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Prolongation of skin graft survival in mice by an azaphenothiazine derivative

Jolanta Artym[a], Maja Kocięba[a], Ewa Zaczyńska[a], Iwona Kochanowska[a], Michał Zimecki[a,*], Wojciech Kałas[a], Leon Strządała[a], Ewa Zioło[a], Małgorzata Jeleń[b], Beata Morak-Młodawska[b], Krystian Pluta[b]

[a] Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla Str. 12, 53-114, Wrocław, Poland
[b] The Medical University of Silesia, School of Pharmacy with the Division of Laboratory Medicine, Department of Organic Chemistry, Jagiellońska 4 Str, 41-200, Sosnowiec, Poland

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ABSTRACT

Azaphenothiazines are predominantly immunosuppressive compounds. We evaluated the efficacy of an azaphenothiazine derivative, 6-chloroethylureidoethyldiquino[3,2-*b*;2′,3′-e][1,4]thiazine (DQT) in prolongation of survival of skin allografts between BALB/c and C57Bl/6 mice. The mice were treated intraperitoneally (i.p.) with 100 μg of DQT on alternate days, on days 1–13 of the experiment (7 doses). The effect of DQT on a two-way mixed lymphocyte reaction (MLR) in the human model, as well as its effect on production of TNF α and IL-10 in a whole blood cell culture, stimulated by lipopolysaccharide (LPS), were evaluated. In addition, DQT effects were investigated regarding the proportion of T cell subsets in human peripheral blood lymphocytes (PBMC) by flow cytometry. Lastly, the effect of DQT on expression of signaling molecules involved in pro apoptotic pathways was determined by RT PCR. The results showed that DQT significantly extended skin graft survival. The compound also strongly suppressed two-way MLR in the human model at a concentration range of 2.5–5.0 μM. In addition, DQT inhibited LPS-inducible TNF α, but not IL-10 production. The compound preferentially caused a loss of the CD3-CD8+CD11b + PBMC cell subset, and transformed CD3+CD8+^{high} into CD3+CD8+^{low} cells. Lastly, we demonstrated significant increases in expression of caspases (in particular caspase 8) and of p53 in a culture of Jurkat T cells. We conclude that the immunosuppressive actions of the compound in allograft rejection may be predominantly associated with induction of cell apoptosis and inhibition of TNF α production. The apoptosis could be predominantly selective for the CD3-CD8+CD11b + cell phenotype.

1. Introduction

Although use of classical immunosuppressors in prevention of graft rejection has been a success of modern medicine, the search for new immunosuppressive drugs remains a constant challenge for medicinal chemistry and pharmacology. The mechanism of action of currently applied immunosuppressors [1–3] has been established [4–6] and new experimental protocols based on the additive actions of these compounds with other preparations, including antibodies, have been investigated [7–10]. Besides application of classical immunosuppresors, novel approaches aimed at inhibition of graft rejection have been also undertaken [11–16]. Phenothiazines with aminoalkyl substituents serve as an important source of valuable drugs [17]. Modification of phenothiazines with azine rings results in formation of azaphenothiazines [18,19]. Recently, we synthesized a series of azaphenothiazines by replacing benzene rings with pyridine and quinoline rings [20–24]. Some of these compounds potently inhibited phytohemagglutinin (PHA)-induced proliferation of human peripheral blood mononuclear cells (PBMC), tumor necrosis alpha production and growth of tumor cell lines. Two of these compounds (6-chloroethylureidoethyldiquino[3,2-b;2′,3′-e][1,4]thiazine and 6-acetylaminobutyl-9-chloroquino[3,2-*b*]benzo[1,4]thiazine) proved to be suppressive in the models of delayed type hypersensitivity to ovalbumin and carrageenan induced footpad tests in mice [25]. These compounds were also effective in the amelioration of contact sensitivity to oxazolone [26] and experimentally induced psoriasis [27]. In addition, 6-acetylaminobutyl-9-chloroquino[3,2-*b*]benzo[1,4]thiazine inhibited pathological changes in dextran sulfate induced colitis in mice [28]. The mechanism of action

* Corresponding author at: Department of Experimental Therapy, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla Str.12, 53-114, Wrocław, Poland.
E-mail address: zimecki@iitd.pan.wroc.pl (M. Zimecki).

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of 6-chloroethylureidoethyldiquino[3,2-b;2′,3′-e][1,4]thiazine (DQT) has been partially investigated and besides suppressive actions on PHA-induced PBMC proliferation and tumor cell line growth [21] the compound strongly inhibited cytokine production in cultures of epidermal and human blood cells [27,29].

The hitherto collected data on immunosuppressive activity of DQT, including its antiproliferative and antitumor properties [21], inhibition of Th1-type immune response [25,26] and amelioration of pathological changes in experimental psoriasis [27] and colitis (unpublished data), prompted us to evaluate the potential value of DQT in prolongation of mouse skin allografts and attempt to explain its mechanism of action relevant to this model.

2. Material and methods

2.1. Mice

C57Bl/6 (H-2^b) and BALB/c (H-2^d) female mice, 12 weeks old, delivered by the Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, were used for the study. The mice were housed in a cage at 21–22 °C with a 12/12 h light/dark cycle and were fed a commercial, pellet food and water *ad libitum*. The local ethics committee at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland, approved the study (permission # 64/2015 and 79/2015).

2.2. Reagents

6-chloroethylureidoethyldiquino[3,2-b;2′,3′-e][1,4]thiazine was obtained via the procedure described previously [22] and was denoted in this article as DQT and represents compound #5 described in previous articles [25–27]. DMSO (dimethyl sulfoxide) was from Fluka, Fetal Calf Serum (FCS) from HyClone, Lymphocyte Separation Medium 1077, RPMI-1640 and Hanks' medium, antibiotic solution and trypsin solution containing EDTA were from Biowest, lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), sodium pyruvate, 2-mercaptoethanol from Sigma-Aldrich, isoflurane (Forane®) was from AbbVie, Poland, buprenorphini hydrochloridum (0.3 mg/ml; Vetergesic Vet) from Orion Pharma, Poland.

2.3. Preparation of the compound for biological assays

The compound DQT was dissolved in DMSO and subsequently: i) diluted in a RPMI-1640 cell culture medium for *ex vivo* tests or ii) diluted in 0.9% NaCl to obtain a dose of 100 μg in 0.2 ml and given to mice intraperitoneally (i.p.).

2.4. Skin grafts in mice

The mice were subjected to 3% isoflurane atmosphere anesthesia, after which their backs were shaved and skin grafts (2 × 2 cm) were taken, devoid of fat tissue and blood vessels, and cross transplanted between the two strains, using absorbable synthetic surgical sutures (polyglycolic acid, USP 5-0/EP1, 3/8 circle 12 mm; Atramat Ltd, Brussels, Belgium). Each of the experimental groups consisted of 18 mice (9 C57Bl/6 and 9 BALB/c mice).

The mice were treated every second day with 100 μg per mouse of DQT i.p. from day 1 to 13 of the experiment (entirely 7 doses). The control mice received 0.2 ml of appropriately diluted DMSO (the solvent for the compound) in 0.9% NaCl. Buprenorphine (0.1 mg/kg b.w.) was applied subcutaneously (s.c.) every 8 h from day 0 to 5 according to the ethics committee's recommendation. The state of skin transplants was monitored daily on day 1–17 of the experiment to determine symptoms of the skin graft rejection. In order to statistically process data, the following scores were assigned to the specific symptoms (Table 1). The average day of the completion of the skin graft rejection and the survival curves were calculated on the basis of the acquired data.

2.5. Human peripheral blood mononuclear cells isolation and culture

Venous blood from a single donor was withdrawn into heparinized syringes and diluted twice with PBS. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Lymphocyte Separation Medium 1.077 according to the supplier's instructions. Isolated cells were cultured in a RPMI-1640 medium supplemented with 10% FCS, antibiotics, 1 mM sodium pyruvate and 2-mercaptoethanol (referred further to as culture medium) and incubated in a cell culture incubator at 37 °C, 5% CO_2.

2.6. Two-way mixed lymphocyte reaction

The compound dissolved in DMSO was further diluted in the culture medium. For the assay, combinations of untreated PBMC from two individuals were used. 2×10^5 cells from each individual (total volume of 200 μl) were combined in each well of 96-well flat bottom culture plates. The compound was used at a final concentrations of 0.625–5.0 μM. The controls were represented by 2×10^5 cells/well of each blood donor. Appropriate DMSO controls were also prepared. After a 5-day incubation in a cell culture incubator (37 °C, 5% CO_2), the degree of cell proliferation was determined by the MTT colorimetric method and the results presented as mean optical density (OD) values at 550/630 nm wavelength ± standard error (SE). Alternatively, the results of the MLR test were determined by using Trypan blue cell exclusion test. After 5-day incubation 150 μl of the culture medium was removed from each well, 150 μl of trypsin solution with EDTA was added and incubated for 20 min at 37 °C. Then the cultures were vigorously agitated with a pipette in order to detach adherent cell and disintegrate cell colonies, followed by an inspection of the results in a converted microscope. Then, the cell suspensions were diluted 5× with ice-cold PBS containing 02% Trypan blue and placed in a Bürker hemocytometer. A person performing enumeration of cells was blinded with regard to identity of experimental groups. A mean value from 3 inspected fields was calculated and the final result was presented as a mean value of viable cells/ml from quadruplicate cultures ± SE. The experiments were performed 3 times using different donor combinations with similar results.

2.7. Determination of T cell antigens

For cell antigen expression studies PBMC were plated on a 24-well plate at a density of 5×10^5 cells/ml culture medium and treated with 10 μM DQT. After a 24 h culture, the cells were examined for expression of CD4 (PE-CF594-conjugated anti-CD4; 1:66; Becton Dickinson), CD8 (PE-conjugated anti-CD8; 1:20; Becton Dickinson), CD3 (BB515-conjugated anti-CD3; 1:66; Becton Dickinson) and CD11b (PerCP/Cy5.5 anti-mouse/human CD11b Antibody, Clone M1/70, Biolegend, San Diego, CA, USA) by simultaneous staining in 4 °C for 30 min. The cells were washed and harvested using a LSRFortessa™ cell analyzer (Becton Dickinson). The experiments were performed 3 times with similar results.

2.8. Viability test

A MTS colorimetric assay was used to establish cytotoxicity of DQT. Human PBMC were plated on a 96-well plate at a density of 10^5 cells/200 μl culture medium and were treated with DQT at a concentration range of 1.0–20 μM. After a 24 h culture, the cells were incubated with MTS containing PMS (CellTiter 96 AQueans Non-Radioactive Cell Proliferation Assay; Promega) for 2 h at 37 °C. The absorption (OD values) of a color product was determined at 490 nm wavelength and was

Table 1
Score assignment for signs of skin graft rejection.

Score	Description of symptoms of skin graft rejection
0.5	No symptoms of rejection (skin graft well blooded, palpably warm with edges straight, soft, neither shrunk nor rolled)
1.0	Early symptoms of rejection (limited blood supply, edges stiff and protruding, middle area palpably soft and warm)
1.5	Late symptoms of rejection (middle area and edges stiff)
2.0	Full rejection (shrinking of whole graft, necrosis, no blood supply, graft scabbed or complete lack of graft)

presented as % viability of appropriate DMSO control (mean values ± SE). The experiments were performed 3 times with similar results.

2.9. Lipopolysaccharide-induced TNF α and IL-10 production in whole blood cell culture

Human whole blood was diluted 10× with a RPMI-1640 medium supplemented with antibiotics and distributed to 48-well culture plates in 1 ml aliquots. The cultures were stimulated with LPS (100 ng/ml) and the studied compounds were added at a studied concentration (0.7–5.6 μM). The control cultures contained DMSO at an appropriate concentration. After an overnight incubation, the supernatants were harvested and frozen at −80 °C until cytokine determination. TNF α and IL-10 levels were determined in the supernatants by using ELISA kits from Thermo Fisher Scientific, in a presence of TNF α or IL-10 standard, and originally expressed in pg/ml. The inhibition (in percentage) of TNF α and IL-10 production was calculated by comparison with appropriate DMSO controls. The experiments were performed 3 times with similar results.

2.10. Cultures of Jurkat cells and gene expression determination with Real Time PCR

Jurkat cells – a human T cell line – were maintained in a RPMI-1640 medium supplemented with 10% FBS, sodium pyruvate, antibiotics and 2-mercaptoethanol. Cells (at a density of 5×10^5/ml) were cultured overnight with DQT (at a concentration 2.5 μM) in a cell culture incubator (37 °C in 5% CO_2). Total RNA isolation from Jurkat cells was carried out with the TRIzol Reagent (Ambion) according to the manufacturer's recommendations. The air-dried RNA pellet was dissolved in 20–30 μl of sterile diethylpyrocarbonate-treated Mili-Q water and stored at −20 °C. Single stranded complementary DNA (cDNA) was synthesized from 1 μg of total RNA using Super verte Kit oligo (dT)15 (Novazym). The expression of genes for GAPDH, caspases -3, -7, -8 and -9, Bcl-2, Fas, NF-κB1 and p53 was determined with AmpliQ 5 × HOT EvaGreen® qPCR Mix Plus (noROX) (Novazym) in "The CFX Connect Real-Time PCR Detection System" (BIO RAD). The sequences of primers are listed in Table 2.

2.11. Statistics

The results are presented as mean values ± SE. Brown-Forsyth's test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (one-way ANOVA) was applied, followed by post hoc comparisons with the Tukey's test to estimate the significance of the difference between groups. Nonparametric data were evaluated with the Kruskal-Wallis analysis of variance. In the skin graft test the experimental groups consisted of 18 mice. The survival rates were compared using the Kaplan-Meier method and analyzed by the log-rank test. Significance was determined at $P < 0.05$. The statistical analysis was performed using STATISTICA 7.0 for Windows.

3. Results

3.1. DQT prolongs allograft survival and inhibits mixed lymphocyte reaction

The kinetics of graft survival is presented in Fig. 1. The differences between graft survival in the DMSO control group and DQT mice were most evident on days 11–14 after transplantation. The prolongation of skin graft survival (determined as the day of final graft rejection) between the DMSO control group (on 11.39 day) and DQT-treated mice (on 13.47 day) was statistically significant (Table 3).

We also determined the effects of DQT on the magnitude of two-way MLR in the human blood leukocytes model. The effects of DQT were performed in parallel using both MTT colorimetric method and Trypan blue exclusion test. A representative result from 3 independent experiments (three different donor combinations) was shown in Table 4 and revealed a significant suppressive action of the compound at a concentration of 2.5 and 5.0 μM.

Preferential inhibition of Th1 type cytokines may also contribute to prolongation of graft rejection time. In the whole blood cell culture stimulated with LPS, the compound at a concentration range of 0.7–5.6 μM significantly inhibited TNF α and not IL-10 production (Table 5).

3.2. Effects of DQT on human blood lymphocyte subpopulations

Next, we investigated the effect of DQT on ratios of lymphocyte subpopulations in human PBMC. First, we found that DQT was relatively low in toxicity for PBMC in the 24 h culture (Fig. 2). The decrease in cell viability was 23% and 35% at 10 μM and 20 μM, respectively.

In addition, a flow cytometry analysis revealed that DQT preferentially caused loss from 7% (Fig. 3A) to 1% (Fig. 3B) of CD3-CD8+ cell subset in human PBMC. Of importance, all cells of this cell

Table 2
Primer sequences.

Gene	Foreward (5'-3')	Reverse (5'-3')
GAPDH	ACCACAGTCCATGCCATCCAC	TCCACCACCCTGTTGCTGT
Casp-9	GAGTCAGGCTCTTCCTTTG	CCTCAAACTCTCAAGAGCAC
Casp-8	GCAAAAGCACGGGAGAAAG	GGATACAGCAGATGAAGCAG
Casp-3	TTCAGAGGGGATCGTTGTAGAAGTC	CAAGCTTGTCGGCATACTGTTTCAG
Casp-7	AGTGACAGGTATGGGCGTTC	CGGCATTTGTATGGTCCTCT
p53	AGATAGCGATGGTCTGGC	TTGGGCAGTGCTCGCTTAGT
Fas	GACAAAGCCCATTTTTCTTC	ATTTATTGCCACTGTTTCAGG
Bcl-2	TATAAGCTGTCGCAGAGGGGCTA	GTACTCAGTCATCCACAGGGCGAT
NF-κB1	GTACTCAGTCATCCACAGGGCGAT	GAGTTACCTACCAGGGCTATTC

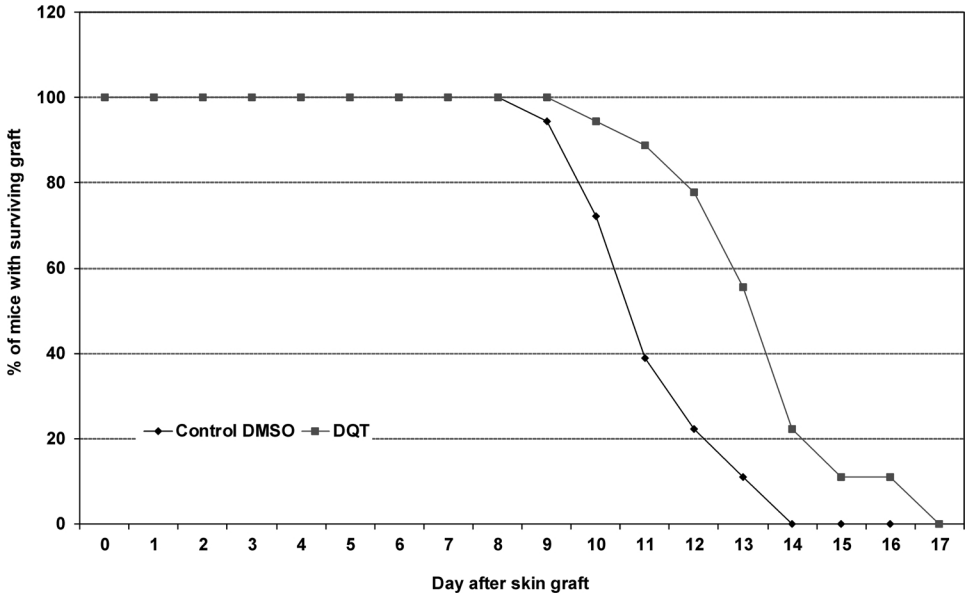

Fig. 1. Survival curves based on complete rejection of skin graft of mice treated with DQT compound. The mice were treated every second day with 100 μg per mouse of DQT i.p. from day 1 to 13 of the experiment. The control mice received appropriately diluted DMSO. The state of skin transplants was monitored daily on day 1–17 of the experiment to determine symptoms of skin graft rejection. Scores (0.5–2.0) indicated in the Table 1 were assigned to the specific symptoms. The results are presented as % of mice with surviving grafts (with scores 0.5–1.5). Statistics: $P < 0.05$ versus control mice treated with DMSO only.

Table 3
Day of complete rejection of skin graft of mice treated with DQT compound. The mice (n = 18) were treated every second day with 100 μg per mouse of DQT i.p. from day 1 to 13 of the experiment. The control mice (n = 18) received appropriately diluted DMSO. The state of skin transplants was monitored daily on day 1–17 of the experiment to determine symptoms of skin graft rejection. Scores (0.5–2.0) indicated in the Table 1 were assigned to the specific symptoms. The average day ± SE of the final skin graft rejection (with score 2.0) was presented.

Group	Day of final graft rejection		± SE
DMSO control	11.39		0.33
DQT	13.47	*	0.37

Table 4
Effects of DQT compound on two-way MLR of human PBMC. For the assay, a combination of untreated PBMC from two healthy donors (D1 and D2) was used. 2×10^5 cells from each individual were combined in each well of 96-well flat bottom culture plates. DQT compound was used at final concentration of 0.625–5.0 μM. Appropriate DMSO dilutions in the culture medium served as control cultures. After 5-day incubation, cell proliferation was determined by the MTT colorimetric method and Trypan blue exclusion test. The results were presented as mean OD values or number of viable cells/ml of culture ± SE. The presented data were confirmed in 3 independent experiments with similar results. Statistics: *, $P < 0.05$ versus DMSO control cultures.

		Cells ($\times 10^6$/ml)		OD values	
Group		mean	± SE	mean	± SE
Control (medium only)		1.457	0.093	0.262	0.006
DMSO control		1.483	0.125	0.259	0.004
DQT (μM)	0.625	1.221	0.091	0.261	0.005
	1.25	1.125	0.097	0.225*	0.004
	2.5	1.021*	0.127	0.145*	0.002
	5.0	0.567*	0.042	0.093*	0.006

subpopulation expressed CD11b antigen, present among others on natural killer cells (Fig. 3A). Such a strong diminishing effect on cell number was not observed with other cell populations, such as CD3+, CD3- or CD4+ (data not shown). In the PBMC culture, treated with DQT, a lower expression of the CD8 antigen in the CD3+CD8+ cell subpopulation, registered as transformed CD3+CD8+^{high} cells (Fig. 3A) into CD3+CD8+^{low} cells (Fig. 3B), was also observed.

Table 5
Effects of DQT compound on cytokine production: TNF α and IL-10 in LPS-treated human whole blood cell cultures. DQT compound was used at concentrations 0.7–5.6 μM. The results are presented as % inhibition in TNF α and IL-10 production in comparison with appropriate DMSO controls. The presented data were confirmed in 3 independent experiments with similar results. Statistics: *, $P < 0.05$ versus DMSO control cultures.

DQT (μM)	TNF α (% of inhibition)	± SE	IL-10 (% of inhibition)	± SE
0.7	36.56*	1.02	10.34	4.09
1.4	35.42*	1.47	4.37	2.00
2.8	38.06*	2.71	3.16	3.02
5.6	34.92*	3.64	0.00	–

3.3. DQT induces proapoptotic signaling in Jurkat T cells

Based on our previous data on induction of apoptosis by the compound in tumor cell lines [27] we evaluated the effect of the compound on expression of signaling molecules, associated with apoptosis, in Jurkat cells (Table 6). The results revealed significant increases in expression of caspases (in particular of caspase 8) and an exceptionally strong increase in p53 expression.

4. Discussion

This investigation is a continuation of our previous *in vitro* and *in vivo* studies on DQT compound revealing its antiproliferative, antitumor and anti-inflammatory properties in various models. Here we demonstrated a significant prolongation of allogenic skin graft survival in mice upon repeatable, intraperitoneal treatment of graft recipients with DQT (Fig. 1 and Table 3). To explain a possible mechanism of DQT action we applied several models, using also human peripheral blood mononuclear cells and Jurkat T cell line.

A mixed lymphocyte reaction is often used for evaluation of efficacy of immunosuppressive agents with a potential application in transplantation [2]. In our experiments DQT strongly inhibited, in a dose-dependent manner, the two-way MLR (Table 4). Importantly, the highest dose used (5.0 μM) had no cytotoxic effect in the PBMC cell culture (Fig. 2).

Sustained TNF α production [30], ability of naive alloreactive T cells to produce TNF α [31] as well as Th1/Th2 cytokine ratio [13] affect allograft survival. In our investigation DQT strongly inhibited TNF α but not IL-10 production (Table 5), suggesting that the selective

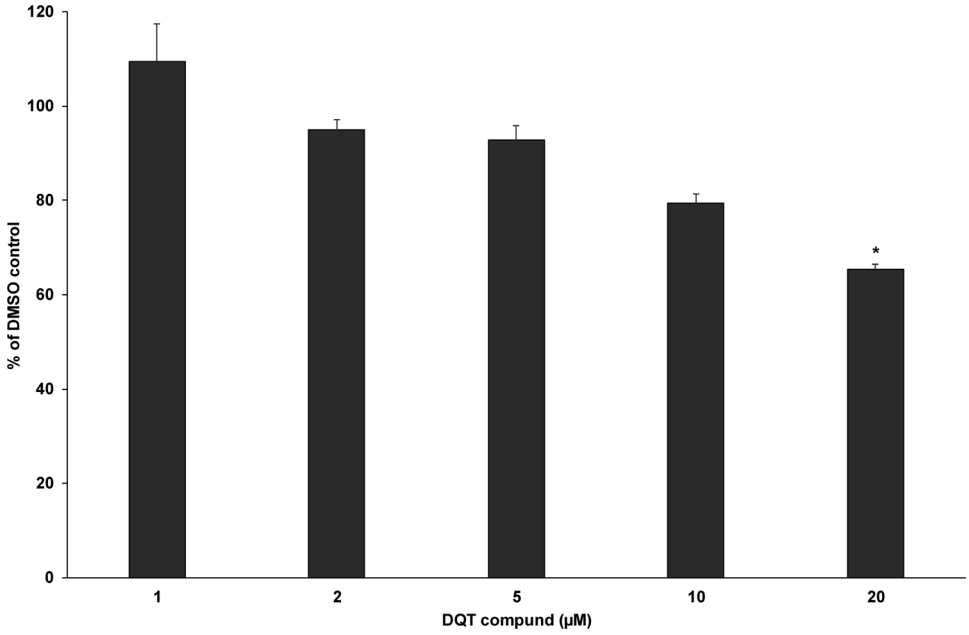

Fig. 2. Effects of DQT compound on viability of human PBMC. Cultured human PBMC were treated with DQT at a concentration range of 1.0–20 μM and viability of cells was determined after 24 h with the MTS test. The results are presented as % of viable cells (mean ± SE) in comparison with appropriate DMSO control cultures (100% viability). The presented data were confirmed in 3 independent experiments with similar results. Statistics: *, $P < 0.05$ versus control cultures with DMSO.

suppression of Th1-type cytokines plays a role in the prolongation of graft acceptance.

The almost complete reduction in the number of CD3-CD8+ PBMC upon exposure to DQT was very intriguing. In our analysis of PBMC subpopulations this cell subset constituted 7% of cells in the PBMC pool, and was reduced to 1% following DQT treatment (Fig. 3A and B). In addition (Fig. 3A), all cells in this subset expressed the CD11b antigen, present on natural killer cells (NK) [32]. Although the role of NK cells in allograft rejection is equivocal [33], this cell subpopulation may contribute to graft rejection using various mechanisms [34]. It is also not excluded that this cell subset may contain CD11b monocyte-derived dendritic cell precursors. These cells differentiate in mice into peripheral dendritic cells, the process correlated with strong priming of allogeneic T cells [35], and thus could play a crucial role in antigen recognition including allografts [36].

The inhibition of MLR by the compound, presented in this investigation, could also result from a decreased recognition of allogeneic cells due to a loss of CD3-CD8+ cells (Fig. 3A and B). The preferential depletion of these cells may provide an additional explanation for efficient inhibition of contact sensitivity to oxazolone, when the compound was applied topically [26]. In addition to the drastic depletion of CD3-CD8+ cells, DQT affected the CD3+CD8+^{high} subset by changing them into CD3+CD8+^{low} cells (Fig. 3A and B). Thus, the compound may also restrict activity of cytolytic, graft-infiltrating CD3+CD8+ T cells [30].

The analysis of signaling pathways in Jurkat T cells, exposed to DQT, revealed preferential increase of expression of proteins associated with apoptosis, such as caspases, Fas and p53. The expression of caspase 8 [37,38] and the p53 protein [39], which play major roles in the process of apoptosis, were particularly elevated by DQT.

Table 6
Expression of genes for selected signaling molecules in Jurkat T cells treated by DQT compound. Gene expression measured with the Real Time PCR technique is presented as a mean absolute value calculated from three trials after normalizing the measurements against GAPDH – a reference protein. Such a procedure enabled comparison of expression of each studied gen in cultures containing DQT with such expression in control cultures. The resulting values describe changes in particular gene expression caused by DQT. These are the absolute values (change coefficients). The presented data were confirmed in 3 independent experiments with similar results.

Bcl-2	Caspases				Fas	NF-κB1	p53
	3	7	8	9			
0.13	7.28	28.26	49.59	7.27	9.45	5.43	120.36

Immunosuppressors enrolled into therapy and these ones under clinical investigations and preclinical studies differ in their mechanism of action. Although classical immunosuppressors, such as calcineurin inhibitors, are very effective, they cause strong systemic immunosuppresssion due to block of IL-2 synthesis, the cytokine essential for expansion of T and B cells. In addition, their side effects include vasoconstriction [40] and increased risk of tumor formation due to inhibition of DNA repair [41]. We are of opinion that the advantage of DQT over calcineurin inhibitors would consist in a higher selectivity of action by depletion of cell populations involved in allograft rejection. DQT, in terms of mechanism of action, bears some similarity to FTY720 which causes a reversible sequestration of alloantigen-specific effector-memory T cells in regional lymphoid tissue, associated with a decrease

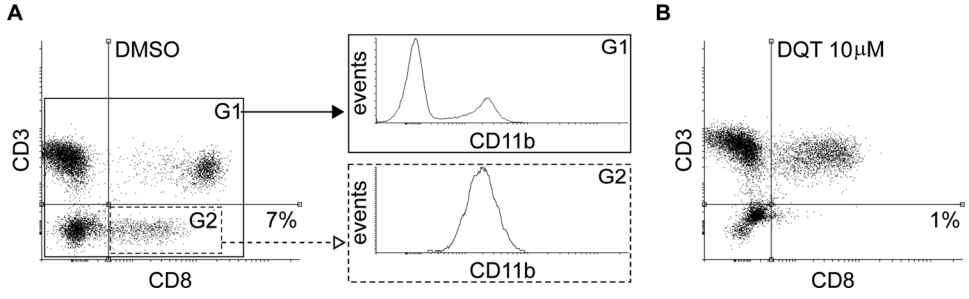

Fig. 3. AB. Effects of DQT compound on cell subsets in human PBMC. Human PBMC were cultured with 0.1% DMSO (A) or with 10 μM DQT (B) and after 24 h were stained with anti-CD3, anti-CD8 and anti-CD11b antibodies. A) Distribution of PBMC populations in control sample. The expression of CD11b on whole population is shown in gate 1 (G1), while expression of CD11b on CD3-CD8+ cells is shown in gate 2 (G2). B) CD3+ and CD8+ population distribution after treatment with DQT.

in T cell infiltration within the allograft and a subsequent prolongation in allograft survival [42]. The decreased number of lymphocytes in the spleen suggests that the spleen may be a target of FTY720 activity, during the early posttransplant period [43]. Interestingly, our unpublished data showed that DQT used in experimentally induced colitis strongly reduced weight of spleen but not of draining lymph nodes. A possibility to combine its action with low doses of cyclosporine to avoid nephrotoxicity, as in the case of FTY720 [44], would be also an interesting option. As this report on DQT efficacy in delaying graft rejection is preliminary it would require application of various experimental protocols. Furthermore, the compound should undergo a long-term toxicity trial to reveal its potential undesirable metabolic effects.

5. Conclusions

In conclusion, several actions of DQT, possibly interdependent, may play a role in the suppression of skin graft rejection by the compound. These actions include induction of cell apoptosis and suppression of TNF α production. A combined depletion of natural killer cells and dendritic cell precursors and restriction of CD3 + CD8 + cytolytic T cell function may also be of significance. The results presented in this work provide additional information on the mechanism of action of DQT azaphenothiazine, which has the potential to be therapeutic.

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