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Research paper

Novel aminotetrazole derivatives as selective STAT3 non-peptide inhibitors

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ABSTRACT

The development of inhibitors blocking STAT3 transcriptional activity is a promising therapeutic approach against cancer and inflammatory diseases. In this context, the selectivity of inhibitors against the STAT1 transcription factor is crucial as STAT3 and STAT1 play opposite roles in the apoptosis of tumor cells and polarization of the immune response. A structure-based virtual screening followed by a luciferase-containing promoter assay on STAT3 and STAT1 signaling were used to identify a selective STAT3 inhibitor. An important role of the aminotetrazole group in modulating STAT3 and STAT1 inhibitory activities has been established. Optimization of the hit compound leads to **23**. This compound inhibits growth and survival of cells with STAT3 signaling pathway while displaying a minimal effect on STAT1 signaling. Moreover, it prevents lymphocyte T polarization into Th17 and Treg without affecting their differentiation into Th1 lymphocyte.

1. Introduction

Signal Transducers and Activators of Transcription (STATs) are a family of seven cytoplasmic transcription factors (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) with critical roles in the regulation of cell growth, cell differentiation, inflammation and immune response [1]. STAT proteins possess six structural regions including a N-domain, a coiled-coil domain, a DNA-binding domain, a linker domain, a Src Homology 2 (SH2) domain involved in protein–protein interaction regulation and a transcription activation domain.

STAT3 activation is mediated mainly by Src proteins or growth factor receptors. Activated Jak kinases phosphorylate tyrosine residues in the intracellular domains of these receptors [2]. STAT monomers bind with their SH2 domains to these receptor-docking sites allowing further interactions of STAT with Jak tyrosine kinases. Jak phosphorylates the tyrosine located at the C-terminal end of the STAT3 SH2 domain resulting in dimer formation through mutual SH2 domain-phosphotyrosine interactions. STAT dimers then translocate into the nucleus to mediate selective transcriptional activity [3].

Besides the transient activation in normal cells, constitutive or inappropriate activation of STAT3 has been found to directly contribute to oncogenesis in a wide variety of cancers [4]. In contrast, STAT1 is thought to play an opposite role via anti-proliferative and pro-apoptotic activities in tumor cells [5]. In addition, a transcriptional response to each STAT protein activation is highly specific and provides polarization of the immune response. An involvement of T helper 17 (Th17) lymphocyte

Abbreviations: STAT, signal transducer and activation of transcription; SH2, Src Homoly 2; Th, T helper; OSM, oncostatin M; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NK, Natural Killer.

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polarization contributes to the initiation of inflammatory diseases as highlighted in colitis or psoriasis models. Here again, the balance between STAT3 and STAT1 was shown to be an important component in the tuning of the immune response as STAT3 activation is associated with Th17 polarization while STAT1 signaling prevents Th17 polarization [6]. Therefore, the development of inhibitors blocking STAT3 transcriptional activity is a relevant clinical issue [7] and the selectivity against STAT1 is crucial for the development of new STAT3 inhibitors with interesting pharmacodynamic profiles.

Since indirect STAT3 blockade by Jak inhibitors is poorly selective and could therefore cause severe adverse effects, several approaches have been taken to directly inhibit STAT3 dimerization [8]. Several peptides and phosphopeptides were designed to mimic the amino acid sequence of STAT3, which interacts with the SH2 domain upon dimerization. However, strategies based on such peptides have shown several limitations including low cell permeability and stability. Therefore, attention has been shifted towards small non-peptidic inhibitors [9] with better pharmacokinetic properties. Here, we aimed to design non-peptidic small molecules inhibiting STAT3 activity with enhanced selectivity against STAT1. For this purpose, a structure-based virtual screening approach was used to identify commercially available chemical compounds with the potential to bind STAT3 to the SH2 domain. A luciferase-containing promoter assay was used to investigate the activity of each candidate on STAT3 and STAT1 signaling. This approach led to the identification of a prospective selective STAT3 inhibitor (**5**). Then, an extensive structure activity relationships study was performed resulting in the molecule **23** and some other derivatives. We demonstrate that an aminotetrazole group included in the hydrophilic moiety impaired the ability of selected chemical compounds to bind STAT1 while maintaining STAT3 inhibition. The inhibitor **23** does not influence STAT1 signaling and is effective *in vitro* in blocking STAT3-dependent physiological functions, such as cancer cell proliferation and Th17 polarization.

2. Results

2.1. Virtual screening for non-peptide inhibitors of STAT3 SH2 domain

The potential interest to target the SH2 domain of STAT3 using non peptide low molecular-weight compounds prompted us to identify prospective inhibitors by a docking-based virtual screening of a commercially available compound library [10] targeting the available crystal structure of mouse STAT3 SH2 domain [11]. The well-known pTyr705-binding site (Lys591, Arg609, Ser611, Ser613) of the Stat3β homodimer was specifically selected for high-throughput docking. Virtual hit selection was based on knowledge-based scoring [12] aimed at identifying compounds, which share key interactions to the Stat3 pTyr705 binding site with the co-crystallized phosphorylated peptide (Ala^{703}-Pro^{704}-$pTyr^{705}$-Leu^{706}-Lys^{707}). A list of 9 out of 52 candidates was selected (Fig. 1A) based on commercial availability and the aqueous solubility of compounds. Compounds were then purchased and tested for their ability to inhibit STAT3 signaling *in vitro*. For this purpose a luciferase promoter assay including STAT3 response elements was used as described previously [13]. Genetically modified HeLa cells with the luciferase STAT3-promoter array were exposed to 50 μM of selected candidate compounds and treated with Oncostatin M in order to activate STAT3. Inhibitions of STAT3 luciferase activity of all studied compounds are shown in Fig. 2. All candidates prevented the induction of luciferase activity following Oncostatin M treatment. Compounds **2** and **6** were cytotoxic on Hela cells (data not shown) and were excluded from further analysis.

2.2. Influence of the selected STAT3 inhibitors on STAT1 activity

The selectivity of selected compounds for STAT3 vs. STAT1 inhibition was tested as follows. HeLa cells containing a luciferase promoter array with STAT1 response elements were used [14]. STAT1 signaling was induced by interferon γ. STAT3 luciferase assays were performed in parallel to determine the selectivity of each inhibitor. The well-known STAT3 SH2 domain inhibitor STA21 [15], was used as a control for STAT3 inhibition. The final concentration of all candidates and STA21 was set to 50 μM. The candidate **5** displayed selectivity close to that achieved with STA21 (Fig. 2). This compound was therefore synthesized in our laboratory to confirm initial screening results and for further experiments. (Fig. 3)

2.3. Structure-activity relationships and development of inhibitor 23

An extensive structure-activity relationships study was performed by synthetizing analogues of **5** where each of three radicals R_1, R_2, R_3 (see Table 1) was substituted by different chemical groups. The stages of chemical synthesis of **5** and **23** are shown in Fig. 3 whereas that of all other derivatives is shown in Figs. S1–S12. Each compound was assessed in STAT3 and STAT1-luciferase reporter assays to evaluate its effect on STAT3 transcriptional activity and selectivity against STAT1. The cytotoxicity of each compound was tested after 24, 72 and 120 h of cell culture using a trypan blue exclusion assay or a viability test based on tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

As a methyl on a phenyl moiety is quickly metabolized, a significant amount of work has been done to substitute this group (R_1 substituent, Table 1.1). Electron donating group (**14**) and hydrophilic groups (**20**, **21**) reduced activity on STAT3 while small electron withdrawing groups retained or increased activity and selectivity against STAT1 (**11**, **12**, **16**, **23**). Among those, the nitro group at the *para* position (**23**) led to an effective STAT3 inhibition and enhanced selectivity. The optimal position of the nitro substituent has been confirmed to be the *para* position (**24**, **25**). An additional nitro group did not improve the inhibitory activity (**26**), however an additional alkoxy substituent on the benzyl moiety (Table 1.2) leads to increase of activity depending on the size of substituent. This last series of compounds led to the identification of compound **30**, which was endowed with high activity but lacked selectivity against STAT1. We also synthesized and tested a series of cycles and heterocycles fused or linked to the benzyl moiety without any convincing result (Table S1).

The methoxy group appeared to be the optimal R_2 substituent, which cannot be removed or replaced (Tables 1.3 and 1.4). In the $R_1 = 4\text{-}CH_3$ series, the activity on STAT3 was enhanced by modulating the hydrophobicity of the halogen (Table 1.5). Bromine at R_3 seems the best compromise between potency and selectivity but this was not reproducible in the nitro series (Table 1.6).

2.4. Importance of the aminotetrazole group

Variations based on the partial or complete modification of the aminotetrazole group were set up to characterize the involvement of each parameter in inhibition and selectivity (Tables 1.7 and 1.8). The importance of the secondary amine was shown by the reduced activity of the methylene derivative **44**. Replacing the tetrazole of **5** with a carboxylic acid in **45** significantly reduced both STAT3 and STAT1 inhibition. Interestingly, non ionizable aldehyde and cyanamide derivatives that share a planar structure displayed the same range of inhibition as the tetrazole analogs. Among the $R_1 = 4\text{-}CH_3$ series, the cyanamide **43** and the aldehyde **42** were both able to inhibit STAT3 transcriptional activity. These experiments suggest