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Design, synthesis, and evaluation of quinazoline T cell proliferation inhibitors

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ABSTRACT

We report here on a class of quinazoline molecules that inhibit T cell proliferation. The most potent compound *N*-*p*-tolyl-2-(3,4,5-trimethoxyphenyl)quinazolin-4-amine (**S101**) and its close analogs were found to inhibit the proliferation of T cells from human peripheral blood mononuclear cells (PBMC) and Jurkat cells, with IC_{50} in the sub-micromolar range. The inhibitor induced G2 cell cycle arrest but did not inhibit IL-2 secretion. The anti-proliferative effect correlated with inhibition of the tyrosine phosphorylation of SLP-76, a molecular element in the signaling pathway of the T cell receptor (TCR). The inhibitor restrained proliferation of lymphocytes with much higher potency than non-hematopoietic cells. This new class of specific T cell proliferation inhibitors may serve as lead molecules for the development of agents aimed at diseases in which T cell signaling plays a role and agents to induce tolerance to grafted tissues or organs.

1. Introduction

Organ transplantation elicits a complex series of immunologic processes that are generally categorized as inflammation, immunity, tissue repair, and structural reinforcement of damaged tissues. Macrophages and T cells mediate inflammation by the secretion of proinflammatory cytokines, for example, interleukin-2 (IL-2), and by the activation of biochemical cascades such as the classic complement cascade. The medical community is in need of novel immunosuppressive agents.[1] Immunosuppressive drugs fall into five groups: (i) regulators of gene expression; (ii) alkylating agents; (iii) inhibitors of de novo purine synthesis; (iv) inhibitors of de novo pyrimidine synthesis and (v) inhibitors of kinases and phosphatases.[2] Glucocorticoids exert immunosuppressive and anti-inflammatory activity mainly by inhibiting the expression of the genes for interleukin-2 and other mediators.[3] Cyclophosphamide suppresses immune responses mediated by B-lymphocytes.[4,5] Methotrexate and its polyglutamate derivatives suppress inflammatory responses through release of adenosine.[5] Mycophenolic acid and mizoribine inhibit inosine monophosphate dehydrogenase.[6,7] Mycophenolic acid induces apoptosis of activated T-lymphocytes.[8,9] A leflunomide metabolite and brequinar inhibit dihydroorotate dehydrogenase.[10,11] Cyclosporine and FK-506/tacrolimus inhibit the phosphatase activity of calcineurin.[12–16] Rapamycin inhibits signal transduction from the IL-2, epidermal growth factor and other cytokine receptors.[17] Immunosuppressive and anti-inflammatory compounds in development include inhibitors of p38 kinase and of the type IV isoform of cyclic AMP phosphodiesterase, which is expressed in lymphocytes and monocytes.[18–20]

Immunosuppressive medications are associated with toxicity due to their non-specific immunosuppressive effects. Reducing immunosuppression can prevent side effects related to over-immunosuppression. However, since the intrinsic immunosuppressive requirements for each donor recipient pair are unknown, immunosuppressive minimization carries a potential risk of under-immunosuppression and consequent acute rejection, premature graft loss and death. A promising future application of immunosuppressive drugs is their use in regimens designed to induce tolerance to allografts. The first step in finding such drugs is a search for agents that inhibit T cell proliferation by novel mechanisms, as the currently used agents, which all possess non-specific broad immunosuppressive effects. In our search for compounds that inhibit T cell proliferation we chose a known chemical scaffold, the quinazoline moiety, which has been utilized to develop tyrosine phosphorylation inhibitors. Quinazolines serve as the backbone for a range of inhibitors. Quinazoline-based tyrosine kinase inhibitors include: the reversible EGFR inhibitors, Gefitinib/Iressa/ZD1839 and Erlotinib/Tarceva; the dual EGFR-HER2 inhibitor, Lapatinib/Tykerb/GW572016; and the irreversible EGFR inhibitor, CI-1033 (reviewed in[21]). Quinazolines also form the basis for inhibitors of serine/threonine kinases, such as CDK,[22] and for a thymidylate synthase inhibitor.[23–26]

In this study, we report on the identification of a new quinazoline family of T cell proliferation inhibitors that operate by a novel mechanism, not yet fully defined. We examined the potency of these compounds on T cell proliferation of peripheral blood mononuclear cells (PBMC). We identified *N*-*p*-tolyl-2-(3,4,5-trimethoxyphenyl)quinazolin-4-amine (**S101**- dubbed **1**) as the most potent

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inhibitor. Here we explore its activity and the structure–activity relationship of a family of compounds related to **1**.

2. Materials and methods

2.1. Chemistry

Thin-layer chromatography was carried out on Merck aluminum sheets, silica gel 60 F_{254} and visualized with UV light at 254 nm. Preparative column chromatography was performed on Merck silica gel 60 (70–230 mesh). High-pressure liquid chromatography was performed on a Merck Hitachi HPLC, which included an L 6200 pump, a D 6000A interphase, L-4250 UV detector and AS-4000 autosampler. Integration employed the HSM HPLC System Manager, Merck KgaA, Darmstadt and Hitachi Instruments, Inc., San Jose. Reversed-phase preparative HPLC was performed with a C-18 column (218TPL022 Vydac).

Mass spectrometry was performed using an LCQDUO, from ThermoQuest of Finnigan, and NMR was on a Bruker AMX 300. Chemical shifts δ are given in ppm referring to the signal center using the solvent peaks for reference: $CDCl_3$ 7.26/77.0 ppm and DMSO-d_6 2.49/39.7 ppm.

Combustion elemental analysis was performed using a Perkin-Elmer 2400 Elemental Analyzer.

All solvents for HPLC analysis and purification were from J.T. Baker, BDH or Bio-Lab Ltd (Israel). Reagents for chemical synthesis were from Frutarom Acros (Geel, Belgium), Fluka (Taufkirchen, Germany), or Sigma–Aldrich (Steinheim, Germany).

Melting point was determined using a Fisher–John melting point apparatus (Fisher Scientific).

2.2. Synthesis

Synthetic procedures and analytical data for compounds **2**–**19**, **21**–**38** are listed in Supporting Information. All tested compounds possessed a purity of not less than 95%.

2.2.1. *p*-Tolyamine

4-Nitrotoluene (7.95 g, 5.8 mmol) in a solution of EtOH/H_2O 9:1 (8 mL) was added dropwise to a mixture of hydrazine hydrate and Raney nickel (7 mL) (14.4 mol) in aq EtOH (10 mL) at 60 °C. After reflux was attained, an additional quantity of hydrazine hydrate (2 mL) was added. The mixture was left to reflux for 25 min, cooled to room temperature, filtered and evaporated to afford *p*-tolyamine (5.2 g, 0.048 mol, 84%). Mp 52–53 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.27 (s, 2H), 6.63 (d, J = 5.9 Hz, 2H), 6.81 (t, J = 7 Hz, 1H), 7.20 (t, J = 7 Hz, 2H).

2.2.2. 2,4-Dichloroquinazoline[27]

$P(O)Cl_3$ (40 mL) was stirred at room temperature for 20 min and then was added to a flask containing 2,4-quinolinedione (10 g, 0.06 mol). The mixture was heated to reflux for 48 h. The brown solution was cooled to 50 °C, poured into cold water (0 °C, 40 mL) while stirring vigorously. The aqueous mixture was maintained at a temperature below 30 °C during the quench. The cold precipitate was filtered, washed with cold water (3× 60 mL) and dried under high vacuum to afford 8 g (0.04 mol) of 2,4-dichloroquinazoline (71%). Mp 118 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.58 (t, J = 7.6 Hz, 1H), 7.84 (t, J = 6.5 Hz, 2H), 8.16 (d, J = 6.9 Hz, H).

2.2.3. (2-Chloro-quinazolin-4-yl)-*p*-tolyl-amine[27]

A mixture of 2,4-dichloroquinazoline (5.8 g, 0.029 mol), *p*-tolyamine (3.5 g, 0.032 mol), and potassium acetate (3.72 g, 0.038 mol) in THF/water (66 mL/30 mL) was stirred at room temperature for 16 h. Water (66 mL) was added to the mixture, and a precipitate was formed. The precipitate was washed with water, filtered, and dried under high vacuum to afford (2-chloro-quinazolin-4-yl)-*p*-tolyl-amine (5.65 g, 0.021 mol, 70%). Mp 198–199 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.27 (s, 3H), 7.21 (d, J = 7.8 Hz, 2H), 7.50 (d, J = 7.2 Hz, 3H), 7.83 (t, J = 8.5 Hz, 2H), 8.50 (t, J = 8.0 Hz, 1H).

2.2.4. 1 *p*-Tolyl-[2-(3,4,5-trimethoxy-phenyl)-quinazolin-4-yl]-amine[27]

A mixture of (2-chloro-quinazolin-4-yl)-*p*-tolyl-amine((2-chloro-quinazolin-4-yl)-*p*-tolyl-amine, 3.76 g, 0.014 mol), ethylene-glycol-dimethyl-ether/water (1 L/120 mL), 3,4,5-trimethoxyphenylboronic acid (2.714 g, 0.014 mol), and sodium bicarbonate (3.6 g) was degassed with argon for 15 min. Pd(dppf)Cl_2 (0.84 g) was added, and the mixture was heated to reflux overnight. After cooling to room temperature CH_2Cl_2 (1 L) and H_2O (500 mL) were added. The organic and aqueous layers were separated, the aqueous layer was extracted with CH_2Cl_2 (2× 500 mL), and the combined organic layers were dried over anhydrous sodium sulfate. The organic solvent was removed under reduced pressure. The crude product was purified by MeOH. Filtration in a Büchner apparatus afforded *p*-tolyl-[2-(3,4,5-trimethoxy-phenyl)-quinazolin-4-yl]-amine (3.15 g, 0.0078 mol 56%). Mp >250 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.33 (s, 3H), 3.74 (s, 3H), 3.91 (s, 6H), 6.81(s, 2H), 7.24 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 7.3 Hz, 2H), 7.58 (t, J = 8.2 Hz, 1H), 7.83 (t, J = 7.2 Hz, 2H), 8.54 (d, J = 7.5 Hz, 1H). Anal. ($C_{24}H_{23}N_3O_3$) C, H, N.

2.2.5. 1 mesylate:[28] *m*-tolyl-[2-(3,4,5-trimethoxy-phenyl)-quinazolin-4-yl]-amine methanesulfonic salt

m-Tolyl-[2-(3,4,5-trimethoxy-phenyl)-quinazolin-4-yl]-amine (**1**, 100 mg 0.25 mmol) was dissolved in THF at room temperature. Methanesulfonic acid (1.2 equiv 20 μL, 0.3 mmol) was added to the mixture. The mixture was mixed at room temperature until a precipitate was observed. The THF was decanted and the precipitate was washed three times with 3 mL of diethyl ether, the ether was removed and the precipitate was dried under nitrogen stream to afford yellow crystals of *m*-tolyl-[2-(3,4,5-trimethoxy-phenyl)-quinazolin-4-yl]-amine methanesulfonic salt (119 mg, 0.24 mmol, 96%). Mp >250 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.33 (s, 3H), 3.74 (s, 3H), 3.91 (s, 6H), 6.81(s, 2H), 7.24 (d, J = 7.7 Hz, 2H), 7.33 (d, J = 6.7 Hz, 2H), 7.58 (t, J = 7.1 Hz, 1H), 7.83 (t, J = 7.8 Hz, 2H), 8.54 (d, J = 8.0 Hz, 1H).

2.2.6. Demethylation of 1: 5-(4-*p*-tolylamino-quinazolin-2-yl)-benzene-1,2,3-triol (20)

An aryl methoxy derivative *p*-tolyl-[2-(3,4,5-trimethoxy-phenyl)-quinazolin-4-yl]-amine (**1**, 30 mg, 7.5 e−5 mol) was dissolved in anhydrous DCM (minimal volume, not fully dissolved); the round flask was then sealed. BBr_3 (76 μL, 0.111 g, 205 g/mol, d = 1.45, 2 equiv/per methoxy group, here 6 equiv were used 5.5 e−4 mol), was added drop wise (from a syringe) and the reaction mixture was stirred at room temperature for 1 h. The degree of conversion was monitored by HPLC until full conversion was achieved, then the reaction was quenched with methanol and evaporated to low volume. Another wash was performed using methanol, followed by a wash with acetonitrile and evaporation, to obtain 5-(4-*p*-tolylamino-quinazolin-2-yl)-benzene-1,2,3-triol (24 mg, 6.6 e−5 mol, 90%). Mp >250 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.35 (s, 3H), 6.75 (s, 2H), 7.22 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 7.5 Hz, 2H), 7.56 (t, J = 8.0 Hz, H), 7.82 (t, J = 8.2 Hz, 2H), 8.24 (d, J = 7.5 Hz, 1H). Anal. ($C_{21}H_{17}N_3O_3$) C, H, N.

2.3. Biology

2.3.1. Culture conditions

Jurkat cells (human, acute T cell leukemia line) and human peripheral blood mononuclear cells (PBMC) were grown in RPMI

medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin. All cultures were incubated in a humidified incubator at 37 °C in 5% CO_2.

NIH 3T3 (mouse embryo fibroblasts), A-431 (human epithelial-like skin carcinoma cells), MDA-MB-468 (human epithelial-like breast carcinoma), PANC-1 (human epithelial-like pancreatic carcinoma), PC-3 (human prostate cancer cells), HEK293 (human embryonic kidney cells), and A375 (human malignant melanoma), were grown in DMEM medium (Biological Industries, Beit Haemek), supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin. MDA-MB-231 (human breast adenocarcinoma) were grown with Leibovitz's L-15 Medium (Biological Industries, Beit Haemek), supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin.

Primary human keratinocytes were maintained in keratinocyte growth medium (KGM): DMEM, 25% Nutrient mixture F-12 (HAM), 10% fetal bovine serum, 5 μg/mL insulin, 0.4 μg/mL hydrocortisone, 0.1 nM cholera toxin, 10 ng/mL epidermal growth factor, 1.8×10^{-4} M adenine, 5 μg/mL transferrin, 2 nM T3, 100,000 U/L penicillin, 100 μg/L streptomycin, and 0.1 mg/mL amphotericin. The primary keratinocytes were cultured from small biopsy specimens.

2.3.2. T cell isolation and stimulation

Blood samples were taken from healthy donors and loaded on a Ficoll density gradient to purify the PBMC population. T cells were purified by human anti-CD3 using an autoMACS instrument (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the manufacturer's instructions. The identity of T cells was confirmed by flow cytometry, as they stained positive for CD3. Cells were stimulated with ConA (10 μg/mL), ODN2006 (0.5 μg/mL; Enzo Life Sciences), or LPS (50 μg/mL; Sigma–Aldrich, Steinheim, Germany), for 72 h in the presence of the inhibitor, and their proliferation was measured in the BrdU incorporation assay as will be later described.

2.3.3. Antibodies

Antibodies used in this study were: Hybridoma C305, specific for the Jurkat Ti beta chain of TCR (from Dr. D. Yablonski, Technion, Israel); anti-ZAP-70 (05-253) from Upstate Biotechnology (Lake Placid, NY); anti-SLP-76 (sc-9062) from Santa Cruz Biotech (Santa Cruz, CA); Hybridoma 4G-10 specific for phosphorylated tyrosine (from our collection); anti-CD28 (MCA709XZ) from Serotec (Cergy Saint-Christophe, France); anti-PGSK3 (9331) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2118) from Cell Signaling Technology (Beverly, MA).

2.3.4. DNA 5-bromo 2′-deoxyuridine (BrdU) incorporation assay

Cells were depleted of fetal calf serum (FCS) for 12 h. They were then stimulated to proliferate by addition of ConA (10 μg/mL) for PBMC from healthy individuals and for Jurkat cells, FCS (10%) for HEK293 and A375 cells, PDGF (30 ng/mL) for NIH 3T3 cells, and EGF (30 ng/mL) for MDA-MB-231, cells (1×10^5) were cultured in 96-well plates for 3 days in the presence or absence of inhibitors at the concentrations specified in the text. Each condition was tested in duplicate wells.

Proliferation was assessed by BrdU incorporation using a colorimetric ELISA kit (Roche, Germany). Subsequent to labeling with 10 μM BrdU, DNA was denatured and the cells were incubated with anti-BrdU-POD, prior to the addition of colorimetric substrate (TMB). The reaction product was quantified by measuring absorbance at 450 nm in an ELISA plate reader (ELx800 BIO-TEK Instruments Inc.).

2.3.5. Mixed lymphocyte reaction (MLR)

In the mixed lymphocyte reaction, stimulator cells cause responder cells to proliferate, owing to alloreactivity.[29] Splenocytes from 2c mice were stimulated by irradiated splenocytes from BALB/c mice in the presence of the evaluated compound at the concentrations specified. Cultures were incubated for 72 h in 96-well plates.

2.3.6. Methylene blue assay

The inhibitor-treated cultures and controls were fixed in glutaraldehyde, 0.05% final concentration, for 10 min at room temperature. After washing, the plates were stained with 0.1% methylene blue in 0.1 M borate buffer, for 60 min at room temperature. The plates were rigorously washed to remove excess dye and dried. The dye taken up by cells was eluted in 0.1 N HCl for 60 min at 37 °C, and the OD of the solution was read at 620 nm. In preliminary titration experiments, linear readings were obtained for 1×10^3 to 4×10^4 cells/well.

2.3.7. IL-2 secretion

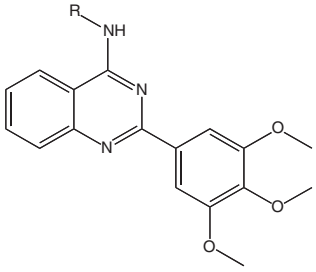
Jurkat or PBMC cells were treated for 12 h with 1 μM, 0.5 μM or no inhibitor. Cells (1×10^6) were plated in 24-well plates (Nunc) and stimulated by C305 (dilution 1:500), anti-CD28 (1:100), PMA (50 ng/ml) or ionomycin (1 μM) for 12 h. Alternately, cells were stimulated for 24, 48, and 72 h in MLR. After stimulation cells were centrifuged at 130*g* for 5 min at room temperature.

Supernatants were collected and IL-2 was assayed using the IL-2 ELISA kit (BioSource International USA, KH0022) according to the manufacturer's instructions. Absorbance was read at 450 nm in an ELISA plate reader (ELx800 BIO-TEK Instruments Inc.).

2.3.8. Cell cycle analysis

The effects of the inhibition of the cell cycle were analyzed with a fluorescence-activated cell sorter (FACS). Cells were grown in 60 mm diameter plates (Nunc) and were treated with inhibitor for 12 and 24 h. After the indicated time, cells were pooled,

Table 1
(3,4,5-Trimethoxyphenyl)quinazoline **1**–**19**



A	R	IC_{50} in PBMC (nM) ± SD	IC_{50} in Jurkat (nM) ± SD
1	4-Methyl phenyl	70 ± 0.05	29 ± 0.23
2	Phenyl	280 ± 0.04	380 ± 0.18
3	4-Trifluoromethoxy phenyl	400 ± 0.00	180 ± 0.11
4	4-Aminobenzyl	380 ± 0.04	480 ± 0.18
5	4-Hydroxy phenyl	300 ± 0.14	230 ± 0.04
6	4-Chloro phenyl	300 ± 0.05	530 ± 0.32
7	4-Methoxy phenyl	500 ± 0.14	1100 ± 0.14
8	3,4-Difluoro-benzyl	200 ± 0.00	730 ± 0.39
9	1H-Indazol-6-yl	450 ± 0.07	600 ± 0.14
10	1H-Indazol-5-yl	600 ± 0.28	700 ± 0.35
11	5-{4-[2-(1H-Indol-3-yl)-ethylamino]}	750 ± 0.21	700 ± 0.35
12	Benzo[1,3]dioxol-5-yl	380 ± 0.04	600 ± 0.00
13	5-[4-(2-Hydroxymethyl-phenylamino)]	850 ± 0.07	580 ± 0.18
14	2,4-Dimethy phenyl	750 ± 0.05	2600 ± 3.39
15	4-Methoxy benzyl	500 ± 0.14	1100 ± 0.14
16	3,4-Dichloro-benzyl	300 ± 0.14	730 ± 0.39
17	3H-Benzoimidazol-5-yl	430 ± 0.11	1500 ± 0.71
18	Benzyl	3250 ± 2.47	600 ± 0.14
19	4-Octyloxy-phenyl	2400 ± 2.26	850 ± 0.07

pelleted by centrifugation, washed in phosphate-buffered saline (PBS), and fixed with cold 70% ethanol for a minimum of 12 h. Cells were re-pelleted and resuspended in PBS. RNase A (50 μg/mL) was added in the dark for 30 min followed by 5 μg/mL of propidium iodide for FACS analysis (Becton–Dickinson FACScan).

2.3.9. Cell stimulation and lysis

Jurkat cells were washed in PBS containing Ca^{2+} and Mg^{2+} (Biological Industries, Beit Haemek), preheated to 37 °C for 15 min, and were either mock stimulated with PBS, or were stimulated for 1 min with C305 (1:500). Cells were then collected and lysed at a concentration of 2×10^{7v}cells/mL in cold lysis buffer containing 1% NP-40, 10 mM Tris (pH 7.8), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL pepstatin A, 1 μg/mL leupeptin, 10 mM sodium pyrophosphate, 0.4 mM EDTA, 0.4 mM sodium orthovanadate, and 50 mM NaF, 50 mM beta-glycerol phosphate pH 7.5 and 20 mM sodium pyrophosphate pH 7.5. After 20 min at 4 °C, the lysates were cleared by centrifugation in a microfuge (Eppendorf, centrifuge 5427R) at 16,000*g* for 10 min at 4 °C. The supernatants were used directly for immunoprecipitation.

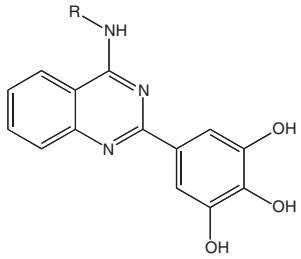
2.3.10. Western blot

Western blots were prepared on nitrocellulose using standard techniques. Membranes were blocked in TBST buffer (10 mM Tris–HCl, pH 7.4 (TBS), 0.2% Tween 20, and 170 mM NaCl) containing 5% low-fat milk, then probed overnight with the appropriate primary antibody. After incubation with horseradish peroxidase-conjugated secondary antibody, immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) detection reagent.

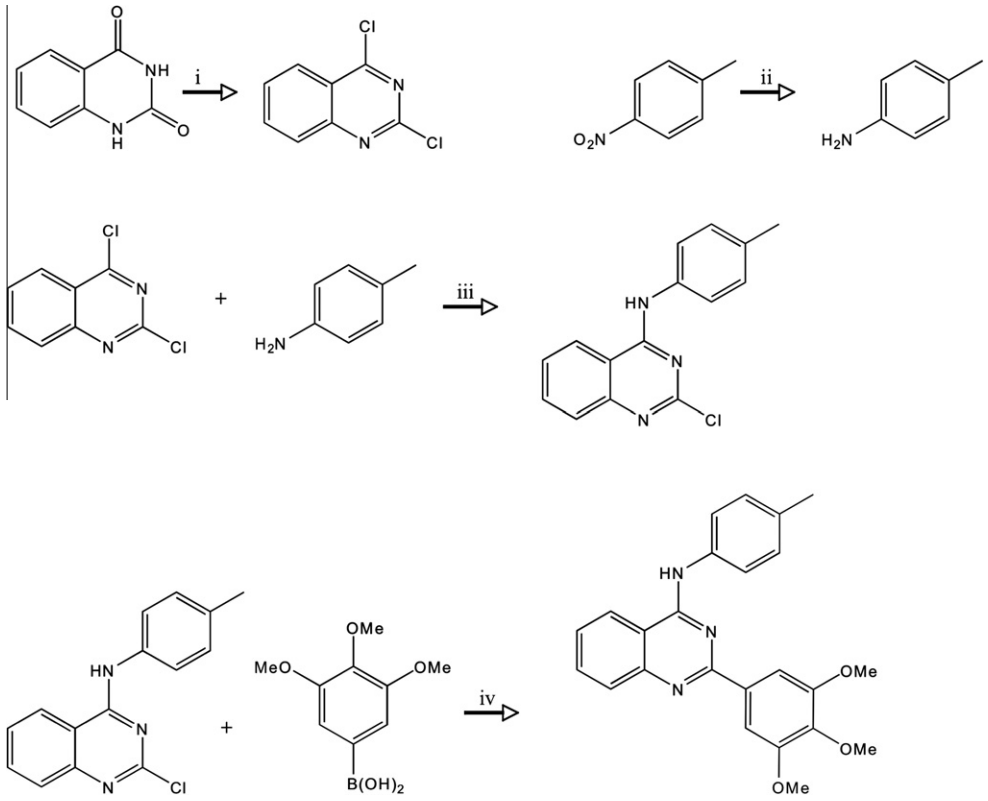
2.3.11. Immunoprecipitation

Bead preparation: 500 μL lysis buffer were mixed with 30 μL protein A (Roche, Germany) and the desired antibody, stirred at room temperature for 1 h, spun down and dried by vacuum. Cell lysates were obtained as described above and incubated with the

Table 2
(3,4,5-Trihydroxyphenyl)quinazoline **20**–**38**



B	R	IC_{50} in PBMC (nM) ± SD	IC_{50} in Jurkat (nM) ± SD
20	4-Methyl phenyl	850 ± 0.21	850 ± 0.21
21	Phenyl	650 ± 0.49	5500 ± 3.50
22	4-Trifluoromethoxy phenyl	2780 ± 0.26	3240 ± 0.31
23	4-Aminobenzyl	570 ± 0.24	670 ± 0.22
34	4-Hydroxy phenyl	970 ± 0.84	1120 ± 0.28
25	4-Chloro phenyl	550 ± 0.21	4250 ± 5.30
26	4-Methoxy phenyl	500 ± 0.14	550 ± 0.21
27	3,4-Difluoro-benzyl	280 ± 0.04	600 ± 0.21
28	1H-Indazol-6-yl	500 ± 0.14	690 ± 0.30
29	1H-Indazol-5-yl	780 ± 0.67	710 ± 0.87
30	5-{4-[2-(1H-Indol-3-yl)-ethylamino]}	1240 ± 0.74	1970 ± 0.74
31	Benzo[1,3]dioxol-5-yl	5500 ± 6.36	800 ± 0.28
32	5-[4-(2-Hydroxymethyl-phenylamino)]	900 ± 0.14	650 ± 0.21
33	2,4-Dimethy phenyl	730 ± 0.04	3700 ± 4.67
34	4-Methoxy benzyl	3260 ± 0.49	5430 ± 0.76
35	3,4-Dichloro benzyl	2600 ± 0.24	3640 ± 0.54
36	3H-Benzoimidazol-5-yl	4340 ± 0.74	2570 ± 0.82
37	Benzyl	7000 ± 3.50	950 ± 0.07
38	4-Octyloxy-phenyl	4530 ± 0.31	4250 ± 1.30



Scheme 1. Synthetic approaches to compounds **1**–**19**. Reagents and conditions: (i) $POCl_3$, Δ; (ii) hydrazine hydrate, Raney nickel, Δ; (iii) potassium acetate, THF/DDW; (iv) ethylene glycol dimethyl-ether/DDW, $Pd(dppf)Cl_2$.

beads for 1.5 h at 4 °C in a tumbling apparatus. The cells were centrifuged and washed three times with lysis buffer (the same lysis buffer used for cell lysis). Sample buffer was added and the samples were boiled for 3 min at 100 °C. The supernatants were subjected to SDS–PAGE and Western blotting.

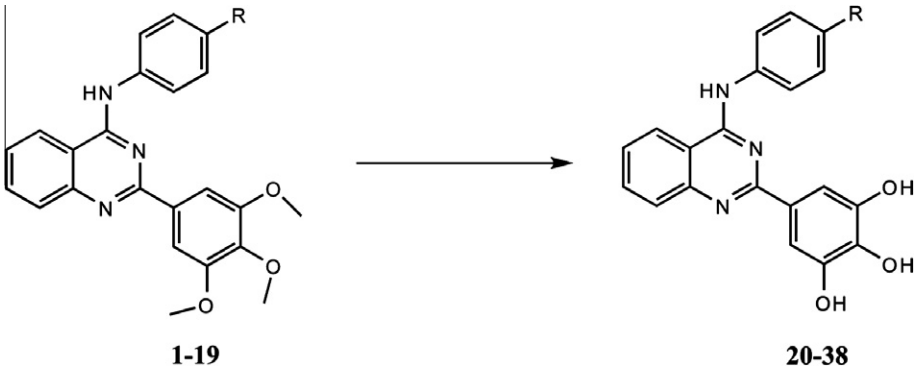
2.3.12. SLP-76 kinase assay

ZAP-70 was immunoprecipitated as described above. The immune complexes were washed two times with lysis buffer and two times with kinase buffer (50 mM Tris, pH 7.5, 100 mM NaCl, and 10 mM $MgCl_2$). The beads were resuspended in 30 μl of kinase buffer containing 3 μg of His-tagged, full-length human SLP-76 protein (kindly provided by Dr. Yablonski, Technion, Israel), and the kinase reactions were initiated by the addition of 10 μl kinase buffer containing 5 μM ATP. The reactions proceeded for 15 min at 30 °C and were stopped by the addition of 6 μL EDTA (100 mM) and sample buffer. The results were analyzed by Western blot.[30]

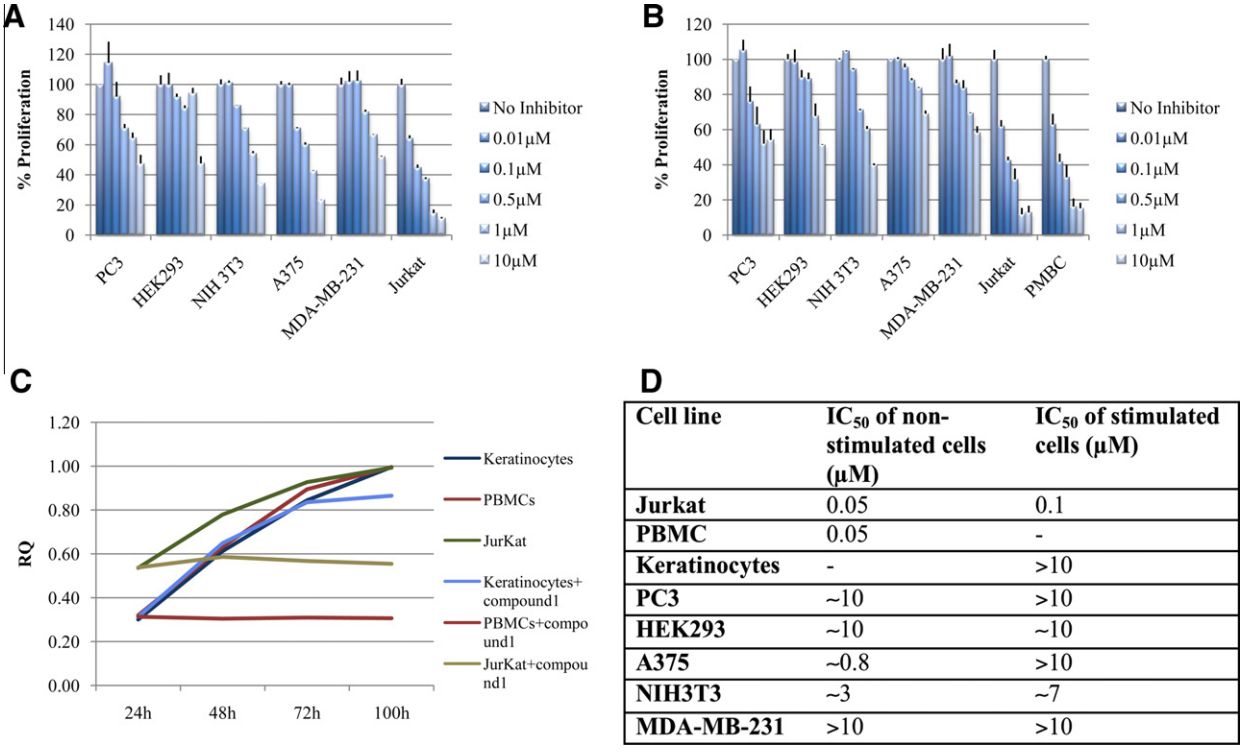
3. Results and discussion

3.1. Chemical synthesis

We hypothesized that we might be able to find T cell proliferation inhibitors among compounds possessing chemical scaffolds used to generate protein kinase inhibitors, since protein kinases play crucial roles in signaling processes and cellular communication. We therefore designed compounds based on the quinazoline moiety, an element that is present in many tyrosine phosphorylation inhibitors and a few serine/threonine kinase inhibitors. In our search for new specific inhibitors of T cell proliferation, we generated two libraries of quinazoline-based compounds. The first library (Table 1, compounds **1**–**19**, Scheme 1) possessed a 3,4,5-trimethoxyphenyl quinazoline skeleton. The trimethoxy-phenyl moiety was tested because it is a common pharmacophore for a variety of inhibitors, including inhibitors of tubulin polymerization[31];



Scheme 2. Synthetic approaches to compounds **20**–**38**. Reagents and condition: BBr_3, methylene chloride, rt.



Cell line	IC_{50} of non-stimulated cells (μM)	IC_{50} of stimulated cells (μM)
Jurkat	0.05	0.1
PBMC	0.05	-
Keratinocytes	-	>10
PC3	~10	>10
HEK293	~10	~10
A375	~0.8	>10
NIH3T3	~3	~7
MDA-MB-231	>10	>10

Figure 1. The effect of **1** on the growth of various cell lines. (A) BrdU uptake of the cells without stimulation. (B) BrdU uptake of the stimulated cells: ConA (10 μg/mL) activated PBMC, serum activated HEK293 and A375, PDGF (30 ng/mL) activated NIH 3T3, EGF (30 ng/mL) activated MDA-MB-231 with increasing inhibitor concentrations. (C) Growth curves of the selected cells without inhibitor and after treatment with 1 μM inhibitor, relevant quantification (RQ) to growth without inhibitor's treatment. (D) IC_{50} values of the non-stimulated and stimulated cells as detailed in (B).

dihydrofolate reductase inhibitor,[32] tyrosine and phosphoinositide kinases[33]; and an HSP-90 inhibitor.[34]

The second library (Table 2, compounds **20**–**38**, Scheme 2) possessed a 3,4,5-trihydroxyphenyl quinazoline skeleton, which was introduced to reduce hydrophobicity. These compounds were obtained by a multistep synthesis, starting from the chlorination of 2,4-quinolinedione by $P(O)Cl_3$ to give 2,4-dichloroquinazoline, followed by the addition of the corresponding aromatic amines. The reaction products were coupled with 3,4,5-trimethoxyphenylboronic acid catalyzed by Pd(II). Compounds **20**–**38** were prepared by the demethylation of compounds **1**–**19**.

3.2. Inhibition of T cell proliferation

Since we were not prejudiced towards any specific protein kinase, we used a biological assay for T cell proliferation. We tested our libraries for inhibition of proliferation of PBMC and Jurkat cells (Tables 1 and 2). Cell proliferation was measured by BrdU uptake. Jurkat cells do not need to be stimulated to proliferate. PBMC cells were stimulated by a non-specific T cell mitogen, Concanavalin A (ConA). All of the compounds that were tested inhibited proliferation at micromolar or sub-micromolar concentrations. The chemical entity **1** possessed the highest inhibition potency, with an IC_{50} of 70 nM for the inhibition of PBMC proliferation and 30 nM for the inhibition of Jurkat cell proliferation (Table 1).

3.3. Structure–activity relationships

We evaluated the role of the aromatic amine ring on the activity (Table 1). Elimination of the methyl group (compound **2**) caused a threefold increase in the IC_{50} of PBMC proliferation and a 10-fold increase in the IC_{50} of Jurkat cell proliferation. The addition of another methyl at position 2 (compound **14**) reduced the potency of the compound 10-fold in both cell lines. A bulky OCF_3 group (compound **3**) increased the IC_{50} by fivefold for PBMC and sixfold for Jurkat. OH or Cl groups at position 4 were similar in their potencies (compounds **5** and **6**). To assess the affect of flexibility and bond expansion, we replaced the aromatic amine ring with a benzyl amine (compounds **2**, **18** and **7**, **15**). This substitution increased the IC_{50} approximately fivefold. 4-Aminobenzyl was found to be more active than benzyl alone (compounds **4** and **18**). Dichloro- and difluoro-benzyls were similar in their abilities to inhibit proliferation. Replacing the ring with indazole, indole, benzodioxol or imidazole (compounds **10**, **11**, **12**, **17**, respectively) failed to improve activity.

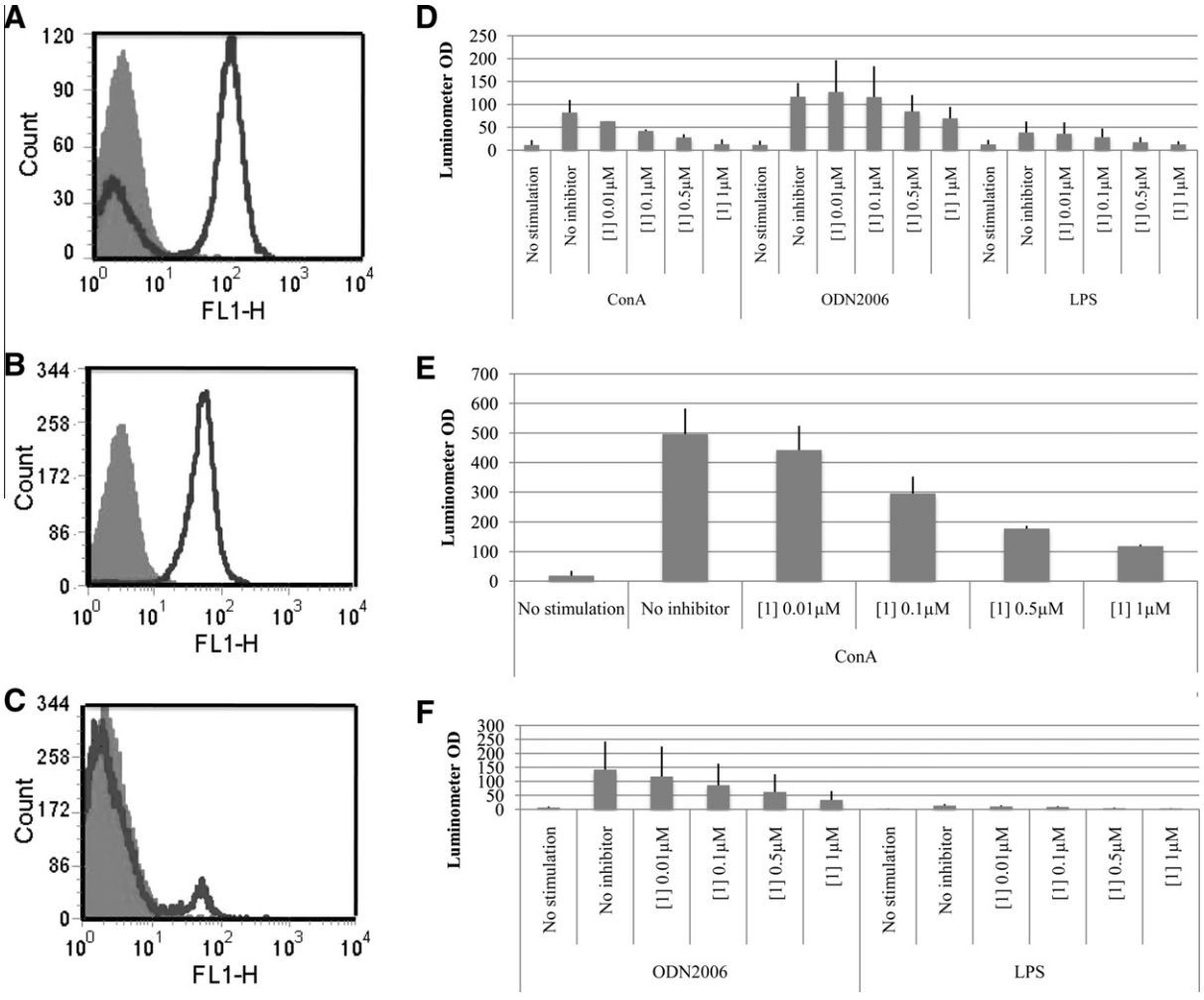


Figure 2. Activation and inhibition of PBMC, CD3+ cells and PBMC depleted of CD3+ cells. Flow cytometry. (A) PBMC; (B) CD3+ cells; (C) PBMC depleted of CD3+ cells. PBMC were stimulated with (10 μg/mL) ConA, (0.5 μg/mL) ODN2006 (Enzo Life Sciences), or (50 μg/mL) LPS (D), CD3+ cells were stimulated with ConA (E), and PBMC depleted of CD3+ cells were stimulated with both ODN2006 and LPS, in the presence and absence of inhibitor (F). Their proliferation was measured by uptake of BrdU and normalized to the maximal proliferation in the absence of inhibitor.

Trihydroxy compounds were less active than their trimethoxy analogs (Table 2), with the exception of compound **27**, in which the methyl group of the aromatic amine in compound **8** was substituted by difluoro-benzyl.

3.4. Compound 1 specifically inhibits the CD3+ cell population

To characterize the mode of action of **1** in more detail, we first examined whether **1** inhibits non-hematopoietic cell lines. We studied the effect of **1** on the growth of four cell lines: NIH 3T3 (mouse embryonic fibroblasts), A-431 (human vulval carcinoma cells), MDA-MB-468 (human breast carcinoma cells), and PANC-1 (human pancreatic carcinoma cells) (Supplementary data, Fig. 1). We also tested the inhibitors on human keratinocytes. The inhibitor slightly affected these cell types at high concentrations, at which the growth of Jurkat and PBMC was strongly inhibited (Fig. 1C). We also examined the effect of **1** on BrdU uptake by stimulated and non-stimulated PC-3 (human prostate cancer), HEK293 (human embryonic kidney), NIH 3T3, A375 (human malignant melanoma), and MDA-MB-231 (human breast adenocarcinoma). The effect of the inhibitor on the proliferation of the various cells had IC_{50} values higher by two orders of magnitude in comparison to the inhibitory IC_{50} for PBMC and Jurkat cells (Fig. 1D).

PBMC consist of a mixture of mononucleated cells, mostly T cells. To identify the cell type that was inhibited by **1**, we first purified T cells from PBMC with an autoMACS apparatus (Miltenyi Biotec), using anti-CD3 antibodies. We were now able to compare three cell populations: total PBMC, CD3+ cells purified from PBMC, and PBMC that had been depleted of CD3+ cells. In order to test the effect of **1** on the various cell populations, we made use of three modes of stimulation: (1) ConA, which stimulates T cells exclusively; (2) ODN2006 (Oligodeoxynucleotide 5′-tcgtcgttttgtcgtttt gtcgtt-3′), which stimulates mainly non-T cells, via Toll-like Receptor-9 (TLR9), which is highly expressed on dendritic cells, B-lymphocytes and natural killer (NK) cells and (3) lipopolysaccharide (LPS), which stimulates mostly B cells, macrophages and some T cells. To test the inhibition by **1** of PBMC proliferation, we used all three stimuli, namely ConA, ODN2006, and LPS. ConA-stimulated proliferation was strongly inhibited by **1**, with an IC_{50} of ~0.1 μM. ODN2006 and LPS-stimulated proliferation of PBMCs was inhibited to a lesser extent, with an IC_{50} of ~0.5 μM (Fig. 2D), which was similar to the IC_{50} of **1** for PBMCs that had been depleted of CD3+ cells, whether stimulated by ODN2006 or LPS (Fig. 2F). The proliferation of isolated CD3+ cells was very strongly inhibited by **1**, with an IC_{50} that was slightly higher than 0.1 μM (Fig. 2E). These data show that **1** specifically inhibits the proliferation of CD3+ T cells, and has a much lesser effect on the

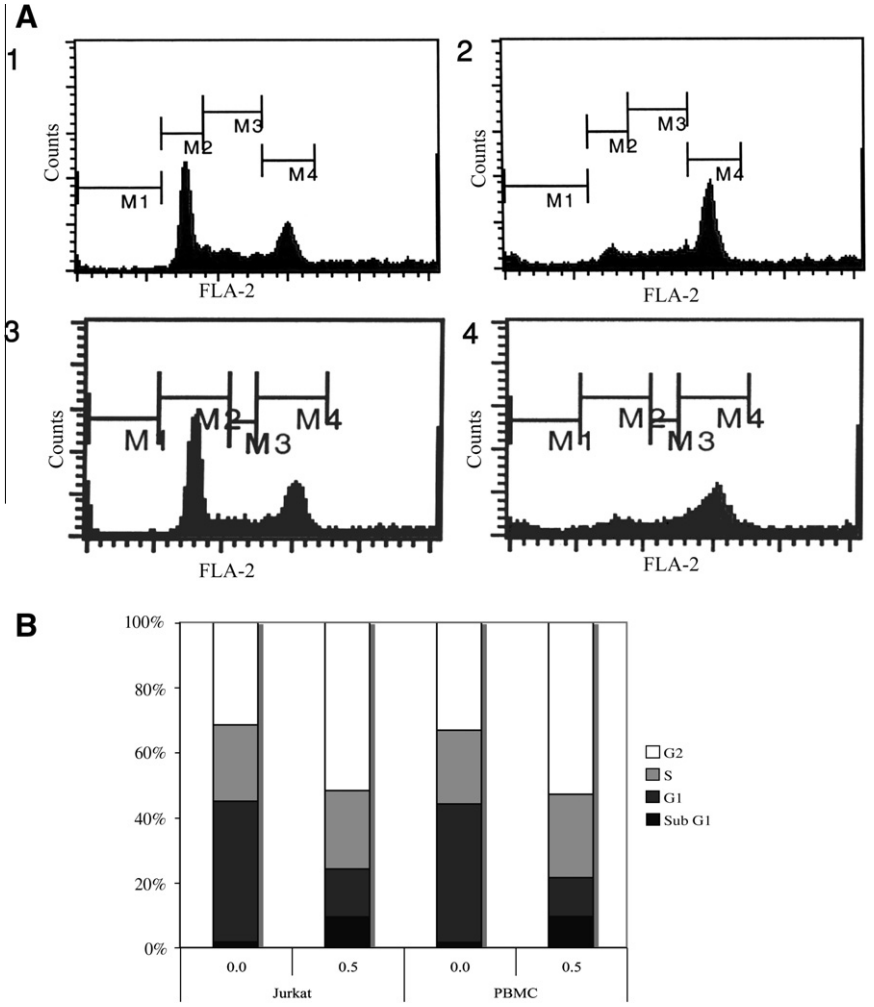


Figure 3. The effect of **1** on the cell cycle of PBMC and Jurkat cells. (A) Cellular G2 arrest and apoptosis as a result of **1** treatment. Distribution in sub-G1 (represented by M1), G1 (M2), S (M3), or G2/M (M4) phase was determined by FACS analysis. Jurkat (1 and 2) 1 without inhibitor, and 2 in the presence of 0.5 μM **1**. PBMC (3 and 4) 3 without inhibitor, and 4 in the presence of 0.5 μM **1**. The *x*-axis shows DNA content; and the *y*-axis shows the number of cells. (B) Graphic quantification of the cell cycle.

non-T cell fraction of the PBMC. These results suggest that **1** affects cell growth factors that are unique to CD3+ T cells.

3.5. Compound 1 specifically arrests T cells in G2

FACS analysis revealed that treatment of PBMC and Jurkat cells with 1 led to arrest at the G2 phase of the cell cycle. The fraction of cells in G2 increased from 30% in the untreated cells to 50% in the treated cells. The sub-G1 population increased from 2% to 9.5%, indicating that some cells underwent apoptosis (Fig. 3).

3.6. Compound 1 inhibited T cell proliferation without affecting IL-2 secretion

Compound **1** showed a remarkable ability to inhibit T cell proliferation in a selective manner, as observed in CD3+ cells, PBMC and Jurkat cells. IL-2 is a cytokine secreted in an autocrine manner by T cells to induce their own proliferation. The production of IL-2 determines whether a T cell will proliferate and become an armed effector cell, where the most important function of the co-stimulatory signal is to promote the synthesis of IL-2.[35] The central importance of IL-2 in initiating adaptive immune responses is well illustrated by the drugs that are commonly used to suppress undesirable immune responses. The immunosuppressive drugs cyclosporine A and FK506 (tacrolimus) inhibit IL-2 production by disrupting the signaling through the T cell receptor, whereas rapamycin (sirolimus) inhibits signaling through the IL-2 receptor. Cyclosporine A and rapamycin act synergistically to inhibit immune responses by preventing the IL-2-driven clonal expansion of T cells.[36,37]

Jurkat cells, which originate from leukemia, divide with no need of exogenous IL-2 stimulation since they produce their own, whereas PBMC proliferation requires exogenous IL-2 for growth. As expected, IL-2 secretion levels were lower in cells isolated from the blood (PBMC) than in Jurkat cells (Fig. 4A and B). We analyzed the effect of **1** on IL-2 secretion in both Jurkat (Fig. 4A) and PBMC (Fig. 4B) cells, using a variety of T cell stimuli. Cells were stimulated with an antibody to the TCR alone or in combination with anti-CD28 or phorbol 12-myristate 13-acetate (PMA). In order to bypass the TCR, we also stimulated the cells with a combination of ionomycin, PMA and anti-CD28 (Fig. 4A). We were surprised to discover that the inhibitor did not act by repressing IL-2 secretion. Irrespective of the mode of stimulation, compound **1** had no effect on IL-2 levels in the medium, up to a concentration of 5 μM inhibitor.

In vivo, T cells are stimulated by neighboring cells. In order to test the effect of **1** on cell-stimulated IL-2 secretion, the supernatants of splenocytes after 24, 48, and 72 h MLR were collected and analyzed for their IL-2 levels (Fig. 4C). Without inhibitor, or in the presence of S145 (compound **20**) levels of IL-2 decreased after 72 h, probably due to the consumption of IL-2 by proliferating cells. This effect was not seen with compound **1**, because the cells did not proliferate. Treatment with cyclosporine A, which is known to repress IL-2 transcription, led to a marked decrease in IL-2 levels

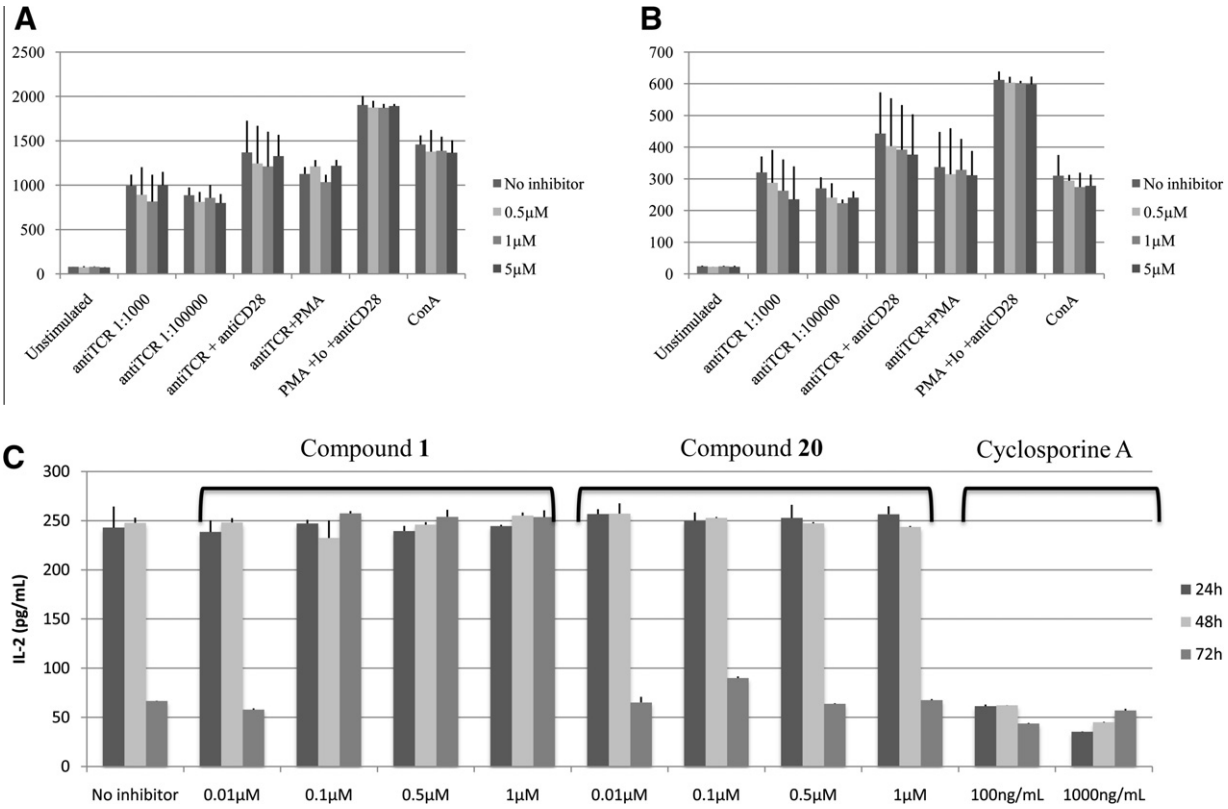


Figure 4. IL-2 secretion after stimulation. IL-2 levels in the growth medium were measured by ELISA after centrifuging out the cells. (A) Jurkat cells. (B) PBMC. Four modes of stimulation were used in order to examine the inhibitor's ability to affect diverse events in the T cell. First, cells were stimulated using anti-TCR antibody, attached to the plate. The coupling of the antibody to TCR initiates a signal transduction cascade, leading to cell proliferation and secretion of IL-2. Second, anti-TCR antibody was used in combination with an antibody to the CD28 molecule. This combination of signaling via the TCR as well as the co-stimulatory effect induced higher levels of IL-2. Third, ionomycin (a calcium ionophore), phorbol 12-myristate 13-acetate (PMA; a PKC activator) and the antibody for CD28, were used in combination. This stimulation activated parallel pathways and gave rise to the highest IL-2 level. Fourth, ConA stimulation served as control to proliferation assays. The experiment was executed with both Jurkat and PBMC. **1** did not affect IL-2 secretion in either cell type, irrespective of the mode of stimulation. PBMC had lower IL-2 levels. (C) MLR; splenocytes from 2c mice were stimulated by irradiated splenocytes from BALB/c mice in the presence of the evaluated compound at the concentrations specified. Cultures were incubated for 72 h in 96-well plates. Supernatants were collected and IL-2 was assayed for 24, 48, and 72 h in the presence of either compound **1** or compound **20**, and Cyclosporine A L.

in the medium, as early as 24 h after initiation of treatment. In the presence of **1**, no visible decrease in IL-2 secretion was detectable even at 1 μM inhibitor (Fig. 4C). At higher concentrations, no viable cells remained. These findings lead us to conclude that the dramatic inhibition by **1** of proliferation is caused by a distinct, novel mechanism that does not involve IL-2 secretion.

3.7. Compound 1 inhibited tyrosine phosphorylation of SLP-76

We were intrigued by the properties of **1** and its analogs, especially by the finding that IL-2 secretion was not inhibited, in contrast to known T cell anti-proliferative agents, and by the effect of **1** on the cell cycle. We therefore began to search for cellular targets of **1**.

The T cell receptor and co-receptor are associated with the Src-family protein kinases, Fyn and Lck. It is thought that binding of a peptide:MHC ligand to the T cell receptor and co-receptor brings together CD4, the T cell receptor complex, and CD45.[38] This allows the CD45 tyrosine phosphatase to remove inhibitory phosphate groups on Fyn and Lck. Phosphorylation of the ζ chains on the receptor enables them to bind and activate the cytosolic tyrosine kinase ZAP-70.[39] ZAP-70 phosphorylates the adaptor protein SLP-76 (Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa), which in turn leads to the activation of PLC-γ by Tec kinases and the activation of Ras by guanine-nucleotide exchange factors. Thus, SLP-76 is one of the key elements in T cell signaling following T cell receptor activation.

Jurkat cells were treated with **1** for 16 h, and SLP-76 phosphorylation was measured by immunoprecipitation of SLP-76 protein followed by Western blotting with an antibody to the phosphorylated tyrosines of the protein (Fig. 5A). We also immunoprecipitated the ZAP-70 kinase from the treated/untreated cells, and tested its ability to phosphorylate recombinant SLP-76 protein as substrate (Fig. 5B). Compound **1** inhibited the activity of ZAP-70, as well as tyrosine phosphorylation of SLP-76, in a dose-dependent manner, indicating that **1** acts upstream of SLP-76. Although in SLP-76 deficient Jurkat cells, no IL-2 is produced[40] at the concentrations we tested (up to 1 μM), SLP-76 phosphorylation was reduced but not abolished. Presumably the remaining SLP-76 activity was sufficient to allow IL-2 secretion.

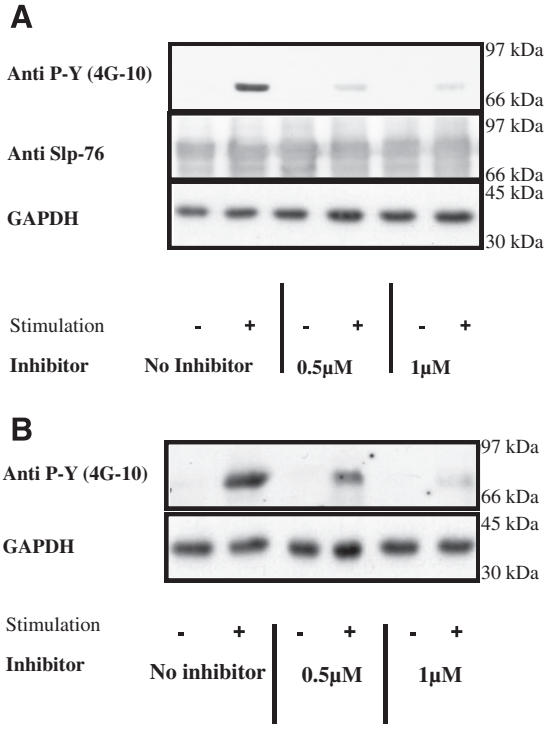


Figure 5. Inhibition of phosphorylation on SLP-76. A. Jurkat cells were treated with inhibitor and SLP-76 was immunoprecipitated from the cell lysate. Phosphorylated SLP-76 was visualized by Western blot using 4G-10 (top) and anti-SLP-76 (middle), GAPDH served as loading control. B. Jurkat cells were treated with inhibitor, lysed and ZAP-70 immunoprecipitated. Recombinant SLP-76 was added and a kinase assay was performed. Phosphorylated SLP-76 was visualized by Western blot, using the anti-PY antibody 4G-10.

4. Conclusion

Clinical transplantation has become a routine procedure, and its successful utilization depends on MHC matching, immunosuppressive drugs, and technical skill. Today, most transplants require generalized immunosuppression of the recipient. This can be toxic and increases the risk of cancer and infection. Induction of specific tolerance, which would suppress the response to the graft without compromising host defense, is now considered the 'holy grail' of immunology.

Compound **1**, described here, shows a remarkable ability to inhibit T cell proliferation in a selective manner. Compound 1 induces G2 arrest, as well as an increase in the sub-G1 population of cells. Our results suggest that **1** affects cell growth factors that are unique to CD3+ T cells. We believe that the reduction in SLP-76 phosphorylation is not the only mechanism by which our inhibitor exhibits proliferation arrest. This information is currently guiding us in the search for the **1** target, directing our search to distinct signaling features of these cells (e.g., specific cytokines, unique signaling molecules, unique kinases). The apparent selective effect of compound **1** on T cells and its lower activity against other cell types indicate that compound **1** may be a potential lead for an inhibitor of graft rejection. In the future we intend to examine if the systemic administration of **1** or one of its derivatives during graft transplantation, can lead to unresponsiveness towards antigens presented by the graft, without harming the body's ability to react toward a pathogen.

T cell proliferation is crucial for development of psoriasis, a common T cell-mediated autoimmune disorder where primary onset of skin lesions is followed by chronic relapses. In an animal model, the blocking of T cell proliferation led to the inhibition of psoriasis development.[41] Thus, **1** can also be considered a potential lead for the development of agents against graft rejection as well as psoriasis and other inflammatory diseases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.004.

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