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NON-PEPTIDE ITAM MIMICS AS ZAP-70 ANTAGONISTS

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Abstract: Non-peptide bidentate ITAM mimics as ZAP-70 antagonists have been prepared by accommodating non-hydrolyzable phosphotyrosine analogues at each end of a non-peptide spacer with a maximal P-P distance of 39 Å. The most potent antagonist **5** had an IC_{50}=0.25μM against ZAP-70 with good cellular activity. Monodentates were ca.10-fold weaker antagonists with improved cell permeability.

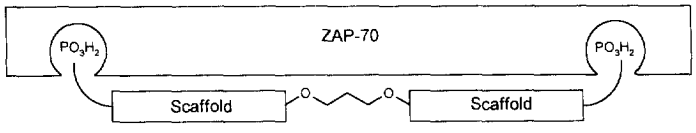
Introduction

Zeta associated protein (ZAP)-70 is a 70 kDa tyrosine kinase and exists exclusively in T cells and natural killer cells and is important for T cell activation. On ligand binding, the intracellular portion of the T cell receptor (TCR) is phosphorylated on two tyrosine residues (Y) within the immunoreceptor tyrosine activated motifs (ITAMs), which provides binding sites for the downstream signalling protein ZAP-70. The tandem SH2 domains of ZAP-70 associate with the obligatorily doubly phosphorylated ITAMs, which contain sequences of 16-17 amino acids pYXX(L/I)X_{7-8}pYXX(L/I) where X is variable. The binding of ZAP-70 to the TCR is believed to be essential for signal transduction, as peptides that block the association of ZAP-70 with the ITAMs also inhibit T-cell signalling events (1). Therefore, ZAP-70 is an attractive target for the development of novel immunosuppressive drugs.

Design and Synthesis

The recent X-ray crystal structure (2) of the tandem SH2 domains of human ZAP-70 in complex with a doubly tyrosine phosphorylated 19-meric peptide derived from the ITAM of the TCR provides information for the design of non-peptide ITAM-mimics as potential ZAP-70 antagonists. Since a major part of the binding energy between ITAM and ZAP-70 is generated by the correct docking of both tyrosine phosphates (pY) on ITAM into the tandem SH2-domains of ZAP-70, one might assume, that the correct distance between these phosphates is more important than the specific peptide sequence between them

Scheme 1



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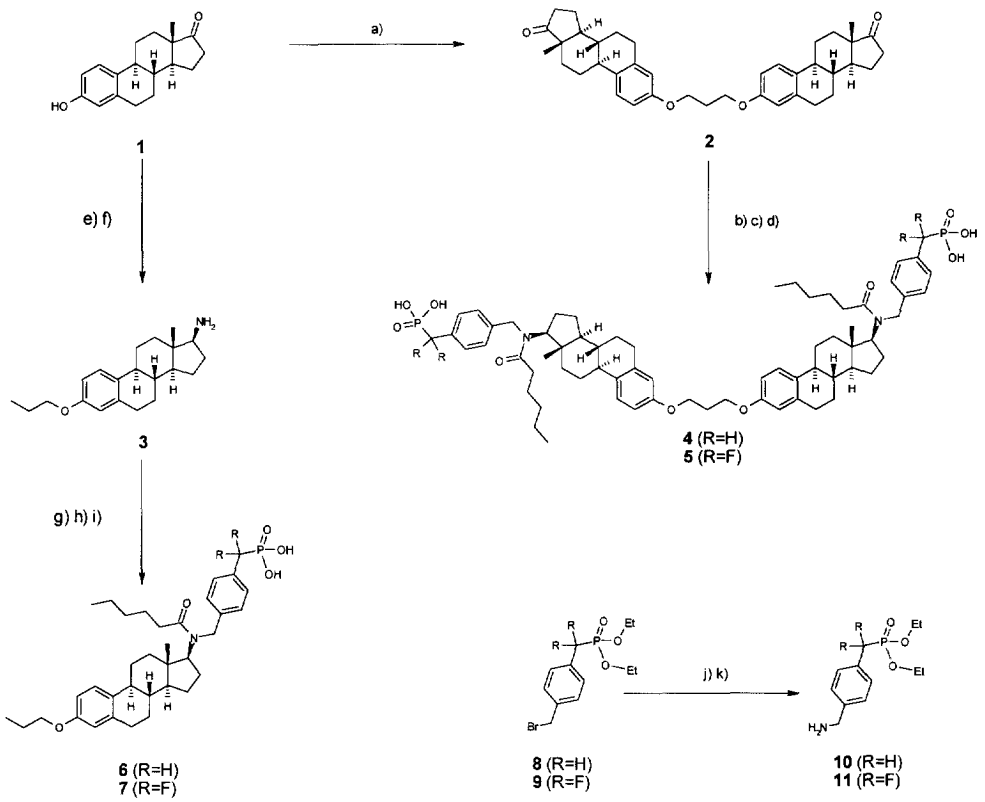
Based on this hypothesis, we embarked on a program to substitute the decapeptide sequence between both pYs by a non-peptide. A rigid scaffold with one functional group at each end was chosen and dimerized to a suitable length via a flexible linker. pY mimics were attached at either end to generate bidentate potential ITAM-mimics with a maximal P-P distance of 39 Å (Scheme 1).

Here we report the synthesis of the first non-peptide ZAP-70 antagonists with estrone as the rigid scaffold (Scheme 2). The latter was dimerized via a propylene bridge and extended at both ends by the novel non-hydrolyzable pY mimics phosphonomethylbenzylamine **10** and phosphonodifluoromethylbenzylamine **11** in a stereoselective reductive amination process to yield the bidentate ZAP-70 antagonists **4** and **5**. **10** and **11** were prepared from bromides **8** and **9** (3). The monodentate ITAM mimics **6** and **7** were prepared by monoalkylation of the β-amine **3** with bromides **8** and **9**.

Scheme 2



a) 1,3-Dibromopropane, 2-butanone, K_2CO_3, rf (reflux) 5 d, 59%. b) **10** or **11** (20 eq.), $NaCNBH_3$ (16 eq.), HOAc (30 eq.), DMF/THF/toluene 1:1:1.5 r.t. (room temperature). 2 d, 98% (R=H); 72% (R=F). c) n-Caproic acid chloride (2.2 eq.), NEt_3 (2.2 eq.), THF, r.t., 2 d. 60% (R=H,F). d) TMS-Br (50 eq.) CH_2Cl_2, r.t., 12 h, evaporate, add

acetone/water 1:1, r.t., 30 min. 76% (R=F), 52% (R=H). e) $H_2NOH.HCl$, NaOH, EtOH, rf, 30 min. Oxim: 77%. f) $LiAlH_4$ (20 eq.), THF, rf, 3 h. 84% exclusively β-amine. g) R=H: **8** (1.4 eq.), 70°C, neat, 30 min. 38%. R=F: **9** (1 eq.), DIPEA (2 eq.), r.t., 30 min., 81%. h) n-Caproic acid chloride (1.2 eq.), NEt_3 (1.2 eq.), THF, r.t., 12 h, 76% (R=H), 61% (R=F). i) TMS-Br (30 eq.) CH_2Cl_2, r.t., 12 h, evaporate, add acetone/water 1:1, r.t., 30 min., 62% (**6**), 75% (**7**). j) R=H: NaN_3 (5 eq.), water/DMF (1:10), 50°C, 2 h, 93%. R=F: NaN_3 (3 eq.), water/DMF (1:10), r.t, 2h, 89%. k) R=H: PPh_3 (1 eq.), CH_2Cl_2/water (1:1), r.t., 15 h, 89%. R=F: PPh_3 (1 eq.), Et_2O/water (1:1), r.t., 12 h, 61%. **10** and **11** decompose within 2 d at r.t. and are stored as HOAc salts.

Results and Discussion

ZAP-70 antagonism was determined using the BIAcore®2000 optical biosensor (Biacore AB) (4). Cellular activity was measured by an IL-2 luciferase reporter gene assay on a Jurkat human T cell line activated by anti-CD28 and anti-TCR antibodies (5). Results are summarised in table 1. The bidentate non-peptide ITAM mimic **4** and its tetrafluoro analogue **5** proved to be ZAP-70 antagonists only ~10 times weaker than the 20-meric diphosphorylated ITAM derived peptide NQLPpYNELNLGRREEpYDVLD **12** (IC_{50}=30nM). The fluorinated antagonist **5** showed a remarkably enhanced ability to penetrate cells compared to the unfluorinated **4**. Monodentates **6** and **7** were 7-10 times weaker than their bidentates **4** and **5** but - due to the presence of only one polar phosphonate group - showed enhanced cellular activity.

Our finding, that the decameric peptide spacer NELNLGRREE in **12** can be replaced by a lipophilic scaffold such as the dimeric estrone confirms our original hypothesis, that the correct distance between the pYs is more important than the specific peptide sequence between them. Of special interest regarding *in vivo* activity are the smaller monodentates **6** and **7**, which show cellular activities in the low μM range. Current work is in progress to determine the specificity of these novel antagonists towards a range of SH2 domains.

Table 1

Compound	ZAP-70 antagonism (4) IC_{50} (μM)	IL-2 inhibition (5) IC_{50} (μM)
4	0.32	26.5
5	0.25	1.9
6	3.2	3.6
7	1.9	0.9
12	0.03	n.t.

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(4) *Biomolecular interaction analysis (BIA).* All measurements were performed using the BIAcore®2000 optical biosensor (Biacore AB). Streptavidin was covalently coupled to CM5-sensorchips by derivatizing the carboxymethylated dextran-hydrogel on the chip surface with N-ethyl-N'-(dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide; thus the signal increased by 800-1000 so-called resonance units (RU). Unreacted groups were quenched during exposure to ethanolamine. Phosphopeptides, N-terminally modified by biotinyl-aminocaproic acid, were then immobilised on the sensor chips to a level of 30-40 RU. The peptide sequence for ZAP-70 was NQLPpYNELNLGRREEpYDVLD (IC50: 30 nM) The tandem SH2 domains of ZAP-70 were cloned and expressed in E.coli as fusion proteins to glutathione-S-transferase (GST), and then purified as described (6, 7). Proteins were injected in buffer A (10 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 0.5 mM DTT, 0.005 % (v/v) Tween 20) at a concentration of 50 nM, and at a flow rate of 10 µl/min for 4 min. Association phase binding reached an equilibrium of 400-800 RU in the absence of antagonist. Inhibitors were titrated from 10^{-7} to 10^{-4} M. Seven seconds into the dissociation phase, monitored for 2 min in free buffer flow, report points were taken to generate dose-response curves for estimating IC_{50} values; ie., the inhibitor concentrations causing a signal decrease of 50 percent. The sensorchip surfaces were very stable and could after sample injection be regenerated using 0.05 % (w/v) SDS.
(5) IL-2 Luciferase reporter gene assay. Antibodies: Goat anti-mouse IgG Fc (Jackson Immunoresearch #115-005-008). Anti-T cell antigen receptor (TCR-1, a/b WT31) (Becton Dickinson # 7770) Anti-CD28 (mouse IgG1 purified from the supernatant of 15E8 clone) Cells: Jurkat human T cell line was stably transfected with the luciferase reporter gene under the human IL-2 promoter (clone 290-H23). Cells are grown in RPMI 1640 containing Glutamax-I (Gibco BRL) supplemented with 10% Fetal Calf Serum (Inotech), 50 mM 2 ß-mercaptoethanol and 1 mg/ml Geneticin (G418 sulphate, Gibco BRL).
Luciferase assay: Microplates were precoated with anti-mouse IgG Fc (10mg/ml) followed by anti-TCR (30ng/ml) and anti-CD28 (1µg/ml) antibodies diluted in PBS w/o Ca, Mg (Gibco BRL). Assays were performed in protein-free hybridoma medium (Gibco BRL). Cells (5.10^4/well) in a total volume of 200 µl were incubated, with or without inhibitory compounds, in precoated plates for 15 to 20 hours at 37°C in a humid atmosphere enriched with 7% CO_2. After activation, plates were centrifuged for 10 minutes at 200g and the supernatants were removed by flicking. Cells were lysed by addition of 20 µl lysis buffer containing 25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1.2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% (v/v) Glycerol and 1% (v/v) Triton X-100. Plates were shaken for 10 minutes on a Titertek shaker. Then plates were scanned in a Luminoskan (Labsystem) where 50 µl of luciferase assay buffer (20 mM Tricine, 1.07 mM magnesium carbonate, 2.67 mM magnesium sulfate, 0.1 mM EDTA, 33.3 mM DTT, 270 µM CoenzymeA, 470 µM Luciferin, 530 µM ATP, pH 7.8) are automatically added.
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