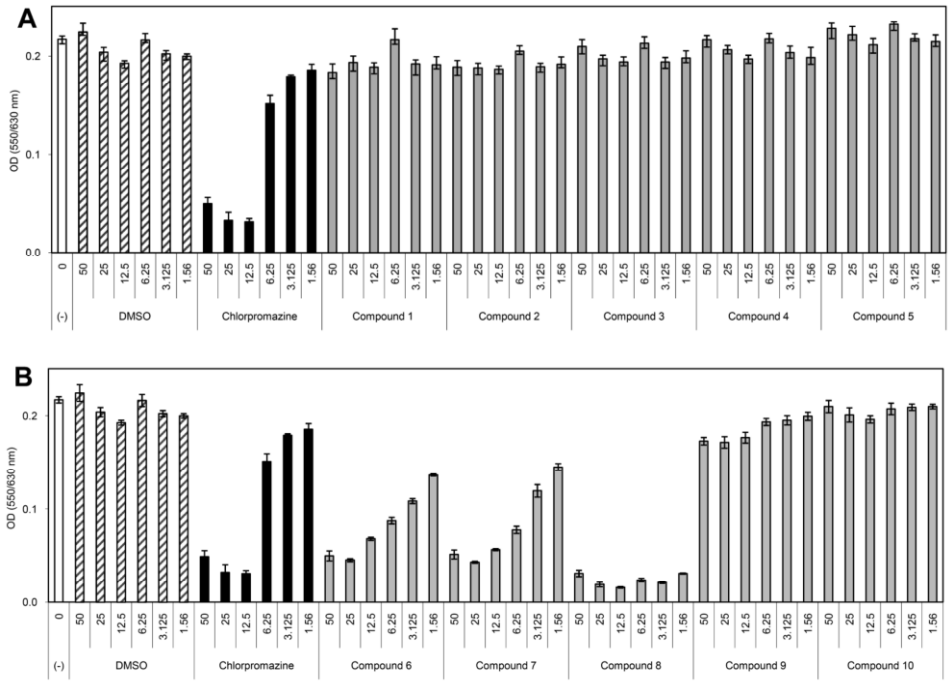

Fig. 5. The effects of compounds **1-5** (A) and **6-10** (B) on the survival of mouse splenocytes. The compounds were incubated overnight at 50-1.56 μg/ml concentrations with mouse splenocytes. The cell viability was measured using the MTT colorimetric method. (-) – no additions. Statistics: A – DMSO (50-6.25) vs chlorpromazine (50-6.25), P = 0.0000; DMSO (50) vs compound **1** (50), P = 0.0108 (ANOVA); B – DMSO (50-6.25) vs chlorpromazine (50-6.25), P = 0.0000; DMSO (50-1.56) vs compound **6** (50-1.56), P = 0.0000; DMSO (50-1.56) vs compound **7** (50-1.56), P = 0.0000; DMSO (50-1.56) vs Compound **8** (50-1.56), P = 0.0000; DMSO (50) vs compound **9** (50), P = 0.0000 (ANOVA).

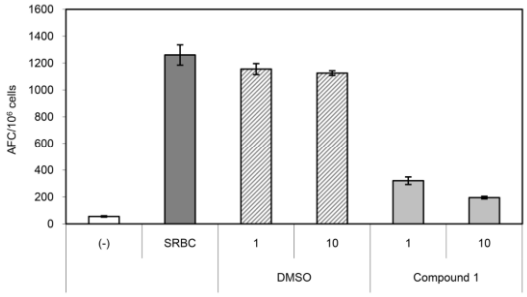

Fig. 6. The effect of compound **1** on the secondary humoral immune response of mouse splenocytes to sheep red blood cells. The compound was added to the splenocyte cultures at 1 and 10 μg/ml. The number of antibody-forming cells was determined after four days. (-) – no additions. Statistics: DMSO (1) vs compound **1** (1), P = 0.0001; DMSO (10) vs compound **1** (10), P = 0.0001 (ANOVA).

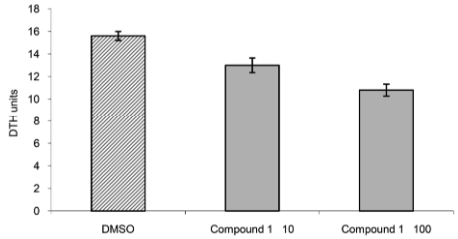

Fig. 7. The effect of compound **1** on the delayed-type hypersensitivity to ovalbumin *in vivo* in mice. The compound was administered to mice at doses of 10 and 100 µg/mouse (p.o.) 1 h before sensitization with OVA. Four days later, the eliciting dose of OVA was administered and 24 h later the DTH reaction was measured. Statistics: DMSO vs compound **1** (10), P = 0.0055; DMSO vs compound **1** (100), P = 0.0001 (ANOVA).

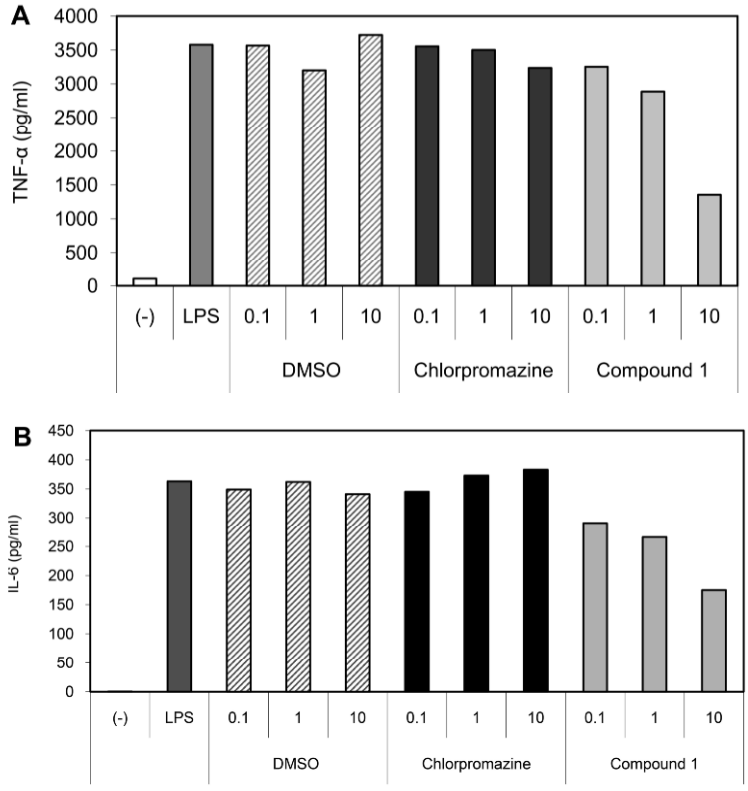

Fig. 8. The effect of compound **1** on the lipopolysaccharide-induced production of tumor necrosis factor-alpha (A) and interleukin-6 (B) in human whole blood cell cultures. Compound **1** and chlorpromazine were added to the human whole blood cultures at concentrations of 0.1, 1 and 10 µg/ml. (-) – no additions. After 24 h incubation, the activities of TNF-α and IL-6 were measured in the supernatants using bioassays.

The effect of compound 1 on LPS-induced cytokine production

In cultures of mouse peritoneal exudate cells (data not shown), compound **1** totally inhibited IL-6 production at 100 μg/ml and moderately inhibited TNF-α production at 10 and 100 μg/ml (34 and 25%, respectively). As shown in Fig. 8, compound **1**, at a concentration of 10 μg/ml, exhibited a distinct (64%) inhibition of TNF-α production in a culture of whole blood human cells. The compound also inhibited IL-6 production in a dose-dependent manner (17, 27 and 49% inhibition for 0.1, 1 and 10 μg/ml concentration, respectively). Chlorpromazine had no effect in that range of concentration.

DISCUSSION

The newly synthesized compounds **1-10** represent two classes of azaphenothiazines – dipyridothiazines (**1-5**) and diquinothiazines (**6-10**) – and show significant anticancer activity against human cancer cell lines. The most active dipyridothiazine was unsubstituted compound **1**, with GI_{50} = 1.70 μg/ml for lung cancer cell line HOP-92. All the diquinothiazines (**6-10**) turned out to be very active (for example, compound **6**: GI_{50} = 1.30 μg/ml against colon cancer cell line COLO205; compound **7**: GI_{50} = 0.08 μg/ml against ovarian cancer cell line IGROV-1; compound **8**: GI_{50} = 0.11 μg/ml against ovarian cancer cell line IGROV-1; compound **9**: GI_{50} = 0.04 μg/ml against melanoma line SK-MEL-5; compound **10**: GI_{50} = 0.24 μg/ml against renal cancer cell line 786-0. Some other cancer lines were similarly sensitive to those compounds [Pluta *et al.*[1]].

It was interesting to study how the incorporation of two nitrogen atoms into the phenothiazine system in the dipyridothiazines (**1-5**) and diquinothiazines (**6-10**), and the addition of two benzene rings in the latter set of compounds influenced their immunological activities, as compared with the activity of a model phenothiazine, chlorpromazine. The results regarding the antiproliferative activities of the studied compounds suggest that the inhibitory activities of compounds **6-10** and the reference drug chlorpromazine could be associated with their toxicity, although the toxicity and proliferation tests were performed on different species. Whereas the toxicity of compounds **6-10** could be attributed to their pentacyclic and hexacyclic ring systems, and to the nitrogen half-mustard unit (in the case of compound **9**), the toxicity of tricyclic chlorpromazine may be rather related to its chlorine atom. On the other hand, the tricyclic compounds **1-5** (with two additional nitrogen atoms incorporated in the phenothiazine system) were virtually devoid of toxicity in the concentration range tested. Despite this, two of those compounds (**1** and **2**, possessing small substituents, i.e. hydrogen and the methyl group) significantly inhibited the proliferative response of PBMC. This inhibition could not be due to the binding of the mitogen (PHA) as shown for other phenothiazines [30], since anti-CD3-induced cell proliferation was also inhibited. It is, however, a likely explanation that the inhibition of both humoral and cellular immune responses by compound

1 (Figs 6 and 7) was due to the suppression of antigen-specific proliferation of T cells in that model, assuming that the antiproliferative activity of the compound is comparable both for human and mouse lymphocytes. That suppressive effect could be associated with its ability to inhibit the activities of TNF-α and IL-6 (Fig. 8A, B), the cytokines essential in generation of the immune response [31, 32]. Such an effect was not observed with 10 μg/ml of chlorpromazine (Fig. 8A, B). It is also important to stress that unlike the reference drug, chlorpromazine [33], the inhibitory activity of compound **1** could not be related to toxicity, since at the studied concentrations, compound **1** was not toxic for splenocytes (Fig. 5). It seems therefore likely that the suppressive effects of chlorpromazine on PHA-induced PMBC proliferation may be associated with the toxicity of that drug ([34] and Figs 2 and 3).

When discussing the results obtained with human peripheral cells, we should bear in mind that only two blood donors were tested, and given the high variability of immune responsiveness between individuals, the effects of the compounds should be confirmed in a study with more blood donors. We demonstrated that that compound **1** was inhibitory in the immunological assays *in vivo* and *in vitro*, and that it likely that its immunosuppressive activity may be associated with the ability to suppress cytokines essential in the initiation of the immune response.

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