Antiproliferative activities and SAR studies of substituted anthraquinones and 1,4-naphthoquinones

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Antiproliferative activities and SAR studies of substituted anthraquinones and 1,4-naphthoquinones

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ABSTRACT

STAT3 is constitutively active in a large variety of cancers. The search for STAT3 inhibitors led to the discoveries of LLLs 3 and 12, which are substituted anthraquinones. LLL12 is an extremely potent compound that exhibits high levels of antiproliferative activity. Herein the synthesis and evaluation of compounds containing either an anthraquinone or 1,4-naphthoquinone moiety are reported. Analogs were evaluated in several cancer cell lines. Interestingly, it was found that the anthraquinones did not follow the same trends as the 1,4-naphthoquinones in regards to potency. LLL12, which contains a sulfonamide at position 1, was found to be the most potent of the anthraquinones. In contrast, the methyl ketone and methyl ester derivatives (LLLs 3.1 and 5.1) were found to be the most potent of the 1, 4-naphthoquinones. Selected 1,4-naphthoquinones were also evaluated in the STAT3 fluorescence polarization assay in order to evaluate their abilities to bind to the STAT3 SH2 domain. They were found to have similar affinities, and their activities suggest that STAT3 is one of their molecular targets.

Signal transducer and activator of transcription 3 (STAT3) has been implicated in numerous solid tumors and hematological malignancies.[1] It is activated in response to a number of cytokines and hormones.[2–5] Following the phosphorylation of a specific tyrosine residue, the STATs undergo homo- and/or hetero-dimerization via the reciprocal interactions of the phosphotyrosine residues and Src homology 2 (SH2) domains, translocate to the nucleus, and activate the transcription of target genes.[6,7] STAT3 is activated upon phosphorylation at Tyr^{705}.[7] It signals as a homodimer or as a STAT1–STAT3 heterodimer.[8]

There currently are not any STAT3 inhibitors on the market, but strategies to target STAT3 have been directed toward its amino-terminal, DNA-binding and SH2 domains. The SH2 domain has received considerable attention, as it is involved in both receptor binding and dimerization. STA-21 was the first reported small molecule inhibitor of the STAT3 SH2 domain.[9] However, STA-21 is structurally complex, making the synthesis of STA-21 analogs for structure–activity relationship (SAR) studies difficult. As a result, the structure was simplified in the design and synthesis of the initial LLL compounds.[10] The anthraquinone moiety was retained, while the fourth ring was discarded.[10] The most potent analog identified was LLL3, which was subsequently evaluated in glioblastoma.[11] LLL3 was shown to inhibit STAT3 DNA-binding in both U373 and MDA-MB-231 cells.[11] Further structural modifications led to the discovery of LLL12, which is a potent small molecule inhibitor of STAT3 (Fig. 1).[12] Over the past few years, LLL12 has been extensively studied.[13–23]

Recently, the antiproliferative activities of substituted 1,4-naphthoquinones were reported.[24] Some of the most potent compounds contained a sulfonamide substituent (PDs 13–15),[24] which is also present in LLL12. The compounds were found to inhibit the STAT3 fluorescence polarization (FP) signal.[24] The K_i values of PD9 and PD18 were derived to be 13.3 ± 0.6 and 18.2 ± 4.8 μM, respectively (Table 1).[24] Herein the synthesis and biological evaluation of additional anthraquinone- and 1,4-naphthoquinone-containing analogs that target STAT3 is reported.

The majority of the compounds contain the anthraquinone moiety (Table 2). They were substituted at the 1, 2, 5 and 8 positions. Position 1 was substituted with ester, amide, acid or sulfonamide groups. Positions 5 and 8 were substituted with hydroxyl or ether groups. This allowed for the capacity for hydrogen bond

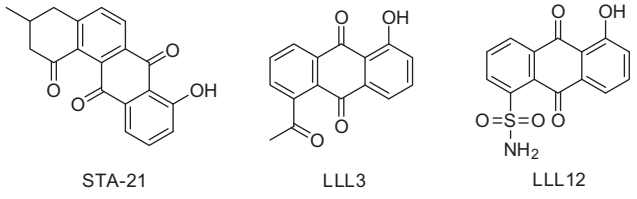

Figure 1. Structures of STA-21, LLL3 and LLL12.

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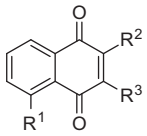
Table 1
Structures of previously reported 1,4-naphthoquinones

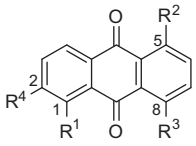


Compound	R^1	R^2	R^3
PD9	OH	$S(CH_2)_2OH$	H
PD13	SO_2NH_2	H	$NHPh(3\text{-}OCH_3)$
PD14	SO_2NH_2	H	NHPh
PD15	SO_2NH_2	H	$NHPh(4\text{-}OCH_3)$
PD18	OH	CH_3	$S(CH_2)_2OH$

acceptance/donation at the two positions and its effects on activity to be studied. With the exception of LLL15, which was substituted at the 2 position, all of the anthraquinones were substituted at either the 5 or 8 position, but not at both. The majority of the 1,4-naphthoquinones were generated as intermediates in the syntheses of the anthraquinones (Table 3). They are less structurally complex than the corresponding anthraquinones. Since they lack the third ring, the contributions to activity of only the substituted 1,4-naphthoquinone portions were able to be evaluated.

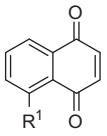
The synthetic procedures for several of the LLL compounds have been previously reported (LLLs 3, 3a, 3.1, 12 and 12.1).[10,12] Many of the novel analogs presented here were synthesized in a similar fashion. The condensation of methyl crotonate with bis(dimethylamino)methoxymethane yielded *tert*-aminodienylester, according to an established procedure (Scheme 1).[25] The combination of the intermediate with juglone produced LLL5 as the single isomer. The ester was then hydrolyzed to produce LLL4. LLL5.1 was synthesized by esterification and subsequent oxidation of 1-naphthoic acid. The oxidation of commercially available 1-naphthalene carboxamide produced LLL6.1, which underwent a regioselective Diels–Alder type reaction with 3-hydroxy-2-pyrone to produce LLL6 (Scheme 2). DCC coupling of LLL4 with β-alanine ethyl ester hydrochloride or glycine methyl ester hydrochloride yielded LLL7 ester or LLL9 ester, respectively, which upon hydrolysis produced LLLs 7 and 9. LLLs 11, 11a, 13 and 13a were produced by the substitution reactions between LLL3 or LLL3a with propyl iodide or benzyl bromide (Scheme 3). Treatment of naphthalene-1-sulfonyl chloride with methylamine hydrochloride produced *N*-methylnaphthalene-1-sulfonamide (Scheme 4). Oxidation and subsequent addition using a reaction protocol that was similar to the synthetic procedure for LLL6 produced LLL14. DCC coupling of β-alanine ethyl ester hydrochloride with anthraquinone-2-carboxylic acid and subsequent hydrolysis of the ester intermediate yielded LLL15.

Table 2
Structures of substituted anthraquinones



Compound	R^1	R^2	R^3	R^4
LLL3	$COCH_3$	OH	H	H
LLL3a	$COCH_3$	H	OH	H
LLL4	COOH	OH	H	H
LLL5	$COOCH_3$	OH	H	H
LLL6	$CONH_2$	OH	H	H
LLL7	$CONH(CH_2)_2COOH$	OH	H	H
LLL7 ester	$CONH(CH_2)_2COOCH_2CH_3$	OH	H	H
LLL9	$CONHCH_2COOH$	OH	H	H
LLL9 ester	$CONHCH_2COOCH_3$	OH	H	H
LLL11	$COCH_3$	OC_3H_7	H	H
LLL11a	$COCH_3$	H	OC_3H_7	H
LLL12	SO_2NH_2	OH	H	H
LLL13	$COCH_3$	OCH_2Ph	H	H
LLL13a	$COCH_3$	H	OCH_2Ph	H
LLL14	SO_2NHCH_3	OH	H	H
LLL15	H	H	H	$CONH(CH_2)_2COOH$

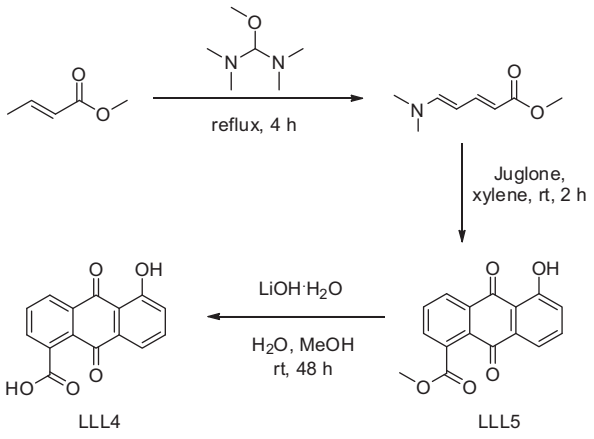
Table 3
Structures of substituted 1,4-naphthoquinones



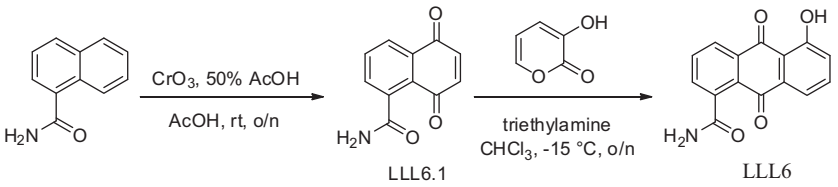
Compound	R^1
LLL3.1	$COCH_3$
LLL5.1	$COOCH_3$
LLL6.1	$CONH_2$
LLL12.1	SO_2NH_2

The antiproliferative and FP assays were carried out according to previously reported procedures.[24] The analogs were evaluated in breast (MDA-MB-231), prostate (DU-145 and PC-3) and colon (HT-29) cancer cell lines at concentrations of 0.5, 5 and 50 μM. The relative potencies of the compounds were fairly consistent across all cell lines (Supplementary Fig. S1–S4). However, there were a few exceptions. At a concentration of 50 μM, LLLs 9 and 10 were more active in DU-145 and HT-29 cells. At a concentration of 5 μM, this trend was also observed for LLL14. LLLs 13 and 12.1 were less active in HT-29 cells. Some compounds, including LLLs 4, 7, 7 ester, 11, 11a and 15, showed minimal activity in all cell lines at all tested concentrations. In contrast, with the exception of LLL12.1 in HT-29 cells, all of the substituted 1,4-naphthoquinones (LLLs 3.1, 5.1, 6.1 and 12.1) nearly abrogated cell growth at a concentration of 50 μM. LLL12 was by far the most active compound in all cell lines.

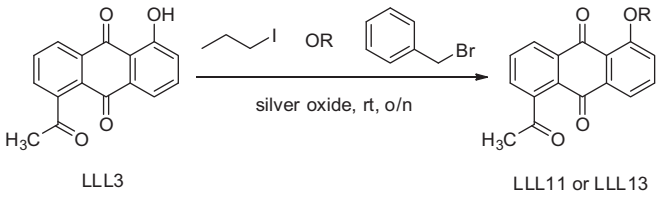
The IC_{50} values of the analogs were also determined in HT-29 and DU-145 cell lines (Tables 4 and 5). The derived values were in line with the observed activities at 0.5, 5 and 50 μM. LLL12 was the most active compound in both cell lines, achieving IC_{50} values of <200 nM. LLL14 was the next most potent compound of those that featured an anthraquinone moiety. However, the methylation of the sulfonamide was clearly not advantageous, as LLL14 was roughly 40 to 60 times less potent than LLL12. Interestingly,



Scheme 1. Syntheses of LLLs 4 and 5.



Scheme 2. Syntheses of LLLs 6 and 6.1.

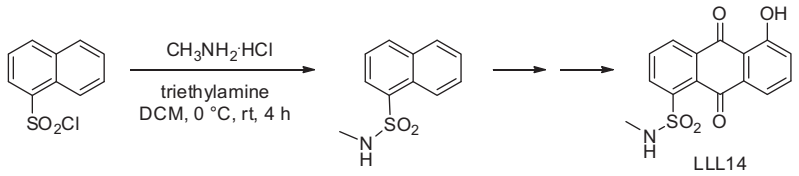


Scheme 3. Syntheses of LLLs 11, 11a, 13 and 13a. The syntheses of LLLs 11 and 13 are shown as examples.

LLLs 3 and 3a exhibited similar potencies in both cell lines. This suggests that there is flexibility as to the position of the hydroxyl substituent, at least as it concerns the methyl ketone analogs. However, it is apparent that having a hydrogen bond donor is more advantageous than substituting with ether substituents, as LLLs 11, 11a, 13 and 13a exhibited no antiproliferative activity. A lack of activity was observed regardless of whether the ether was at the 5 or 8 position, or the identity of the substituent was a propyl ether or benzyl ether. In comparing LLLs 3-6, 7, 7 ester, 9, 9 ester and 12, it was found that besides the sulfonamide moiety, the methyl ketone substituent at position 1 (LLL3) was the most active. Substitution with a carboxylic acid at this position (LLL4) resulted in a total loss of activity. The presence of a methyl ester at this location was not advantageous, as LLL5 was approximately four times less active than LLL3. Amide substitution was somewhat tolerated; LLL6 was roughly half as potent as LLL3. Extending the amide of LLL6 with an acetic acid substituent did not appear to alter activity (LLL9), while extension with methyl acetate resulted in a threefold reduction in activity (LLL9 ester). The presence of larger substituents resulted in total losses of activity (LLLs 7 and 7 ester).

The activities of the four 1,4-naphthoquinones did not follow the same pattern (Table 4). The major difference was that the methyl ketone and methyl ester derivatives were the most active (LLLs 3.1 and 5.1) of the 1,4-naphthoquinone series, while the sulfonamide-containing LLL12.1 was among the least active. Also, LLL6 was more active than LLL5, while LLL5.1 was more active than LLL6.1. LLL6.1, which contains a primary amide substituent, had similar activities to LLL12.1. Interestingly, it also had similar activities to LLL6. In contrast, LLL3.1 was roughly twice as potent as LLL3, and LLL5.1 was four to seven times as potent as LLL5. The 1,4-naphthoquinone derivatives of LLLs 4, 7, 7 ester, 9, 9 ester, 14 and 15 were not evaluated. LLLs 9 and 14 were the only compounds of these that demonstrated noteworthy antiproliferative activities. However, it would be interesting to see if the corresponding 1,4-naphthoquinones would also lack activity, as identical trends were not observed for the analogs of the two series.

Since LLL12 is known to inhibit STAT3 phosphorylation, it was evaluated in the FP assay along with LLLs 3.1, 5.1 and 12.1. As predicted, LLL12 inhibited the FP signal considerably. However, the lone presence of LLL12 in the FP buffer also resulted in high and anomalous FP readings, making it difficult to quantify its ability to inhibit the FP signal. LLLs 3.1, 5.1 and 12.1 all inhibited the FP signal to a similar extent (Fig. 2). This data demonstrates that the evaluated 1,4-naphthoquinone derivatives are able to bind to the STAT3 SH2 domain. However, their comparable activities make it difficult to determine the SARs of these compounds in regards to STAT3 inhibition. The calculated *c*log*P* values for these compounds are all 1.9 or below (ChemDraw Ultra 12.0). As a result, solubility in the aqueous FP buffer was not expected to be a limiting factor in the evaluation of these 1,4-napthoquinones. That proved to be the case.



Scheme 4. Synthesis of LLL14.

Table 4
Antiproliferative activities (IC_{50} values in μM) of the anthraquinones and corresponding 1,4-naphthoquinones in DU-145 and HT-29 cells

R[1]	Anthraquinones			1,4-Naphthoquinones		
	Compound	DU-145	HT-29	Compound	DU-145	HT-29
$COCH_3$	LLL3	11.5 ± 1.8	10.4 ± 0.8	LLL3.1	6.4 ± 0.5	4.2 ± 0.8
	LLL3a	10.2 ± 2.4	8.5 ± 0.6			
$COOCH_3$	LLL5	45.7 ± 7.5	37.3 ± 1.9	LLL5.1	11.3 ± 1.6	5.8 ± 1.4
$CONH_2$	LLL6	17.7 ± 2.2	24.7 ± 1.2	LLL6.1	17.6 ± 4.4	15.6 ± 3.5
SO_2NH_2	LLL12	0.19 ± 0.01	0.11 ± 0.01	LLL12.1	18.1 ± 0.5	18.7 ± 2.9

Cells were plated at 2500 cells/well and allowed to attach overnight. They were then treated with varying concentrations of compounds for 72 h. Antiproliferative activities were determined through use of the MTS assay (Promega). Compounds were tested at least twice in triplicate.[24]

Table 5
Antiproliferative activities (IC_{50} values in μM) of the remaining anthraquinones in DU-145 and HT-29 cells

Compound	DU-145	HT-29
LLL4	>100	>100
LLL7	>100	>100
LLL7 ester	>100	>100
LLL9	19.5 ± 2.3	20.6 ± 1.4
LLL9 ester	70.8 ± 6.5	67.6 ± 9.1
LLL11	>100	>100
LLL11a	>100	>100
LLL13	>100	>100
LLL13a	>100	>100
LLL14	8.7 ± 1.6	6.5 ± 1.4
LLL15	>100	>100

Compounds were evaluated as it is described in Table 4.[24]

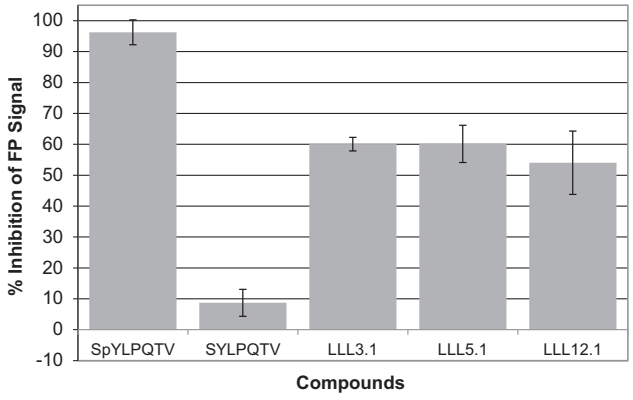


Figure 2. Inhibitory activities of LLL compounds (500 μM) in the STAT3 FP assay. SpYLPQTV and SYLPQTV were used as positive and negative control peptides at a concentration of 100 μM. For all experiments, STAT3 and 5-carboxyfluorescein-SpYLPQTV were used at final concentrations of 150 and 10 nM, respectively. Each compound was tested at least twice in triplicate, according to the previously reported procedure.[24]

The differences in the structural characteristics that led to activity in the anthraquinone and 1,4-naphthoquinone series are intriguing. These data suggest that the analogs of the two series may be binding to biological targets through different interactions. None of the analogs exhibited potencies similar to those of LLL12. However, LLLs 3.1 and 5.1 were found to be relatively potent, which is encouraging. In comparing the potencies of LLL12.1 with PDs 13-15, it is evident that the presence of additional substituents can be advantageous for activity.[24] Therefore, LLLs 3.1 and 5.1 could be functionalized in a similar manner. It is possible that using LLLs 3.1 and 5.1 as lead compounds could result in the discovery of more potent 1,4-naphthoquinones. In addition, further functionalization of the LLL12 core could potentially result in more potent anthraquinones as well.

The sulfonamide portion of LLL12 is predicted to bind to the STAT3 Tyr^{705} site in an interaction that involves at least three hydrogen bonds.[12] The sulfonamide moiety is able to act as both a hydrogen bond acceptor and donor. In contrast, the substituents of the analogs not containing a sulfonamide at this position are only able to act as hydrogen bond acceptors. Therefore, it is intriguing that LLL12.1 is not the most potent of the monosubstituted 1,4-naphthoquinones, at least if one is assuming that these compounds bind to STAT3 in the same way as the anthraquinone analogs. Based on the data obtained from the FP assay, the conclusion can be made that both LLL12 and the 1,4-naphthoquinones are able to bind to the STAT3 SH2 domain. However, it is possible that the smaller sizes of the 1,4-naphthoquinone analogs results in them binding to the SH2 domain with a slightly different geometry. This could make the sulfonamide-containing compounds no longer the tightest binding of the monosubstituted 1,4-naphthoquinones. Still, the 1,4-naphthoquinone derivatives could be acting through additional mechanisms of action (MOAs). Their resulting effects on cellular signaling in cancer cell lines will need to be determined to make further conclusions. Additional studies will hopefully result in the discovery of more potent analogs and further elucidation of their MOA(s).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.09.098.

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