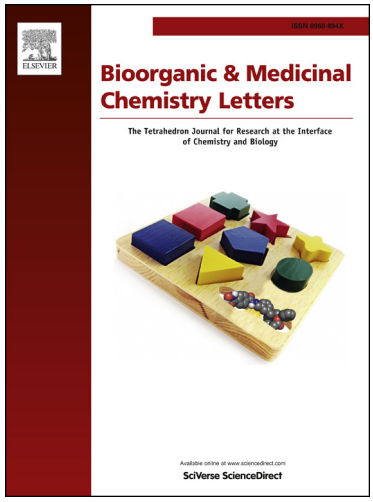

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PII: S0960-894X(15)00767-2
DOI: http://dx.doi.org/10.1016/j.bmcl.2015.07.063
Reference: BMCL 22952

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 27 May 2015
Revised Date: 18 July 2015
Accepted Date: 21 July 2015

Please cite this article as: Kim, K., Kim, S-J., Han, Y.T., Hong, S-J., An, H., Chang, D-J., Kim, T., Lee, J., Surh, Y-J., Suh, Y-G., Identification of small molecule inhibitors of the STAT3 signaling pathway: Insights into their structural features and mode of action, *Bioorganic & Medicinal Chemistry Letters* (2015), doi: http://dx.doi.org/10.1016/j.bmcl.2015.07.063

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Identification of small molecule inhibitors of the STAT3 signaling pathway: Insights into their structural features and mode of action

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
STAT3
Small molecule inhibitors
Apoptosis
Antitumor
Breast cancer

ABSTRACT

A series of novel STAT3 inhibitors consisting of Michael acceptor has been identified through assays of the focused in-house library. In addition, their mode of action and structural feature responsible for the STAT3 inhibition were investigated. In particular, analog **6** revealed promising STAT3 inhibitory activity in HeLa cell lines. The analog also exhibited selective inhibition of STAT3 phosphorylation without affecting STAT1 phosphorylation and cytostatic effect in human breast epithelial cells (MCF10A-*ras*), which supports cancer cell-specific inhibitory properties.

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Signal transducer and activator of transcription 3 (STAT3) is one of the STAT proteins (STATs 1, 2, 3, 4, 5a, 5b, and 6), which relay signals from cytokines and growth factor receptors in the plasma membrane to the nucleus where they regulate gene transcription.[1] Activation of STAT3 signaling is generally dependent on the phosphorylation of specific tyrosine residue by upstream cytokine receptor-associated kinase and growth factors receptor-associated tyrosine kinase. STAT3 undergoes dimerization via reciprocal interactions between the phosphor-tyrosine and SH2 domain and then moves to the nucleus resulting in activation of the target gene transcription, which modulate critical cellular responses, including cell differentiation, proliferation, apoptosis, angiogenesis, metastasis, and immune responses.[2] STAT3 is highly implicated in tumorigenesis in a variety of solid and hematological malignancies through overexpression and constitutive activation.[1b, 3] Although STAT1 and STAT3 are quite similar in terms of their proteins and target DNA sequences, STAT1 plays a major role as a pro-inflammatory, anti-pathogenic, and anti-proliferative factor. The biological function of STAT1 is mostly antagonistic to that of STAT3.[4] Consequently, STAT3 has been validated as a novel cancer drug target and development of selective STAT3 inhibitors have been consistently demanded because the inhibition of abnormally elevated STAT3 activity or expression is an important therapeutic modality.[5]

Recently, we have been working on the identification and mode of action of novel and selective STAT3 inhibitors from our in-house chemical library, which consist of a variety of scaffolds derived through long-term medicinal chemistry works. Thus, we investigated the focused in-house library[6] based on the inhibition of STAT3 transcriptional activity using HeLa/STAT3-luc cells. Subsequently, we identified a series of novel STAT3 inhibitors, which is shown in Figure 1, and investigated their structural features and mode of action to further develop STAT3-selective inhibitors with therapeutic potential. Interestingly, all of the identified inhibitors possess a Michael acceptor as a common electrophilic moiety, which was in agreement with a recent report.[7] It was reported that Michael acceptor of many potent STAT3 inhibitors function as a key moiety to react with the cysteine residues of the active-site. Herein, we describe our recent work on selective STAT3 inhibitors and present insight into their structural features responsible for their inhibitory activities.

We initially investigated the structural feature of the eight identified compounds shown in Figure 1. Enone **6** was prepared from dimethoxyphenyl acetate by a known procedure.[5b] The syntheses of the other enones are outlined in Schemes 1 and 2. For the syntheses of enones **1** and **2**, dimethoxy phenol **9** was subjected to propargylation, and the subsequent bromination of the resulting ether **10** afforded bromo alkyne **11**. A regioselective Claisen rearrangement of **11** followed by a spontaneous cyclization provided benzopyran **12**. Finally, the addition of an aryl anion, prepared from **12**, to aldehyde **13** or **14**[5b] followed by oxidation of the resulting alcohol afforded ketone **1** or **2**, respectively. For the syntheses of enones **3** and **7**, benzopyrans **17** and **18** were initially prepared by a regioselective electrocyclization of **15** from 3-methyl-2-butenal[8] or by the procedure described for **12**, respectively. Adol condensation of acetophenone **17** and **18** with 3,4-dimethoxybenzaldehyde or 2,4,5-trimethoxybenzaldehyde followed by *O*-methylation of the resulting enones **4** and **8** produced **3** and **7**, respectively.

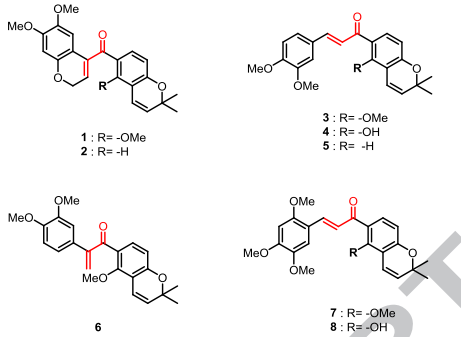
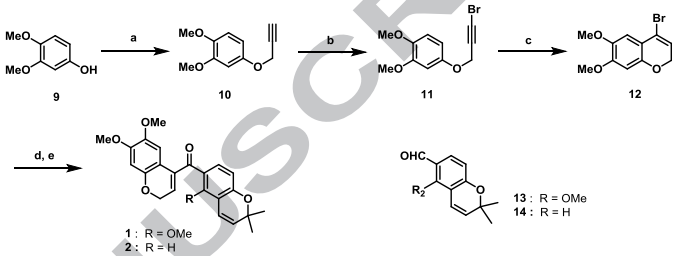
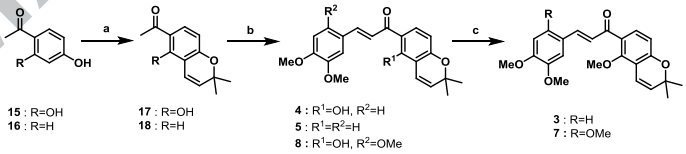


Figure 1. Chemical structures of the STAT3 inhibitors from in-house library



Scheme 1. Reagents and conditions: (a) K_2CO_3, propargylbromide, DMF, 60 °C, 100%; (b) Br_2, NaOH, water, DME, 86%; (c) diethylaniline, 215 °C, 77%; (d) *n*-BuLi, aldehdye **13** or **14**, -78 °C; (e) DMP, DCM, rt, 41% for **1**, 53% for **2** for 2 steps.



Scheme 2. Reagents and conditions: (a) for **17**: 3-methyl-2-butenal, pyridine, acetone, 120 °C, 32 %; for **18**: i) 3-chloro-3-methyl-1-butyne, K_2CO_3, KI, DMF, 60 °C, 100%; ii) diethylaniline, 195 °C, 65%; (b) 3,4-dimethoxybenzaldehyde for **4** and **5**, 2,4,5-trimethoxybenzaldehyde for **8**, KOH, EtOH, reflux, 57 % for **4** and **5**, 49% for **8**; (c) K_2CO_3, iodomethane, acetone, 60 °C, 81% for **3**, 88% for **7**.

The identified enones exhibited different STAT3 inhibitory potencies, although they all consist of a key enone system as a Michel acceptor. Analog **6** exhibited the most potent inhibition of STAT3 transcription as shown in Figure 2.

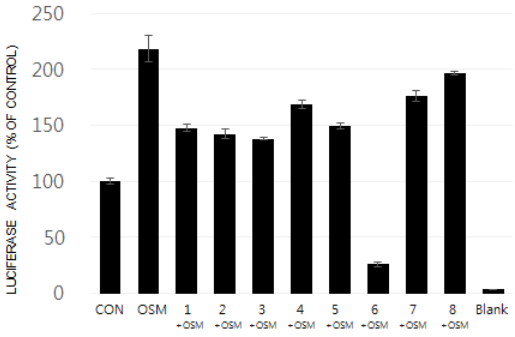


Figure 2. STAT3 transcriptional activities of the identified enones. HeLa/STAT3-luc cells were pretreated with DMSO or each compound (10 μM) for 24 h, stimulated with oncostatin M (OSM) 10 ng/mL for 5 h, and then assayed for the luciferase reporter gene activity.

We assumed that the high inhibitory activity of enone **6** was likely due to the favorable conformation indicated by the sterically less-hindered exo-olefin for interaction with the

nucleophilic cysteine residue. This was well supported by our molecular modeling study of the identified inhibitors as shown in Figure 3. To investigate the conformational space of the enones, molecular dynamics simulations were carried out using the simulated annealing protocol.[9] The structures with the lowest energy are shown in Figure 3. Compared to enones **1** and **3**, enone **6**, which contains an exo-olefin, appeared to have a sterically less-hindered electrophilic carbon for Michael type 1, 4- addition. The nucleophilic attacks on the other enones by the cysteine residue were less likely to occur due to the steric hindrance. Figure 3 (b) shows overlaid structures of the final ten ensembles with enone **6** having the lowest energy among the 500 conformers generated in the simulated annealing process.

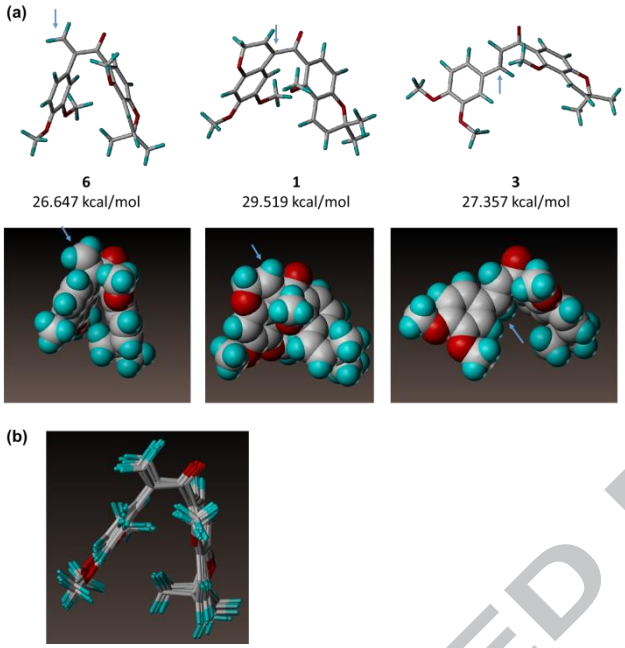


Figure 3. (a) The lowest energy conformations of the structures of enones **1**, **3**, and **6**. (b) Overlaid structures of the final ten ensembles with the lowest energy conformations of **6** obtained by the SYBYL molecular modeling program.

To further confirm that the enone system of **6** was a crucial moiety for interaction with the thiol residue, we prepared analogs **19**, **20**, and **21**, which are devoid of a Michael acceptor, by deletion (**19**) or reduction (**20**) of the enone-olefin[5b] and carbonyl group (**21**). As we anticipated, analogs **19**, **20**, and **21** did not suppress STAT3 transcription as shown in Figure 4. Obviously, structural changes in the α,β-unsaturated carbonyl moiety resulted in the complete loss of STAT3 inhibitory activity. This result supports that the enone system of **6** plays a crucial role for the STAT inhibitory activity of **6** based on its interaction with the cysteine residue of STAT3.

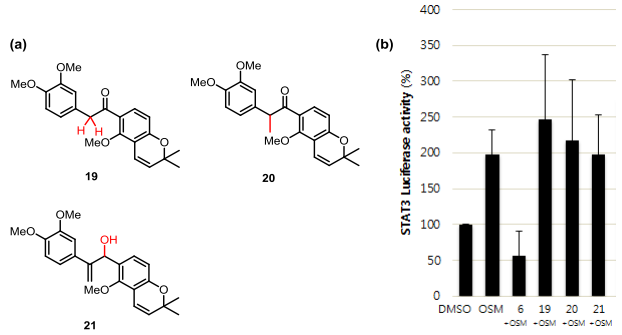


Figure 4. (a) Chemical structures of the analogs without a Michael acceptor. (b) Effects of structural modifications on STAT3 transcription activity. HeLa/STAT3-luc cells were pretreated with DMSO or each analog (10 μM) for 24 h, stimulated with oncostatin M (OSM, 10 ng/mL) for 5 h, and then assayed for the luciferase reporter gene activity.

To investigate the mode of action for **6**, STAT3 phosphorylation was examined by Western blot analysis using H-ras transformed MCF10A (MCF10A-*ras*) human breast epithelial cells, which seemed to serve as an adequate model for studies on mammary carcinogenesis (Figure 5). Enone **6** exhibited suppression of STAT3 phosphorylation while **19** and **20**, which are devoid of enone system, showed no suppressive effect on STAT3 phosphorylation.

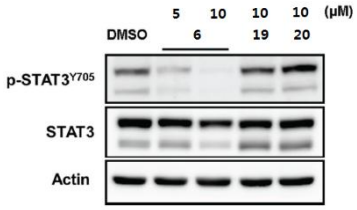
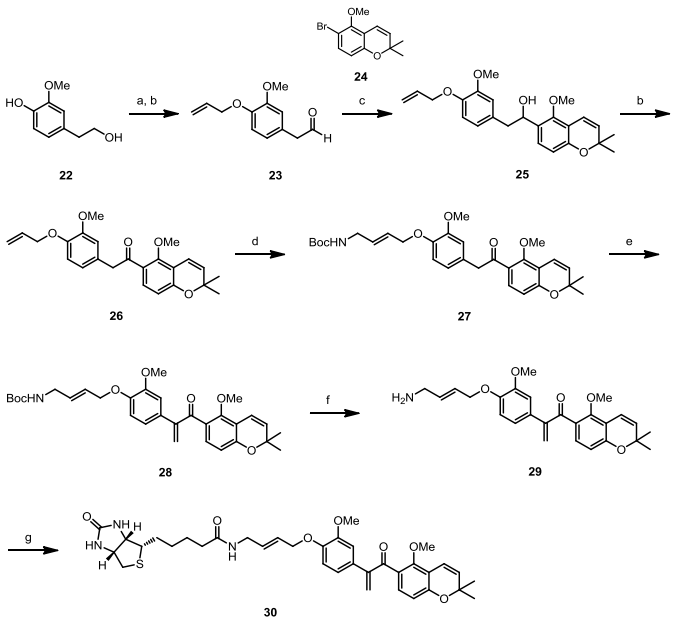


Figure 5. Immunoblot analysis on STAT3 phosphorylation inhibition by **6**. MCF10A-*ras* cells were treated with 5 and 10 μM of **6** and with 10 μM of **19** and **20** for 24 h. (b) Effect of the thiol reducing agents on the suppression of STAT3 phosphorylation by **6**. MCF10A-*ras* cells were treated with 0.5 mM of DTT for 1 h followed by treatment with 10 μM of **6** for 24 h.

In order to confirm direct interaction between STAT3 and **6**, we prepared biotinylated-**6** (**30**) as shown in Scheme 3. Allylation of phenol **22** followed by Dess-Martin oxidation of the alcohol produced aldehyde **23**. Anionic addition of benzopyran **24**[6b] to aldehyde **23** followed by oxidation of the resulting alcohol (**25**) yielded ketone **26**. Cross-metathesis (CM) of **26** with *tert*-butyl *N*-allylcarbamate using Grubbs' second generation catalyst afforded the Boc-protected amine **27**. Aldol condensation of **27** with paraformaldehyde produced enone **28**, which was treated with hydrochloric acid to yield amine **29**. The crude amine was directly treated with Hünig base and then with biotin, activated with *N*-hydroxysuccinimide, to produce **30**. Finally, binding of **30** to STAT3 was confirmed by immunoblot analysis as shown in Figure 6. From these results, the inhibition of the STAT3 transcriptional activity by **6** through the disruption of dimerization and translocation was concluded.



Scheme 3. Reagents and conditions: (a) allyl bromide, K_2CO_3, acetone, reflux, 93%; (b) Dess-Martin periodinane, $NaHCO_3$, CH_2Cl_2, 0 °C to rt, **23**: 69%, **26**: 70%; (c) **24**, *n*-BuLi, THF, -78 °C to rt, 55%; (d) *tert*-butyl *N*-allylcarbamate, Grubbs II catalyst, CH_2Cl_2, reflux, 40%; (e) $(CH_2O)_n$, K_2CO_3, DMF, rt, 72%; (f) 4*N* HCl in dioxane, CH_2Cl_2, rt; (g) (+)-biotin *N*-hydroxysuccinimide ester, iPr$_2$NEt, DMF, rt, 30% for 2 steps.

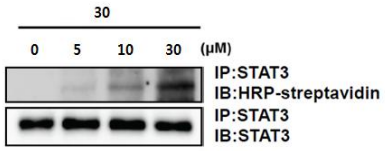


Figure 6. Immunoblot analysis of biotin-conjugate **30** binding to STAT3. MCF10A-*ras* cells were incubated with **30** for 24 h. The binding of **30** to STAT3 was detected using HRP-streptavidin.

Next, we investigated the selective inhibition of STAT3 by **6** and subsequent induction of cell death in human breast cancer cell lines. As anticipated, Western blot analysis shown in Figure 7 supported that enone **6** selectively inhibited phosphorylation of STAT3 but not STAT1.

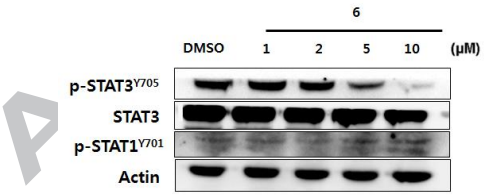


Figure 7. Immunoblotting analysis for the selective inhibition of pSTAT3 by enone **6**. MCF10A-*ras* cells were treated with **6** of the indicated concentrations for 24 h, and the expressions of p-STAT3, STAT3 and p-STAT1 were analyzed by Western blot analysis.

Finally, the anti-proliferative effects of the identified STAT3 inhibitors were examined on the untransformed MCF10A and MCF10A-*ras* cells. As shown in Figure 8, enone **6** exhibited selective cytotoxic effects to MCF10A-*ras* cells at 2 μM after 24 h of treatment, whereas other enones were not cytotoxic in both cells. Moreover, enone **6** showed effective suppression in the viability of MDA-MB-231 cells rather than MCF7 cells (Figure 9). Considering that both MCF10A-*ras* and MDA-MB-231 cell lines express high levels of pSTAT3 whereas MCF10A and MCF7 cell lines express lower levels of pSTAT3,[10] a possible mechanism for the selective cytotoxicity of **6** in cancer cells rather than in normal cells is likely due to the enhanced expression of STAT3 in cancer cells, leading to a greater reduction in cell proliferation by enone **6**.

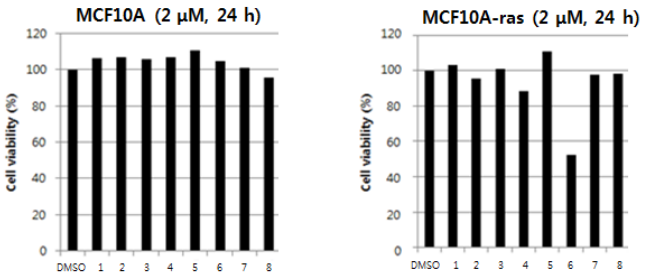


Figure 8. Evaluation of the cytotoxicities in MCF10A and MCF10A-*ras* cells by the identified STAT3 inhibitors. Both cells were treated with each inhibitor for 24 h.

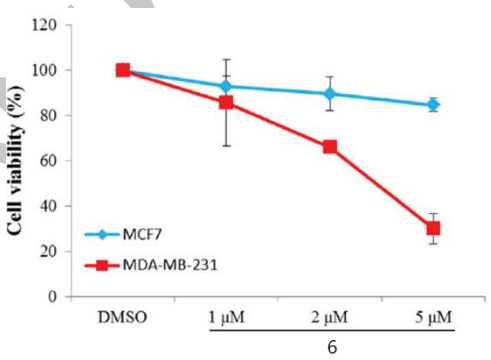


Figure 9. Effect of enone **6** on the viability of MCF7 and MDA-MB-231 cells. Both cells were treated with **6** at the indicated concentrations for 24 h.

In summary we report the discovery of novel STAT3-selective inhibitors through the screening of a focused library. In particular, enone **6** exhibited a promising inhibition in STAT3-driven luciferase expression in HeLa cells. We confirmed that the enone moiety of **6** is essential for the direct interaction with STAT3 via Michael addition. Most notably, enone **6** selectively inhibited the activation of STAT3 without affecting STAT1 and showed selective viability suppression in human breast cell line harboring constitutively active STAT3. Intensive studies on enone **6** including elucidation of its precise inhibition and development of more potent STAT3 inhibitors based on the current results are making good progress.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant for the Global Core Research Center (GCRC) funded by the Korea government (MSIP) (No. 2011-0030001) and the National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MSIP) (No. 2013R1A1A2063282).

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Graphical Abstract

Identification of small molecule inhibitors of the STAT3 signaling pathway: Insights into their structural features and mode of action

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