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Suramin inhibits the CD40–CD154 costimulatory interaction: A possible mechanism for immunosuppressive effects

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ABSTRACT

Suramin is a symmetric polysulfonated naphthylamine–benzamide urea derivative approved for the treatment of trypanosomiasis and onchocerciasis and a known P2 (ATP/UTP purine receptor) antagonist. Here, we report its ability to inhibit the important CD40–CD154 costimulatory interaction required for T cell activation and the development of an effective immune response. *In vitro*, it inhibited the binding of both human and murine CD154 (CD40L) to their receptor (CD40) even in the presence of protein-containing media and prevented the CD154-induced proliferation of human B cells as well as the corresponding increase in surface expression of CD86, CD80, CD40, and MHC class II in a concentration-dependent manner. Furthermore, in isolated human islets, it also decreased the CD154-induced release of inflammatory cytokines such as IFN-γ, interleukin-6 (IL-6), and IL-8. Suramin was selected for investigation because it has been reported to be an inhibitor of the interaction of TNF-α with its receptor and CD154 is a member of the TNF-family. However, it turned out to be a considerably, about 30-fold, more effective inhibitor of the CD40–CD154 protein–protein interaction than of the corresponding TNF interaction. Its median inhibitory concentration ($IC_{50} \approx 50$ μM) is somewhat higher than for the P2-receptor, but well within the range of its therapeutic concentration levels. Suramin shows considerable polypharmacology, but its interference with the positive costimulatory interaction might provide a possible, not yet identified mechanism for its ability to suppress T cell activity and induce immunosuppression, which might also have limited its clinical usefulness in the treatment of AIDS and cancer.

1. Introduction

Costimulatory interactions are an important therapeutic target for treatments aiming to modulate immune responses [1,2]. According to current knowledge, the activation of T cells requires two signals (or three, if growth signals are included): engagement of the T cell receptor (TCR) with the MHC–peptide complex (the antigen-specific signal 1) and ligation of costimulatory molecules on T cells with their respective ligands on antigen-presenting cells (APCs) (signal 2). Accord-

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Abbreviations: ANOVA, analysis of variance; BrdU, bromodeoxyuridine; BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; FBS, fetal bovine serum; HRP, horseradish peroxidase; IFN, interferon; MLR, mixed lymphocyte reaction; MHC, major histocompatibility complex; PBS, phosphate buffered saline; PPI, protein–protein interaction; RT, room temperature; TNF, tumor necrosis factor.

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ingly, costimulatory blockade is one of the most actively investigated pathways to mitigate immune responses in transplant patients and even in autoimmune diseases with the main focus being on the CD28–CD80/CD86 and the CD40–CD154 pathways [1,2]. Blocking of CD154 from interacting with its receptor CD40 [3–5] is a highly effective immunomodulatory therapy [6]. It is of particular interest for our research since administration of anti-CD154 monoclonal antibody (mAb) consistently allows for allogeneic islet engraftment and long-term insulin independence in various animal models including nonhuman primate (NHP) models assuming adequate numbers of functional pancreatic islets are used [7].

CD154 (CD40L, gp39, TRAP) is a member of the tumor necrosis factor (TNF) superfamily of cell surface interaction molecules, mainly expressed on activated ($CD4^+$) but not resting T cells, and also on activated B cells, activated platelets, and other cells [3–5]. Its receptor, CD40 is a 45–50 kDa type I membrane protein expressed on primary B cells, monocytes, macrophages, dendritic cells, and even pancreatic duct and β-cells [8]. Although CD40 and CD154 are expressed as cell surface proteins, natural soluble forms have been described.

During the investigation of the CD40–CD154 interaction, we identified suramin as an unexpectedly effective inhibitor of this interaction. Suramin, a symmetric polysulfonated naphthylamine–benzamide urea derivative (Fig. 1), is approved for the prophylactic treatment of African sleeping sickness (trypanosomiasis) and river blindness (onchocerciasis) caused by parasitic infections. It is a known P2 (ATP/UTP purine receptor) antagonist ($IC_{50} \approx 5–10\ \mu M$) [9], a known inhibitor of the binding of a range of tumor growth factors, and it has various other biological activities as well [10,11]. Starting in the mid-1980s, it was evaluated as a possible reverse transcriptase inhibitor in clinical trials in patients with acquired immunodeficiency syndrome (AIDS), where it failed to show sufficiently positive effects [12]. Since it showed promising activity in HIV-associated neoplasms such as Kaposi's sarcoma and non-Hodgkins lymphoma, and because of its ability to inhibit the actions of various growth factors, such as fibroblast growth factors (FGF), platelet derived growth factors (PDGF), transforming growth factors α and β (TGF), and insulin like growth factor I (IGFI), it has also been investigated for antitumor activity mainly for adrenocortical and prostate cancers [11], ultimately, however, with only modest effects [13]. Documented toxicities of suramin include, among others, adrenal and renal insufficiency, coagulation factor abnormalities, immunosuppression, and poly-neuropathy [10,14]. Suramin has also been shown to inhibit the growth of several lymphoid cell lines, peripheral blood lymphocytes, and T cells as well as to cause profound and prolonged thymic atrophy and splenic lymphocyte depletion in normal mice [15–18]. It also dose dependently inhibited the allogeneic mixed lymphocyte reaction (MLR) [19]. Here, we report its ability to inhibit the binding of CD154 to its receptor (CD40), even in the presence of protein-containing media, and to prevent the CD154-induced proliferation of B cells as well as the corresponding increase in surface expressions of CD86, CD80, CD40, and MHC class II in a concentration-dependent manner. Suramin's ability to interfere with the CD40–CD154 costimulatory interaction identified here might account for its immune-suppressive effects, which might also have limited its clinical usefulness in the treatment of AIDS and cancer.

2. Materials and methods

2.1. Materials

Suramin and tartrazine, which was used as a negative control, as well as other chemicals and reagents used were obtained from Sigma–Aldrich (St. Louis, MO); the monoclonal anti-human CD154 (clone 40804), anti-human TNF-α antibodies (clone 1825), and the soluble mouse CD40:Fc human chimera were obtained from R&D Systems (Minneapolis, MN). Anti-mouse CD154 antibody (MR1) was obtained from Taconic (Hudson, NY). Other receptors (hCD40, TNF-R1), ligands (hCD154, mCD154, TNF-α) and enhancer for ligands were obtained from Alexis Biochemicals (San Diego, CA).

2.2. Inhibition of CD40–CD154 and TNF-α–TNF-R1 interactions

A 96-well plate-based cell-free *in vitro* binding inhibition assay was developed following a modification of a similar assay described in the literature [20]. Microtiter plates (Nunc F MaxiSorp) were coated overnight at 4 °C with 100 μL/well of receptor diluted in PBS pH 7.2. Plates were blocked with

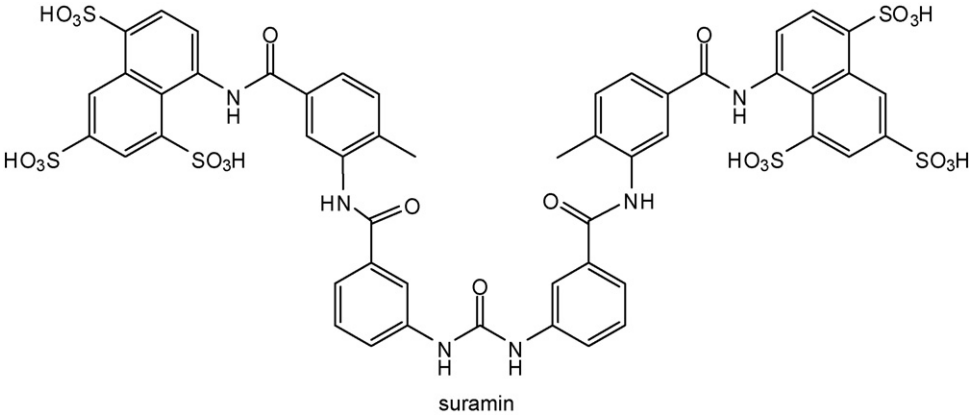

Fig. 1 – Structure of suramin, usually used as its corresponding hexasodium salt.

200 μL/well of blocking solution (PBS pH 7.2, 0.05% Tween-20, 1% BSA) for 1 h at room temperature (RT). After washing twice with washing solution (PBS pH 7.4, 0.05% Tween-20), the corresponding FLAG-tagged ligand diluted in 100 mM HEPES, 0.005% BSA pH 7.2 or in growth media (see below), was added to the wells (total volume of 100 μL/well) together with different concentrations of compounds tested. After 1 h incubation at RT, the plates were washed three times with washing solution. Bound ligand was detected with 200 μL/well of secondary antibody anti-FLAG M2 – peroxidase HRP conjugate (Sigma–Aldrich, St. Louis, MO) diluted in washing solution. After 1 h incubation at RT, the plates were washed four times and 120 μL/well of the HR-peroxidase substrate TMB (3,3′,5,5′-tetramethylbenzidine; Sigma–Aldrich) were added. After approximately 30 min incubation in the dark, the reaction was stopped with 30 μL of 1 M H_2SO_4. The amount of bound ligand was directly proportional to the absorbance read at 450 nm. In the assay using human proteins, the predetermined concentrations of hCD40 (CD40:Fc human chimera recombinant; Alexis Biochemicals) and hCD154 (soluble human, FLAG-tagged, recombinant; Alexis Biochemicals) used were 0.3125 μg/mL and 0.00875 μg/mL, respectively; whereas, in the assay with murine proteins, the concentration of mCD40 (mouse CD40:Fc human chimera, soluble, R&D Systems) and (FLAG-tagged) mCD154 (recombinant mouse CD40L, soluble, Alexis Biochemicals) were 0.3125 μg/mL and 1.12 μg/mL, respectively. The concentration of TNF-R1 (soluble recombinant; Alexis Biochemicals) and TNF-α (FLAG-tagged, soluble recombinant; Alexis Biochemicals) used were 0.6 μg/mL and 0.02 μg/mL, respectively.

2.3. Inhibition of CD154-induced human B cell proliferation

Inhibition of cell proliferation was determined using the colorimetric cell proliferation ELISA BrdU kit from Roche Applied Science (Indianapolis, IN). Human $CD19^+$ B cells (StemCell Technologies Inc., Vancouver, Canada) were cultured in 96 wells tissue culture plates in 100 μL/well at a cell density of 5×10^5 cells/mL in growth media (IMDM medium supplemented with 5% FBS, 100 U/mL penicillin and streptomycin 100 μg/mL and 1× of insulin-transferrin-selenium-G supplement; all materials from Invitrogen, San Diego, CA) and 10 μg/mL of recombinant human IL-4 (R&D Systems) containing 0.1 μg/mL of hCD154 (with addition of 2 μg/mL of enhancer for ligands) and various concentrations of test compounds. Cells were incubated at 37 °C, 90% humidity, and 5% CO_2 for 48 h after which BrdU labeling solution was added as recommended, and cells were cultivated for another 48 h. After the labeling period, detection of incorporated BrdU was carried out following the instructions of the colorimetric cell proliferation ELISA BrdU kit. Each experimental condition was tested in triplicate. Cursory cell viability evaluations were performed after 48 and 96 h of cultivation using trypan blue staining on a hemacytometer. In addition, potential proliferation inhibition due to general cytotoxicity was evaluated in the same cells using a standard *in vitro* toxicology assay kit based on metabolism of MTT (TOX1-Kit; Sigma–Aldrich). Cells were cultivated in the presence (or absence) of the test compound in RPMI media 1640 (Invitrogen, San Diego, CA) supplemented with 5% FBS, 100 U/mL penicillin, and streptomycin 100 μg/mL for 24 h and then for 12 h in the presence of MTT before processing.

2.4. Flow cytometry analysis

Human $CD19^+$ B cells were cultivated in the same conditions described above in the presence of CD154, enhancer for ligands, and different concentrations of compounds. After 20 h of activation, the cells were washed once with Pharmingen stain FBS buffer and stained with anti-CD40-PE (clone MAB89), anti-HLA-DR-PE-Cy™7 (clone L243), anti-CD86-FITC (clone 2331) and anti-CD80-PE-Cy™5 (clone B7-1) as recommended by the manufacturer. Cells were washed two times with stain FBS buffer, re-suspended in this buffer containing DAPI, and analyzed using a BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA). The flow cytometer as well as the antibodies and reagents used were obtained from BD Pharmingen (San Diego, CA), except anti-CD40-PE, which was from Beckman-Coulter, Inc. (Miami, FL). DAPI stain exclusion was used to assess cell viability [21,22], and expression of cell surface markers was quantified in live cells only after gating for DAPI.

2.5. CD154-induced cytokine release in isolated human islets

Human pancreatic islets were obtained from the Human Islet Cell Processing Facility at the Diabetes Research Institute (University of Miami, Miller School of Medicine) or from the Islet Cell Resource basic science islet distribution program. The glands were cold-preserved in University of Wisconsin solution. Islets were isolated by using a modification of the automated method [23] with different lots of the enzyme liberase HI (Roche, Indianapolis, IN) and a standard purification step [23]. Islets were cultured identically (37 °C, 5% CO_2) in CMRL medium-1066 (Invitrogen, Carlsbad, CA), niacinamide (10 mM, Sigma, St. Louis, MO), ITS (BD Biosciences, San Jose, CA), Zn_2SO_4 (15 μM, Sigma), GlutaMAX™ (2 mM, Invitrogen), HEPES (25 mM, Sigma), fetal bovine serum (10%, Invitrogen), and penicillin–streptomycin (100 IU/mL–100 μg/mL, Invitrogen). Human islets were cultured in suspension in ultralow attachment plates at a density of 1×10^3 Islet Equivalents per mL of CMRL-based media. Islets were stimulated for 24 h at 37 °C with CD154 (1 μg/mL; with addition of 1.5 μg/mL of enhancer for ligands). Suramin (100 μM) was added 20 min before the addition of CD154. Concentrations of IL-6, IL-8, and IFN-γ in supernatants from controls and treated islets were determined, using Multi-Plex cytokine kits following the manufacturer's protocol (Bio-Plex platform technology; Bio-Rad Laboratories, Hercules, CA). The obtained values were normalized by islet total DNA content (Quant-iT Pico Green dsDNA Assay Kit, Invitrogen).

2.6. Data fitting and statistics

All binding inhibition and cell proliferation assays were tested in triplicates per plates and the average data were used to

establish dose–response curves; assays were repeated at least three independent times. Data were converted to percent inhibition and fitted with standard log inhibitor vs. normalized response models using GraphPad Prism 5.01 (La Jolla, CA, USA) to establish median inhibitory IC_{50} values:

$$B = 100\frac{C}{C + IC_{50}} = 100\frac{1}{1 + 10^{(\log IC50 - \log C)}}$$

Proliferation, viability, and cytokine release data were analyzed by one-way repeated-measures analysis of variance (ANOVA) followed by Tukey's multiple comparison test as a *post-hoc* test for individual differences using GraphPad Prism 5.01 and a significance level of $p < 0.05$ for all comparisons.

3. Results

3.1. Inhibition of human CD40–CD154 interaction

Inhibitory activity was quantified using a 96-well plate-based cell-free *in vitro* binding inhibition assay measuring the amount of bound soluble CD154 to plate-coated CD40 in the presence of increasing concentrations of suramin. Anti-CD154 monoclonal antibody (mAb) and tartrazine were used as positive and negative controls, respectively. Tartrazine, an FDA approved food colorant (FD&C Yellow no. 5), was used as negative control because it contains aromatic sulfone moieties similar to those present in suramin, but consistently showed no CD40–CD154 inhibitory activity. While the antibody showed less than nanomolar potency (i.e., 0.25 nM ≈0.04 μg/mL), tartrazine showed no significant inhibitory effect. Suramin concentration-dependently inhibited the binding of CD154 to its receptor (CD40) with a median inhibitory concentration IC_{50} of approximately 15 μM in HEPES buffer ($\log IC_{50} = -4.84_{\pm 0.05}$; Fig. 2). Because nonspecific protein binding could be a problem, especially for protein–protein interaction (PPI) inhibitors, the inhibitory activity in FBS-containing cell-growth media was also determined. Both the antibody and suramin lost some activity compared to HEPES – suramin losing somewhat more, but still maintaining adequate activity: the anti-CD154 antibody showed an IC_{50} of approximately 0.4 nM (0.07 μg/mL) and suramin an IC_{50} of 65 μM ($\log IC_{50} = -4.16_{\pm 0.04}$; Fig. 2). In both cases, concentration-dependences were adequately described by standard log inhibitor vs. response models.

3.2. Inhibition of murine CD40–CD154 interaction

Suramin's inhibitory activity in the murine CD40–CD154 receptor–ligand system was also tested. The experimental setup used was similar to the one described above, but using mouse CD40 and CD154 and a corresponding mouse anti-CD154 antibody. Whereas the human mAb was inactive in this system, the murine antibody was active with the expected less than nanomolar potency (and, vice versa, the murine antibody was inactive in the human system). Suramin inhibited binding here with a potency similar to the one shown in the human system (in fact, slightly higher) having an IC_{50} of approximately 3 μM in HEPES ($\log IC_{50} = -5.58_{\pm 0.05}$) and about an order of magnitude higher in media (Fig. 3).

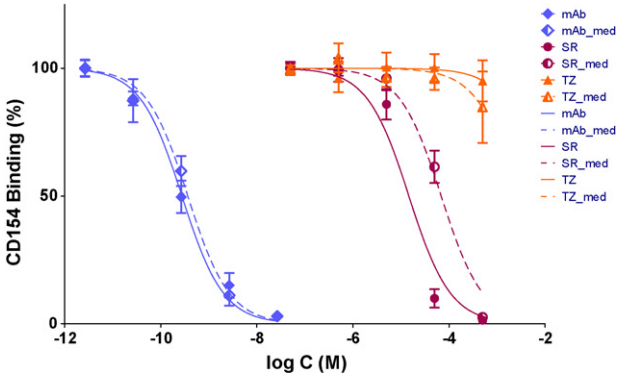

Fig. 2 – Inhibition of the human CD40–CD154 protein–protein interaction by suramin in HEPES buffer (SR, filled circle and continuous line) and in cell growth media (semi-open circle and dashed line) with an anti-CD154 monoclonal antibody (mAb, diamond) and tartrazine (TZ, triangle) as positive and negative controls, respectively for comparison.

3.3. Inhibition of human TNF-α–TNF-R1 interaction

Because suramin shows considerable polypharmacology, it was important to test whether it shows any selectivity towards inhibiting this specific PPI, and the results are not just the effect of some nonspecific binding or nonspecific inhibitory activity. Since CD154 is a member of the TNF family of cell surface interaction molecules, the inhibition of the interaction between TNF-α and its receptor TNF-R1 was measured for comparison. Again, the experimental setup used was similar to the ones described above, but using plate-coated TNF-R1 and TNF-α and a corresponding anti-TNF-α antibody as positive control. Suramin inhibited the binding of TNF-α to its receptor with an IC_{50} of about 500 μM in HEPES ($\log IC_{50} = -3.31_{\pm 0.03}$; Fig. 4) and about five-fold higher in media; hence, it shows considerable (about 30-fold) selectivity

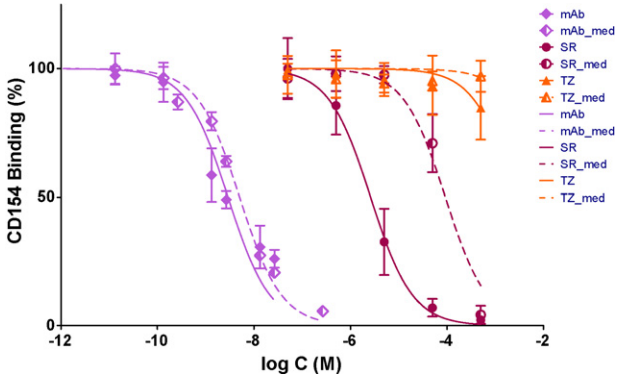

Fig. 3 – Inhibition of the murine CD40–CD154 protein–protein interaction by suramin in HEPES buffer (SR, filled circle and continuous line) and in cell growth media (semi-open circle and dashed line) with the mouse anti-CD154 monoclonal antibody (mAb, diamond) and tartrazine (TZ, triangle) as positive and negative controls, respectively for comparison.

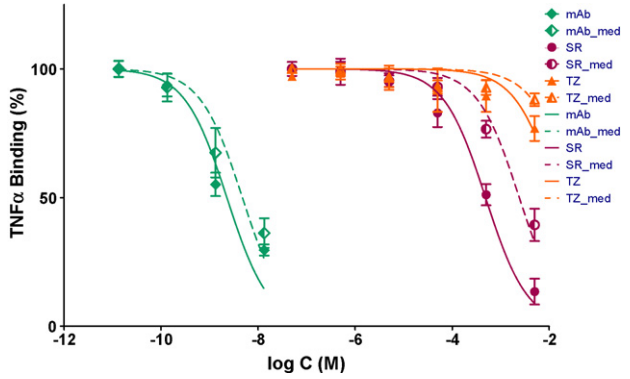

Fig. 4 – Inhibition of the human TNF-α–TNF-R1 protein–protein interaction by suramin in HEPES buffer (SR, filled circle and continuous line) and in cell growth media (semi-open circle and dashed line) with an anti-TNFα monoclonal antibody (mAb, diamond) and tartrazine (TZ, triangle) as positive and negative controls, respectively for comparison.

toward the CD40–CD154 system. The IC_{50} obtained here for suramin in inhibiting the binding of TNF-α to its receptor is in general agreement with the one published by Mancini et al. (650 μM in PBS with 0.1% BSA) [24].

3.4. *Inhibition of CD154-induced human B cell proliferation*

CD40 stimulation is an important signal for B cell proliferation, and in the presence of cytokines such as IL-4, it is sufficient to also induce isotype switching and Ig production. Soluble CD154 (CD40 ligand) can dose-dependently induce the proliferation of human B cells as measured by standard proliferation assays. Here, the proliferation of human $CD19^+$ B cells in the presence of soluble CD154-induced CD40 stimulation was used to assess the inhibitory effect of suramin in comparison with a positive (mAb) and a negative (tartrazine) control using a standard BrdU proliferation assay in 96 wells tissue culture plates. The assay confirmed that this effect is concentration-dependently inhibited by the corresponding mAb in the nM range, and also by suramin with an IC_{50} (approximately 95 μM; $\log IC_{50} = -4.02_{\pm 0.05}$; Fig. 5) in good agreement with its inhibitory potential for CD40–CD154 binding in cell culture media (≈65 μM). Tartrazine, which was inactive in the binding assay, was used as a negative control, and it indeed had no proliferation inhibiting activity. The viability of cells, assessed by trypan blue exclusion, was around 60–70% and was not significantly affected by any of these compounds at the concentrations tested. To eliminate the possibility that inhibitory effects are due to nonspecific cellular toxicity, standard cytotoxicity evaluations were performed. While cytotoxicity could certainly be an issue at higher concentrations of suramin, no significant toxicity on B cell was observed for concentrations up to 250 μM (Supplementary Data, Figure S1).

Most B lymphocytes are in the resting state (G_0), which, upon stimulation with antigen or mitogen, undergo a series of events leading to activation, entry into the cell cycle, and differentiation into memory or antibody secreting plasma cells [25]. The important role of CD40–CD154 interactions in the regulation of costimulatory activity on B cells is now well recognized [5]. Even before cell proliferation is evident, a variety of biochemical events, which can last hours or even days, occur immediately after CD40-mediated B cells activation. Some of these events include increase in cell size and expression of cell surface molecules such as CD69 (early event), MHC class II, CD80, CD86, and CD134 (later expressed in plasma cells) [25]. To further corroborate that the inhibitory capacity of suramin is due to blockage of the CD40–CD154 interaction, the expression of cell surface molecules was analyzed by flow cytometry in CD154-activated B cells cultivated in the presence of various concentrations of suramin, mAb, and tartrazine. By gating for live cells, possible effects due to cytotoxicity are eliminated. Nevertheless, for all concentrations studied, viabilities for suramin treated cells as

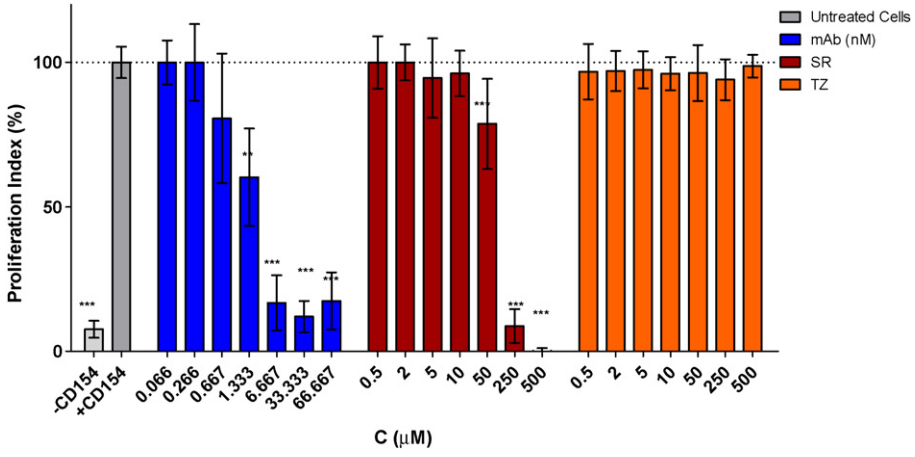

Fig. 5 – Concentration-dependent inhibition of CD154-induced human B cell proliferation by suramin (SR) as well as by anti-CD154 mAb and tartrazine (TZ) as positive and negative controls, respectively. Data (average ± S.D.; for $n = 3$ independent experiments with triplicates for each condition) were analyzed by ANOVA with Tukey's *post-hoc* test and *, **, and * indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively compared to cells with CD154 (+CD154).**

determined by DAPI exclusion [21,22] were not significantly different ($p > 0.05$) from those of the corresponding controls both for unstimulated and CD154-stimulated B cells indicating again no significant B cell toxicity for suramin at these concentrations. The results shown in Fig. 6 clearly indicate that CD154-mediated upregulation of MHC class II, CD40, CD80, and CD86 is inhibited by suramin in a concentration-dependent manner, and they remain at levels comparable to those observed in non-activated B cells in the groups treated with suramin at concentrations above 100 μM. Similar results were obtained with cells cultivated in the presence of mAb, used as a positive control. The presence of tartrazine had no effect on the expression of any of the cell surface protein tested showing expression profiles comparable to CD154-activated cells. Typical results demonstrating inhibited expression of CD86 and CD80 by suramin are showed in Fig. 7. In this experiment, the presence of suramin alone in cultures of non-activated B cells (−CD154) had no noticeable effects on the expression of CD86 and CD80 (Fig. 7) as well as CD40 and MHC class II (Fig. 6).

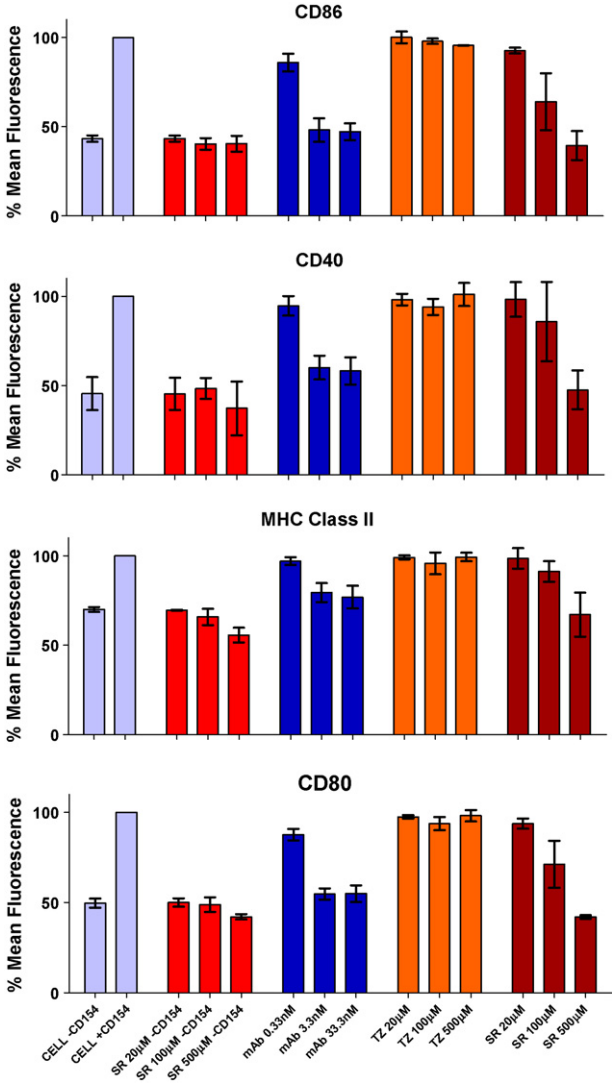

Fig. 6 – Expression levels of CD86, CD80, CD40, and MHC class II on CD154 activated human B cells treated with various concentrations of suramin (SR) as well as by anti-CD154 mAb and tartrazine (TZ) as positive and negative controls, respectively as indicated by the mean fluorescence levels. Control and suramin alone treated cells not activated by CD154 are denoted as 'Cell – CD154' and 'SR – CD154', respectively. Data shown are average ± S.D. for $n = 3$ independent experiments after normalization with CD154-stimulated cells as 100% for each marker on live cells after gating by staining with DAPI.

3.5. *CD154-induced cytokine release in isolated human islets*

Ligation of CD40 is known to mediate a variety of immune and inflammatory responses, such as the expression of adhesion molecules, cytokines, matrix-degrading enzymes, prothrombotic activities, and apoptotic mediators [4]. In pancreatic islets, the insulin secreting cells are susceptible to functional impairment and cell death when exposed to proinflammatory cytokines. Interaction of CD40 with soluble CD154 activate proinflammatory pathways in pancreatic islets, causing increased secretion of interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein (MIP)-1β [8] in ductal cell-depleted islet cultures. Consequently, the ability of suramin to inhibit CD154-induced cytokine release in human pancreatic islets was tested here to further confirm its ability to interfere with the CD40–CD154 interaction. Soluble CD154 (1 μg/mL ≈ 7 nM) did indeed increase the release of IL-6, IL-8, and IFN-γ in human islet culture. Addition of suramin (100 μM) to the human islet culture 20 min before the stimulation with CD154 decreased the CD154-induced cytokine release by the islet cells (Fig. 8).

4. Discussion

CD154 is a member of the TNF family with its extracellular region displaying an approximate 25% sequence identity to TNF proteins [26]. Suramin has been shown to inhibit the interaction of trimeric TNF-α with its receptor (TNF-R1, CD120a, p55) by binding to the ligand and possibly provoking deoligomerization of the trimer [24,27]. In fact, this is why it was selected for investigation here; however, it was found to be surprisingly active in inhibiting the CD40–CD154 interaction compared to its ability to inhibit the TNF-α–TNF-R1 interaction: it was more than 30-fold more active (IC_{50} of 15 μM vs. 500 μM). Its inhibitory effect seems to be sufficiently specific to be only moderately affected by the presence of proteins in the media: its IC_{50} value increased only about four-fold in the presence of cell growth media (65 μM vs. 15 μM; Fig. 2). In the meantime, whereas the human and murine anti-CD154 antibodies are completely specific, each one being active in the nanomolar range in its corresponding system, but completely inactive in the other one, suramin has about the same potency in both systems, as one could expect for a less specific, small molecule inhibitor (Fig. 2 vs. Fig. 3). Suramin inhibited the soluble CD154-induced proliferation of human B cells as well as the expression of cell surface molecules such as

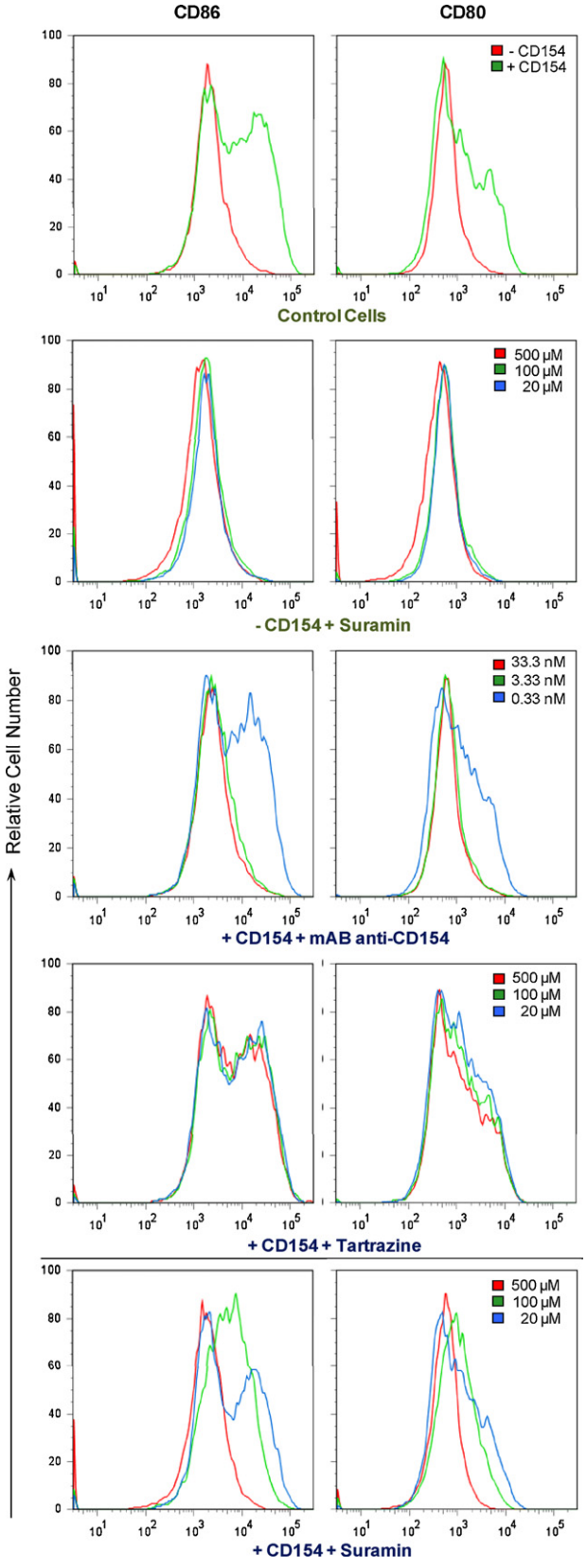

Fig. 7 – Expression of CD86 and CD80 on CD154-activated human B cells treated with various concentrations of suramin, anti-CD154 mAb, and tartrazine. Histograms show the intensity of fluorescence for CD86 and CD80 (left and right column, respectively) obtained in a typical experiment in live cells gated by staining with DAPI.

CD86, CD80, CD40, and MHC class II in concentration-dependent manner, further confirming its ability to interfere with the CD40–CD154 costimulatory PPI. These seem to be specific effects not related to general cytotoxicity as confirmed by the cytotoxicity tests and by the presence of effects in live cells as seen in the flow cytometry assay. While a few peptide inhibitors of the CD40–CD154 interaction have been reported recently in the literature with activities ranging from quite high (IC_{50}s of 50–100 nM [28]) through medium (IC_{50}s of 50–100 μM [29,30]) to very low ($IC_{50} > 1$ mM [31]), suramin seems to be the first small molecule compound with adequate CD40–CD154 inhibitory activity identified so far in the public literature besides a series of dipyridine derivatives claimed in a patent [20].

In addition to the role played by the CD40–CD154 interaction in immune responses, there is increasing evidence for its role in atherosclerosis [32], cardiovascular disease [33], acute coronary syndrome, thrombosis, inflammation [34], inflammatory bowel disease [35], or even metabolic syndrome. Consequently, inhibition of the CD40 signaling can be beneficial in pathogenic processes of chronic inflammatory diseases, such as autoimmune diseases, neurodegenerative disorders, graft-vs.-host disease, cancer, and atherosclerosis. In addition to the cell membrane associated form, CD154 also exists as a truncated soluble protein, sCD154 (sCD40L). Considering that CD40–CD154 interaction has also been shown recently to activate proinflammatory pathways in pancreatic islets [8] and that secretion of proinflammatory cytokines almost certainly has a negative effect on the function and survival of transplanted islets, strategies that prevent or inhibit CD40 expression and its signaling pathways in β-cells are of particular interest for us to optimize islet transplantation. Hence, we also explored the potential of suramin to inhibit the CD154-induced cytokine release in human pancreatic islets and found that suramin at 100 μM indeed decreased the levels of IL-6, IL-8, and IFN-γ back to values comparable to those of the control (Fig. 8). This certainly further emphasizes the possible effect of suramin on the CD40–CD154 interaction, but in the complex milieu of the inflammatory response of isolated human islets and with such a polypharmacologically active compound as suramin, one cannot exclude the possibility of the involvement of some other pathway(s). Binding of CD154 to CD40 activates a number of downstream signals through TNF receptor-associated factors (TRAFs) acting as adaptor molecules that bind to the cytoplasmic tail of CD40 and subsequently recruit kinases and other effector proteins [4]; the most important transcription factor that is being activated by CD40 signaling is nuclear factor kappa B (NFκB). There is also a possibility that some of the effects of CD154, and in particular its inflammatory effects, are mediated by other receptors; Mac-1 has been suggested recently as a possibility [36]. Suramin has been reported recently to dose-dependently suppress TNFα and IL-6 production with a suppression of macrophage NF-κB activity as a suggested mechanism [37].

Suramin can be considered a 'colorless dye', since it is structurally related to certain polysulfonated azo dyes such as trypan blue or Evans blue, but it does not contain the aryl azo moiety causing their vivid color. In fact, it was developed by Bayer early in the 20th century exactly to avoid the problem of

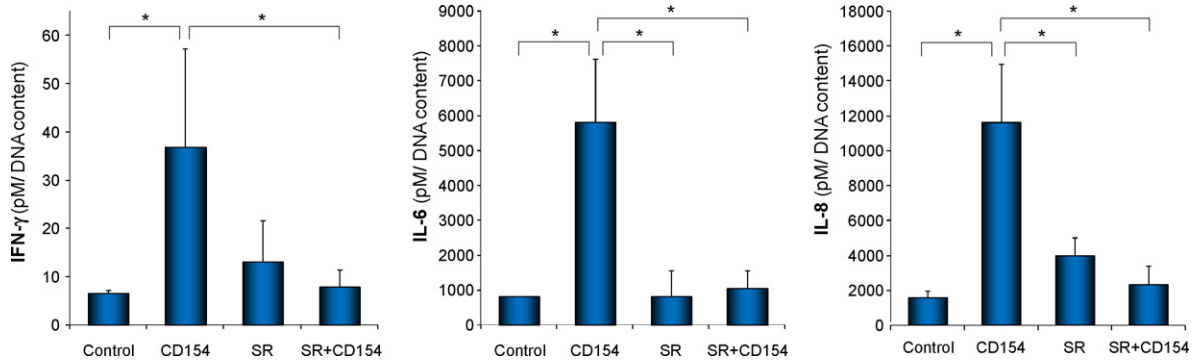

Fig. 8 – *In vitro* inhibition of soluble CD154-induced cytokine release (IFN-γ, IL-6, and IL-8) in human islets by suramin (SR, 100 μM). Data shown are average ± S.D. for $n = 3$ human islet preparations treated in duplicates for each condition. They were analyzed by ANOVA with Tukey's *post-hoc* test and * indicate significant differences at $p < 0.05$.

coloring while also improving the trypanocidal activity originally noticed by Ehrlich and co-workers for some symmetrical polysulfonated naphthylamine azo dyes [10,11]. More recently, it was evaluated as a possible treatment in patients with AIDS [12] and adrenocortical and prostate cancers [11], ultimately, however, with only modest effects [13]. Suramin can cause adrenal and renal insufficiency, coagulation factor abnormalities, immunosuppression, and poly-neuropathy [10,14]. Immunosuppression is one of its known side-effects [10,15], and it has been shown to suppress T cell activity [18,38]. It dose dependently inhibited the murine and human allogeneic MLRs with IC_{50}s around 40 μM [19]. It has been shown to inhibit the growth of several lymphoid cell lines (140 μM, *in vitro*) and to cause profound and prolonged thymic atrophy and splenic lymphocyte depletion in normal mice (30–60 mg/kg, i.p.), a selective lymphocytotoxicity that was suggested as a possible mechanism accounting for the persistent immune-suppression reported in suramin-treated AIDS patients [15]. In rats, it reduced myocardial inflammation in experimental autoimmune myocarditis (10–40 mg/kg, s.c.). This was associated with reduction of T cell activation and modulation of Th1/Th2 balance [39]. Suramin inhibited the proliferation of U937 cells and mitogen-induced T cell proliferation in a dose-dependent manner [18] and with an activity level ($IC_{50} \approx 50$ μM) in good agreement with that seen here. It concentration-dependently inhibited the binding of IL2 to its cell surface receptor, which was also suggested as a possible mechanism accounting for at least a portion of its immunosuppressive effects [16]. However, this inhibition occurred only at relatively high concentration, being clearly present at 700 μM, but not significant at 70 μM. The serum levels of suramin during therapeutic use are in the 10–200 μM range [40,41], well within the activity levels observed here.

Obviously, one need to remember that suramin exhibits strong polypharmacology and exerts multiple biological effects as it is an antagonist of various receptors and an inhibitor of several enzymes involved in signal transduction. In addition to being a well-known P2 purinergic receptor antagonist ($IC_{50} \approx 5$–10 μM) [9,10] and inhibitor of the binding of a range of tumor growth factors [10,11], it also inhibits the function of G-proteins [42], protein kinase C, and protein–tyrosine phosphatases (PTPases) [43]. Suramin can inhibit the receptor–G-protein coupling, and more recently, some direct evidence has also been found that it can block the association of G-protein α and βγ subunits by interfering with the Gα–Gβγ association with an estimated IC_{50} in the 5 μM range [44], suggesting that it can act as a PPI inhibitor in other cases too. Accordingly, at concentration levels where it seems to inhibit the CD40–CD154 PPI (50–100 μM), it is likely to have effects on other receptors, enzymes, and PPIs as well; therefore, it is difficult to clearly outline/isolate the mechanism behind its pharmacological and immunological effects. Suramin has also been claimed to inhibit complement activation and blood clotting [10]. Somewhat contrary to the present observations, suramin was reported to have adjuvant properties promoting the expansion of antigen-specific Th1 and Th2 cells *in vivo*, possibly, through a G-protein-mediated mechanism [45]. It was also reported to enhance the expression of costimulatory molecules (CD40, CD80, and CD86) and MHC class II in mouse splenocytes for concentrations up to 35 μM, for which we found no evidence here in human B cells (Figs. 6 and 7).

In conclusion, suramin seems to be an effective small molecule inhibitor of the CD40–CD154 costimulatory PPI. Its activity to inhibit this interaction is somewhat less, but in a comparable range to its ability to inhibit the P2 purinergic receptor or the function of certain G-proteins, and it is well within the range of the concentration levels achieved during clinical use. Its ability to interfere with this costimulatory interaction might provide a plausible mechanism for some of its immunosuppressive effects, which might have limited its usefulness in the treatment of AIDS and cancer, but might prove useful as a starting point in the search for effective small-molecule costimulatory blockers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.01.001.

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