UNIVERSITÀ DEGLI STUDI DI MILANO
FACOLTÀ DI FARMACIA

Tricyclic pyridazinone derivatives: development on new STAT3 inhibitors

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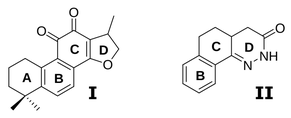
Introduction

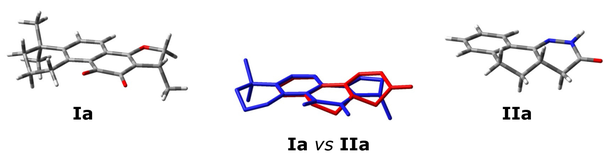
Signal transducer and activator of transcription 3 (STAT3) is a member of Stat family which comprises seven isoformes (STAT1-4, 5a, 5b, 6). They directly relate signals from the cytoplasmic membrane to the nucleus[1] and regulate transcription of target genes. The Stats have several retained domains and among them, Src homology (SH2) domain, located between DNA binding domain and C terminal domain, is fundamental for Stats activation. It is triggered by many cytokines and growth factors including epidermal growth factor (EGFR), platelet-derived growth factor (PDGF), IL-6, as well as oncogenic proteins such as Src and Ras and in addition numerous carcinogens (e.g. cigarette smoke, diesel exhaust) (Figure 1)[2]. STAT3 activation is regulated by phosphorylation of *Tyr705* in SH2 domain by intrinsic kinases such as EGFR kinases, Janus activated kinases (Jak) or kinases associated to the receptors (Src, Abl). After *Tyr705* phosphorylation, STAT3 dimerizes, forming homodimers and/or heterodimers with Stat1 by reciprocal phosphotyrosine SH2 interactions. The dimers translocate into the nucleus, bind to response elements in gene promoters and enhance the transcription of target genes. STAT3 is constitutively activated in a wide variety of human solid and blood tumors following disregulation of cytokine receptors, growth factor receptors and Jak activity. Numerous published reports have shown that blocking constitutively activated STAT3 signalling leads to apoptosis of tumors cells[3-5] but has no effect in normal cells[6-7]. This selective inhibition might reflect an irreversible dependence of tumor cells on high level of STAT3 for growth and survival, whereas normal cells might be able to withstand lower level STAT3 activity or use alternative signaling pathways. Indeed, STAT3 has been proven to be a creditable molecular target for cancer therapy. On these bases the design, the synthesis of new molecules as STAT3 inhibitors, and the studies of their interaction with the target are very important for the development of new anticancer drugs endowed with interesting pharmacodinamic properties and reduced side effects.

Modeling studies

A conformational study of the reference compound Cryptotanshinone **I** and of a generic compound with pyridazinone structure **II** was carried out. Attention was focused, for **I**, on the flexibility of the **A** and **C** rings, and, for **II**, on the possible inversion of **C** ring.

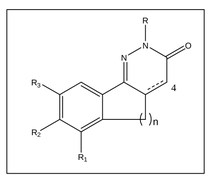


All calculations were carried out using the Gaussian03 program package. The conformational space of the compounds was explored through optimizations at the B3LYP level with the 6-31G(d) basis set and the energy of the optimized conformations was recalculated in water, at the same level as above, using a polarizable continuum solvent model (PCM).



Both molecules are quite rigid and characterized by planarity, requisite supposed to be important for the inhibitory activity[8] . In order to highlight analogies between the two structures, we superimposed the preferred conformations of **Ia** (blue) and **IIa** (red) through rms fitting of the atoms of the **BCD** tricyclic moiety. The overlap shows that the two compounds match very well.

Biological evaluation



Compound	n	C4	R	R_1	R_2	R_3	% Inhibition (2 μM)[a]
1	1	CH_2	$(CH_2)_3COOEt$	H	H	H	28,25
2	1	CH	$(CH_2)_3COOEt$	H	H	H	< 1
3	2	CH_2	$(CH_2)COOEt$	H	H	H	3,1
4	2	CH_2	$(CH_2)_2COOEt$	H	H	H	29,95
4a	2	CH_2	$(CH_2)_2COOH$	H	H	H	< 1
5	2	CH_2	$(CH_2)_3COOEt$	H	H	H	46,25
5a	2	CH_2	$(CH_2)_3COOH$	H	H	H	7,72
6	2	CH_2	$(CH_2)_4COOEt$	H	H	H	20,91
7	2	CH_2	$(CH_2)_5COOEt$	H	H	H	24,49
8	2	CH_2	$(CH_2)_6COOEt$	H	H	H	22,36
9	2	CH	$(CH_2)_2COOEt$	H	H	H	0,76
9a	2	CH	$(CH_2)_2COOH$	H	H	H	0,18
10	2	CH	$(CH_2)_3COOEt$	H	H	H	< 1
10a	2	CH	$(CH_2)_3COOH$	H	H	H	< 1
11	2	CH	$(CH_2)_4COOEt$	H	H	H	29,02
12	2	CH_2	$(CH_2)_3COOEt$	H	Cl	H	9,3
13	2	CH_2	$(CH_2)_3COOEt$	OCH_3	H	OCH_3	23,35
14	2	CH_2	$(CH_2)_3CH_3$	H	H	H	21,77
15	2	CH_2	$(CH_2)_4CH_3$	H	H	H	< 1
16	3	CH_2	$(CH_2)_3COOEt$	H	H	H	< 1
17	3	CH	$(CH_2)_3COOEt$	H	H	H	15,16
Cryptotanshinone							25

[a] The STAT3 inhibitory activity was evaluated through a modified procedure of dual-luciferase assay[11] in human colorectal carcinoma cells HCT-116, characterized by uncontrolled expression of STAT3.
The activity was expressed as % of inhibition, at different concentrations, after 24h treatment with the tested compounds and Cryptotanshinone[9] , which was used as reference.

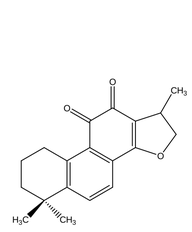
Conclusions

Based on the structural analogy between Cryptotanshinone and a tricyclic pyridazinone moiety we investigated on previous studies, we have now synthesized a series of novel derivatives as possible STAT3 inhibitors.

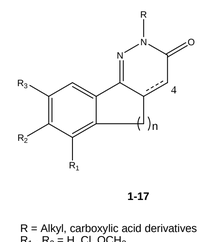
Preliminary biological results showed that several compounds were provided with activity similar or even better (**5**) than that of the model in the luciferase assay. The nature of the side chain seems to be responsible for the most significant differences in potency. In particular hydrolysis of the esters **4** and **5** to their corresponding acids (**4a** and **5a**) brought about a complete loss of activity, since docking studies showed for all the compounds quite similar poses, this results is probably due to pharmacokinetic reasons.

Aim

During our ongoing researches[8] aimed at the design and the synthesis of new non peptide small molecules as potential STAT3 inhibitors, we have focused our attention on an interesting natural compound, Cryptotanshinone, studied by our Korean colleagues for its potent inhibitory activity versus STAT3[9]. Since molecular modeling studies suggested a structural similarity between Cryptotanshinone and a series of pyridazinone derivatives investigated by our research group in previous studies[10], we designed, synthesized, and performed biological evaluation of new several derivatives (**1-17**) reported below.



Cryptotanshinone



1-17

R = Alkyl, carboxylic acid derivatives
R_1, R_2 = H, Cl, OCH_3
n = 1-3

In details, we explored the effects of substituents both at N-2 and at different positions of thearomatic ring as well as the size of the central ring. The synthetic procedures to obtain compounds **1-17** are reported in **Schemes 1** and **2.**

Chemistry (I)

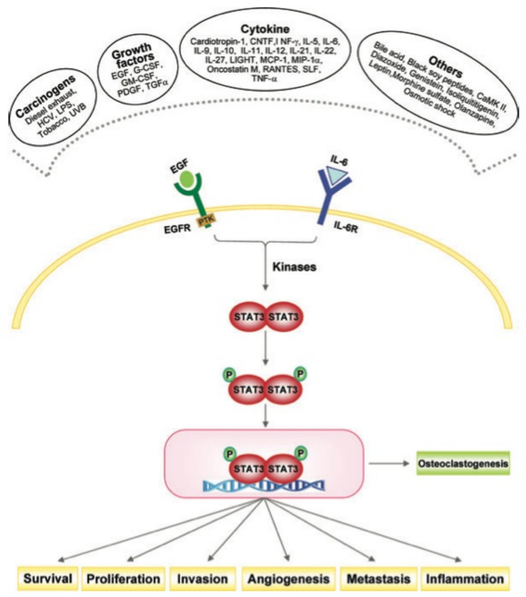
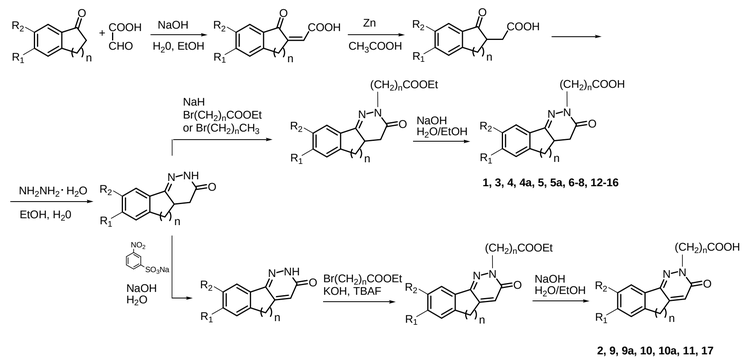
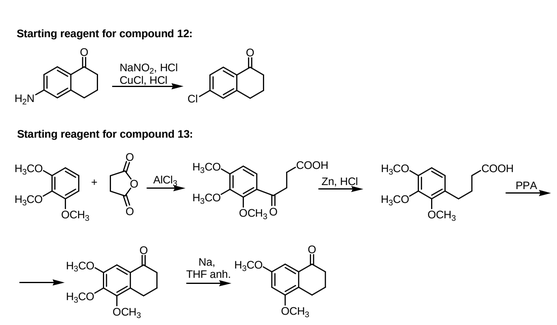


Figure 1. STAT3 activation signaling pathway[2]



Scheme 1. General procedure for the synthesis of all compounds.

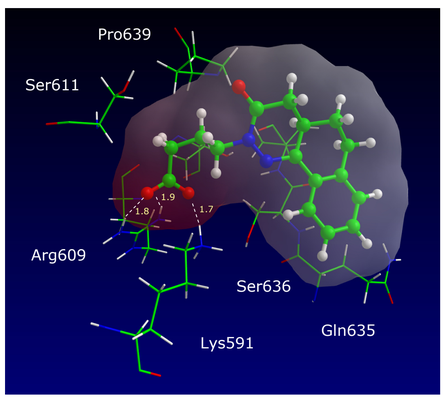
Chemistry (II)



Scheme 2. Synthesis of the starting reagents for compounds **12** and **13**.

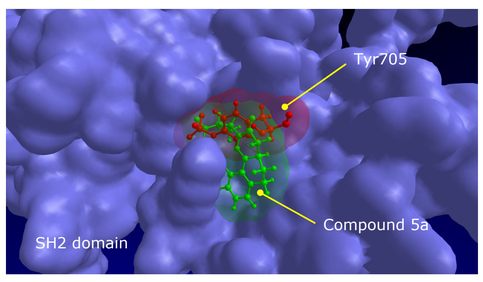
Docking studies

The STAT3 structure, co-crystallized with a DNA fragment[12], was downloaded from the Protein Data Bank[13] (PDB-ID 1BG1) and was optimized by NAMD2[14] (30.000 steps, conjugate gradients). All considered compounds were built by VEGA ZZ[15], docked to STAT3 by GriDock[16], selecting the SH2 domain as target region and the resulting complex were minimized to avoid the unfavorable interactions.



This picture shows the main residues involved in the STAT3 – compound **5a** interaction. Since the poses of all docked molecules are quite similar, **5a** can be considered as reference model. The carboxylic group is inserted in the positively charged pocket lined by *Arg609* and *Lys591*. Both aminoacids are know to play a pivotal role in the STAT3 dimerization process, because they interact with the negatively charged phosphorylated *Tyr705* of the other subunit. The red surface highlights the area with the best electrostatic binding energy.

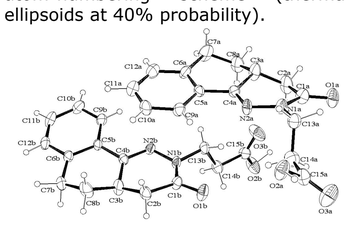
Comparing the pose of the compound **5a** (green) with the position of the phosphorylated Tyr705 (red) of the second STAT3 subunit, it appears clear how it can compete in the dimerization process.



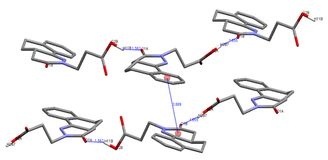
The same docking study was also performed for the Cryptotanshinone and although it is able to bind the SH2 domain, its interaction energy (-5.65 kcal/mol) is not so good as that of compound **5a** (-7.30 kcal/mol).

Crystallography

ORTEP[17] view of **4a** and the relative atom-numbering scheme (thermal ellipsoids at 40% probability).



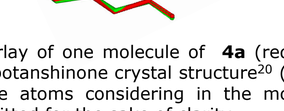
Intermolecular interactions



Strong O-H…O hydrogen bonds and π-π stacking between phenyl rings.

Crystals were grown by evaporation of a methanolic solution. Data collection: Enraf Nonius CAD-4 diffractometer using MoKα (λ=0.71073Å) radiation at 293(2)K. The structure was solved by direct methods[18], refinements were carried out with SHELX-97[19]. All non-H-atoms were refined anisotropically and hydrogens were introduced at calculated positions, in their described geometries.

Superimposition of the two independent molecules forming in the asymmetric unit of **4a** (in green the A labelled ones).



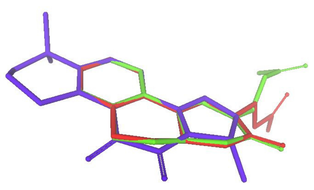
Significant torsion angles (°) highlighting the main structural differences in the two indipendent molecules are:

N2-N1-C13-C14: 76(1) ; [-100(1)]

O2-C15-C14-C13: 65(1) ; [-170(1)]

[the value for the A labelled molecule]

Overlay of one molecule of **4a** (red and green the A labelled ones) onto the Crypotanshinone crystal structure[20] (blue) obtained through r.m.s. fitting of the same atoms considering in the modeling calculations. Hydrogen atoms are obmitted for the sake of clarity.



The high level of similarity of the crystal structures supported the molecular modeling results.

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