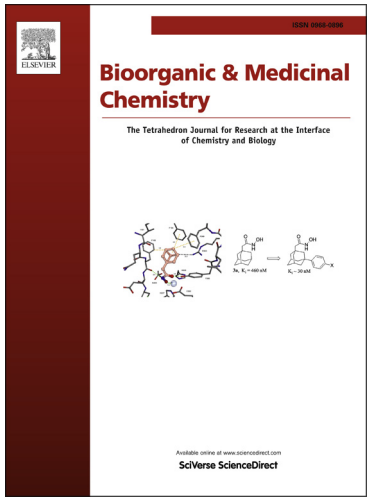

Anticancer activity and SAR studies of Substituted 1, 4-Naphthoquinones

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Anticancer activity and SAR studies of Substituted 1, 4-Naphthoquinones

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Abstract

In this paper, we report the structure activity relationship studies of substituted 1,4-naphthoquinones for its anticancer properties. 1,4-naphthoquinone, Juglone, Menadione, Plumbagin and LLL12.1 were used as lead molecules to design PD compounds. Most of the PD compounds showed improved antiproliferative activity in comparison to the lead molecule in prostate (DU-145), breast (MDA-MB-231) and colon (HT-29) cancer cell lines. PD9, PD10, PD11, PD13, PD14 and PD15 were found to be the most potent compound with an IC_{50} value of 1-3 μM in all cancer cell lines. Fluorescent polarization assay was employed to study the inhibition of STAT3 dimerization by PD compounds. PD9 and PD18 were found to be potent STAT3 dimerization inhibitors.

1. Introduction

1,4-Naphthoquinones are one of the most important and widely distributed chemical class in the quinone family. Chemically, naphthoquinones can generate reactive oxygen species such as superoxides and hydroxyl radicals. In addition, the quinone moiety can also serve as an electrophile and can react with different biological targets in various species including human. 1,4-naphthoquinones are reported to have a variety of pharmacological properties, including antibacterial,[1] antifungal,[2,3] antiviral,[4-6] anti-inflammatory,[7,8] antiartherosclerotic,[9] and anticancer effects.[10-12]

Recently, 1,4-napthaquinones such as Menadione, Juglone and Plumbagin have gained importance for their anticancer activity. Juglone, from its natural source, *Juglans mandshuria,* has been used for the treatment of cancer in traditional Chinese medicine.[13] Plumbagin, derived from the plant genus Plumbago, has been reported to have antibacterial, antifungal as well as anticancer activities. It has been shown to exhibit cytotoxic properties in various cancer cell lines as well as *in vivo* studies in animal models.[14-17] Menadione (Vitamin K3) and its derivatives have been reported to have anticancer effect in hepatic, breast, blood, bladder and oral cancer cell lines.[18]

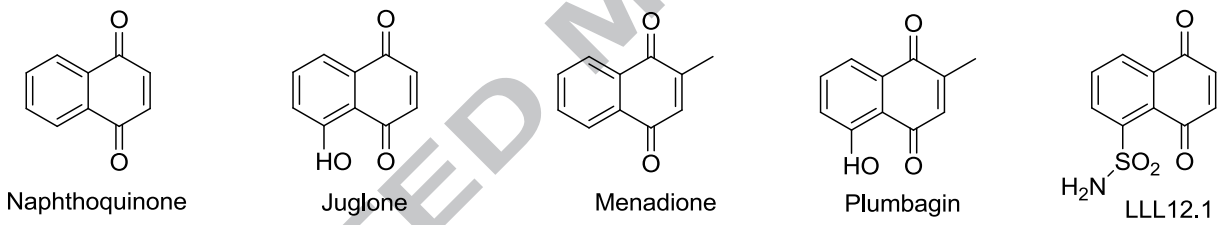


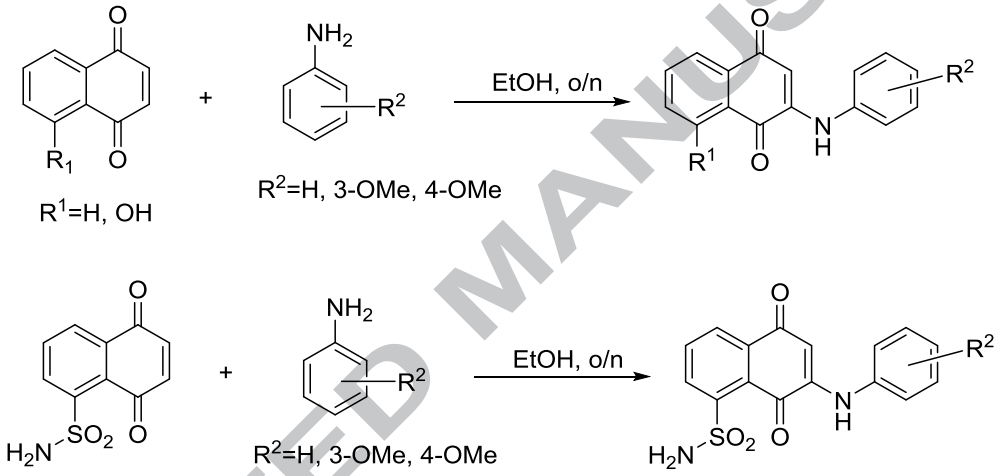
Figure 1. Structure of substituted 1,4-napthaquinones

The goal of this study is to design more potent analogs with 1,4-naphthoquinone scaffold and study the structure activity relationship (SAR) for anticancer activity. 1,4-naphthoquinone, Juglone, Menadione, Plumbagin and LLL12.1 (**Figure 1**) were used as lead molecules.[19] A total of 18 analogs were designed and synthesized, and their antiproliferative activities in prostate (DU-145), breast (MDA-MB-231) and colon (HT-29) cancer cell lines were determined (**Table 1**). The active analogs were further tested for their ability to inhibit STAT3 dimerization using fluorescent polarization (FP) assay.

2. Results and Discussion

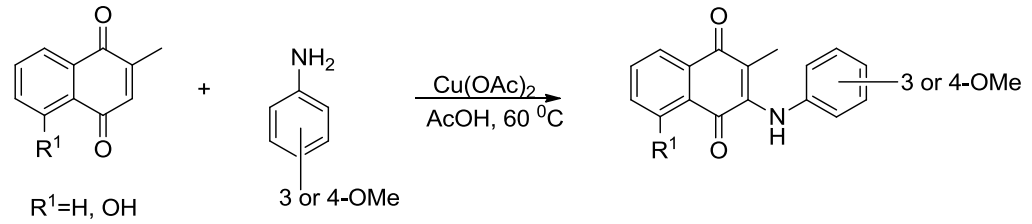
2.1 Chemistry

PD1 and PD2 were designed based on 1,4-naphthoquinone, while Juglone was used to design compounds PD3-5, PD9 and PD10. PD6-8 were designed based on Menadione; PD16-18 were based on Plumbagin and PD11-15 were designed using LLL12.1 as the lead compound. PD1-4, PD6, PD8 and PD18 are known molecules reported in literature,[20-26] while the other PD compounds are novel molecules. PD1-5 synthesis was accomplished by reacting either naphthoquinone or Juglone with aniline or anisidines in ethanol at room temperature (**Scheme 1**) according to reported procedure,[27] but in absence of $CeCl_3$. PD13-15 was synthesized from LLL12.1 using similar reaction conditions.

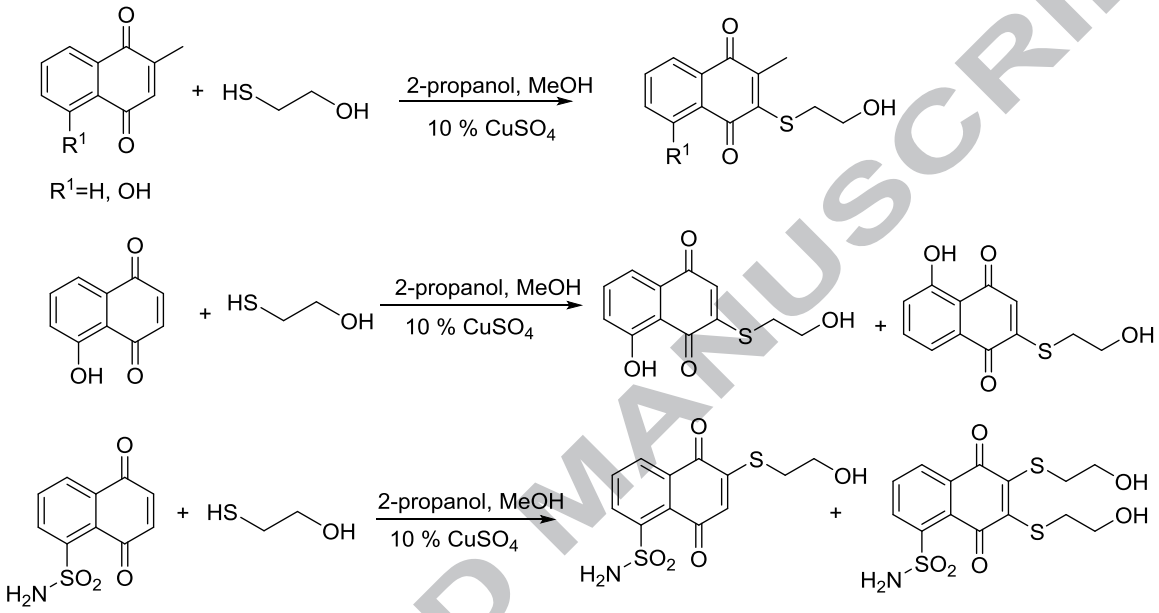


Scheme 1. Synthesis of PD1-5, PD11, PD13-15

Synthesis of PD6, PD7, PD16 and PD17 were carried out by reacting appropriate naphthoquinone with appropriate aniline in acetic acid using 10% copper acetate as catalyst (**Scheme 2**). It has been shown that adding copper acetate in acetic acid readily promotes oxidative coupling of anilines to naphthoquinones.[28] Since plumbagin and menadione didn't give sufficient yield with the earlier reaction protocol in ethanol, we decided to utilize copper chemistry for the synthesis of the compounds. PD8-11, PD12 and PD18 were synthesized by reacting the appropriate naphthoquinone with 2-thioethanol in a solvent mixture of methanol and 2-propanol (**Scheme 3**).



Scheme 2. Synthesis of PD6, PD7, PD16, PD17

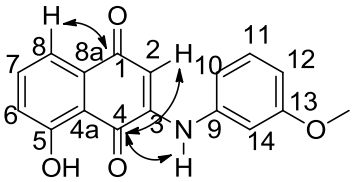


Scheme 3. Synthesis of PD8-12 and PD18

Juglone has been shown to predominantly yield one isomer on reaction with thioglycolic acid.[29] A few examples also exist, where Juglone have been shown to undergo Michael addition at position 2, such as the addition of dimethylamine to Juglone and the reaction of various 2,3-dihalogen juglones with aniline and *p*-toluidine.[22,30,31] The structural assignment of the product obtained from the addition reaction of 5-substituted naphthoquinones was achieved using two-dimensional NMR spectroscopy (2D NMR).

The reaction of various anilines with Juglone predominantly yielded the regioisomer with the addition product at position 3. The other isomer (2-position) was obtained in trace amounts in this case. The structural assignment of PD5 is used to illustrate this point and can be extrapolated as a representation of other Juglone analogs. In the structural assignment of PD5, HMBC correlation is observed between C-1 (δ_c 182.77 ppm) and H-8 (δ_H 7.45 ppm, doublet) which differentiates between C-1 (δ_c 182.77 ppm) and C-4 (δ_c 186.35 ppm). H-8 is also confirmed by COSY, which also confirms H-6 and H-7 by showing correlation to each other. HMBC correlation observed between C-4 and the NH proton (δ_H 9.2 ppm) further confirms the structure of PD5. HMBC correlation is also observed between the

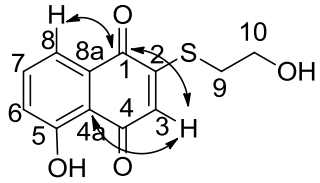
hydrogen bonded carbonyl C-4 and H-2 (δ_H 6.0 ppm), which further confirms this regiochemistry. H-2 also shows HMBC correlation with C-8, C-8a and C-9, respectively (**Figure 2**).



Atoms	δ_H (ppm)	Atoms	δ_C (ppm)
H-2	6.08 (s, 1H)	C-1	182.77
H-6	7.25 (d, 1H)	C-4	186.35
H-7	7.73 (t, 1H)		
H-8	7.45 (d, 1H)		

Figure 2. ^{1}H and ^{13}C NMR assignments of PD5

Reaction of Juglone with thioethanol resulted in both isomers, PD9 & PD10, in almost equal quantity indicating no regioselectivity. HMBC correlation is observed between C-1 (δ_c 182.00 ppm) and H-8 (δ_H 7.56 ppm, doublet) which differentiates between C-1 and C-4 (δ_c 187.42 ppm). HMBC correlation is also observed between C-1 and H-3 proton (δ_H 6.82 ppm) (**Figure 3**). In case of PD10, which is the other regioisomer of PD9, the observed HMBC correlation between C-4 and H-8; and other evidences confirms its structure.

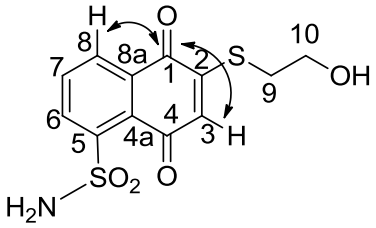


Position	δ_H (ppm)	Position	δ_C (ppm)
H-3	6.82 (s, 1H)	C-1	182.00
H-6	7.36 (d, 1H)	C-4	187.42
H-7	7.76-7.68 (m, 1H)		
H-8	7.56 (d, 1H)		

Figure 3. ^{1}H and ^{13}C NMR assignments of PD9

In case of PD11, HMBC correlation is observed between C-1 (δ_c 181.75 ppm) and H-8 (δ_H 8.29 ppm, doublet). HMBC correlation is also seen between C-1 and H-3 (δ_H 6.90 ppm), which confirms the

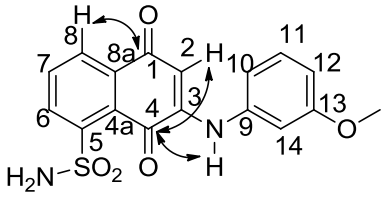
structure of PD13. HMBC correlation of H-7 with C-8a (δ_C 143.5 ppm); and H-3 with C-4a (δ_C 129.96 ppm) further confirms this regiochemistry of PD11 (**Figure 4**).



Position	δ_H (ppm)	Position	δ_C (ppm)
H-3	6.90 (s, 1H)	C-1	181.75
H-6	8.44 (d, 1H)	C-4	181.45
H-7	8.05 (t, 1H)	C-4a	129.96
H-8	8.29 (d, 1H)	C-8a	143.49

Figure 4. ^{1}H and ^{13}C NMR assignments of PD11

In case of PD13, HMBC correlation is observed between C-1 (δ_C 181.86 ppm) and H-8 (δ_H 8.27 ppm, doublet). H-8 is also confirmed by COSY, which also confirms H-6 (δ_H 8.38 ppm) and H-7 (δ_H 8.04 ppm) by showing correlation with each other. HMBC correlation is also observed between C-4 (181.57 ppm) and H-2 (δ_H 6.17 ppm). HMBC correlation observed between C-4 and the NH proton (δ_H 9.49 ppm) further confirms the regiochemistry (**Figure 5**). COSY shows H-11 (δ_H 7.36 ppm, triplet) is coupled with SO_2NH_2 and correlates with H-10 (δ_H 7.0 ppm, doublet) and H-12 (δ_H 6.8 ppm, doublet). As a result, H-14 is merged with H-10 in ^{1}H NMR and appears at 6.96 ppm.



Position	δ_H (ppm)	Position	δ_C (ppm)
H-2	6.17 (s, 1H)	C-1	181.86
H-6	8.38 (d, 1H)	C-4	181.57
H-7	8.04 (t, 1H)		
H-8	8.27 (d, 1H)		

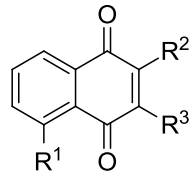
Figure 5. ^{1}H and ^{13}C NMR assignments of PD13

2.2 Biological activity

2.2.1 Antiproliferative activity

PD compounds were tested for their antiproliferative activity in prostate, breast and colon cancer cell lines (**Table 1**). The 1,4-napthaquinone with R^1 substitution as electron donating hydroxyl group (Plumbagin or Juglone) had similar antiproliferative activity when compared to the unsubstituted 1,4-napthaquinone, while R^1 substitution with electron withdrawing sulfonamide group (LLL12.1) led to a decrease in activity in all cell lines. The R^3 substitution of unsubstituted 1,4-napthaquinone with aromatic amine (PD1 & PD2) showed no major change in activity, except in breast cancer cells where decreased activity was observed. The R^3 substitution of Juglone with aromatic amine (PD3-5) increased its antiproliferative activity in both prostate and breast cancer cells, whereas 2-thioethanol substitution at R^2 or R^3 position of Juglone showed an increase in activity across all cell lines. The R^3 substitution of Menadione (PD6-8) with aromatic amines or 2-thioethanol led to a decrease in activity with the exception of 2-thioethanol derivative (PD8), increased activity was observed in breast cancer cells. Similarly, R^3 substitution of Plumbagin (PD16-17) with various anilines led to a decrease in activity in prostate and colon cancer cells. 2-thioethanol substituted Plumbagin derivative (PD18) showed increased activity in breast and colon cancer cells. R^3 substitution of LLL12.1 with aromatic amine (PD13-15) led to a 6-12 fold increase in antiproliferative activity in breast cancer cell lines; and 12-20 folds in colon cancer cells in comparison to LLL12.1. The substitution of LLL12.1 with 2-thioethanol (PD11) at R^2 position led to a moderate increase in activity, while the di-substituted LLL12.1 analog (PD12) showed a decrease in activity. These results indicate the general trend that substitution at either R^2 or R^3 of the 1,4-napthoquinone ring increases antiproliferative activity, while di-substitution at both R^2 and R^3 decreases activity.

LLL12.1 is the precursor of the potent STAT3 inhibitor, LLL12, which binds to the SH2 domain of STAT3 protein and prevent its dimerization.[19] Thus, the selected PD compounds were further evaluated for their abilities to inhibit STAT3 dimerization using fluorescent polarization (FP) assay.



1,4-Naphthoquinone

Compound	R^1	R^2	R^3	DU-145 (IC_{50} µM)	MDA-MB-231 (IC_{50} µM)	HT-29 (IC_{50} µM)
Plumbagin	OH	CH_3	H	5.23 ± 0.24	21.24 ± 0.51	4.19 ± 0.06
Juglone	OH	H	H	11.67 ± 0.05	7.15 ± 1.43	7.71 ± 0.88
1,4-Naphthoquinone	H	H	H	10.89 ± 2.29	4.48 ± 0.11	12.76 ± 1.12
Menadione	H	CH_3	H	9.86 ± 0.22	12.68 ± 0.50	9.69 ± 0.42
LLL12.1	SO_2NH_2	H	H	14.39 ± 0.61	16.13 ± 0.17	24.62 ± 1.40
PD-1	H	H	-NHPh	5.52 ± 1.25	> 100	31.25 ± 3.95
PD-2	H	H	-NHPh(4-OMe)	6.02 ± 1.37	> 100	12.64 ± 0.14
PD-3	OH	H	-NHPh	3.13 ± 0.7	7.41 ± 0.06	10.02 ± 0.99
PD-4	OH	H	-NHPh(4-OMe)	2.85 ± 0.43	2.67 ± 0.14	6.88 ± 0.30
PD-5	OH	H	-NHPh(3-OMe)	2.27 ± 0.38	3.31 ± 0.03	5.51 ± 0.36
PD-6	H	CH_3	-NHPh(4-OMe)	8.12 ± 0.77	>100	19.0 ± 6.65
PD-7	H	CH_3	-NHPh(3-OMe)	15.16 ± 1.78	> 100	> 100
PD-8	H	CH_3	$-SCH_2CH_2OH$	22.3 ± 1.55	3.27 ± 1.09	17.5 ± 2.85
PD-9	OH	$-SCH_2CH_2OH$	H	3.62 ± 0.05	2.66 ± 0.29	2.17 ± 0.59
PD-10	OH	H	$-SCH_2CH_2OH$	3.37 ± 0.29	1.69 ± 0.05	2.11 ± 0.78
PD-11	SO_2NH_2	$-SCH_2CH_2OH$	H	2.85 ± 0.17	9.68 ± 1.50	9.68 ± 1.73
PD-12	SO_2NH_2	$-SCH_2CH_2OH$	$-SCH_2CH_2OH$	19.5 ± 4.75	22.9 ± 0.70	26.6 ± 2.50
PD-13	SO_2NH_2	H	-NHPh(3-OMe)	1.47 ± 0.08	2.22 ± 0.03	1.89 ± 0.14
PD-14	SO_2NH_2	H	-NHPh	2.44 ± 0.23	3.39 ± 0.64	1.97 ± 0.05
PD-15	SO_2NH_2	H	-NHPh(4-OMe)	1.22 ± 0.01	1.73 ± 0.26	1.05 ± 0.08
PD-16	OH	CH_3	-NHPh(4-OMe)	14.22 ± 2.28	21.40 ± 1.64	15.65 ± 11.3
PD-17	OH	CH_3	-NHPh(3-OMe)	10.28 ± 1.64	20.51 ± 0.86	> 100
PD-18	OH	CH_3	$-SCH_2CH_2OH$	8.16 ± 2.35	5.09 ± 1.21	2.16 ± 0.50

Table 1. Antiproliferative activity of PD compounds

2.2.2 Validation of the fluorescence polarization assay

In order to obtain a full saturation curve, increasing concentrations of STAT3 (0-1200 nM) were incubated in the presence of 10 nM 5-FAM-SpYLPQTV. The K_d value was determined to be 136 nM, which is similar to the value of 150 nM obtained by Schust and Berg using the peptide 5-FAM-GpYLPQTV-NH_2.[32] The slight increase in binding that was observed is expected due to the presence of 10% DMSO in the buffer, which has been shown to slightly decrease the K_d value. The unlabeled peptide SpYLPQTV showed greater than 90% inhibition of the FP signal at concentrations as low as 2 µM, with a

calculated IC_{50} of 329 nM, which is similar to the value of 300 nM for the unlabeled peptide GpYLPQTV-NH_2 observed by Zhang and colleagues (**Figure 6**).[33] At a concentration of 100 µM the unlabeled, non-phosphorylated peptide SYLPQTV showed only 9 ± 4% inhibition. The small amount of inhibition observed is presumably due to non-specific binding. These data demonstrates that only the unlabeled, phosphorylated peptide inhibits the binding of the fluorescent probe to the STAT3 SH2 domain, and that the assay is functioning properly.

a) **b)**

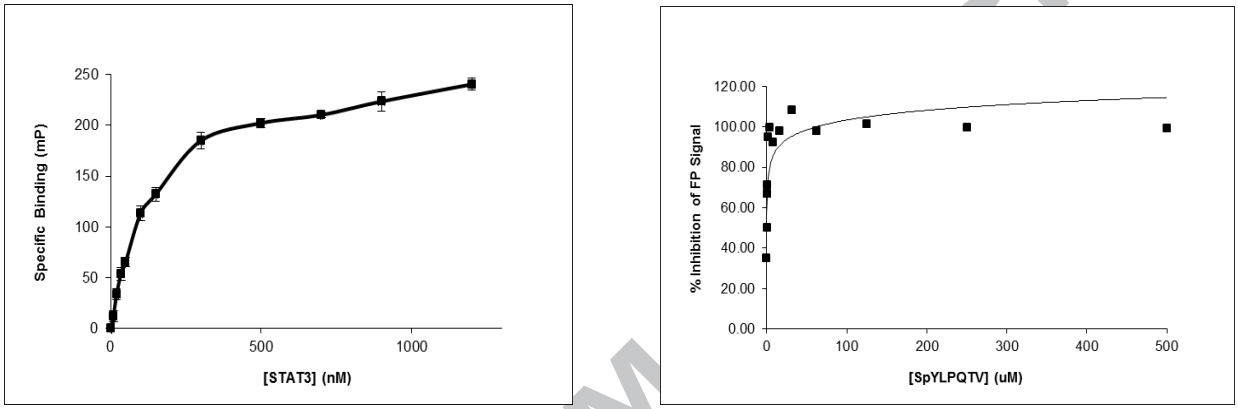


Figure 6. a) Saturation curve: Changes in specific binding that were observed when 10 nM 5-FAM-SpYLPQTV was incubated with increasing concentrations of STAT3. Specific binding is equal to the contribution to the signal of bound ligand. **b)** Percent inhibition of the fluorescence polarization signal when various concentrations of unlabeled peptide (SpYLPQTV) were incubated with 150 nM STAT3 and 10 nM 5-FAM-SpYLPQTV

2.2.3 Inhibition of the fluorescence polarization signal in the presence of compounds

PD4 and PD5 were almost entirely insoluble in the fluorescent peptide buffer at a concentration of 500 µM (which explains their lack of activity) while PD7, PD13 - PD17 were partially insoluble. Other compounds had no solubility issues at the pre-mentioned concentration. PD9 and PD13 had slight issues with internal fluorescence at the excitation/emission settings that were used.

PD compounds were first evaluated at 500 µM. At this concentration four of the compounds (PD9, PD10, PD11 and PD18) inhibited the signal by greater than 50 percent and had comparable activity as that of Plumbagin and Juglone (**Figure 7**). PD4, PD5, PD13, PD14 and PD15 had potent activity in the cellular assays, but in contrast showed lower inhibitory activities in the FP assay. Their lack of solubility in the buffer could explain the reduced potency in the assay. PD9 and PD18 were selected for further evaluation due to their potent activity in the cellular assays and their suitability for the FP assay. The K_i

values of PD9 and PD18 were derived to be 13.3 ± 0.62 µM and 18.2 ± 4.78 µM, respectively, which is similar to the activities of other potent compounds that have been reported through the use of this assay.[33-35] This data demonstrates the ability of these compounds to bind to the STAT3 SH2 domain and inhibit dimerization.

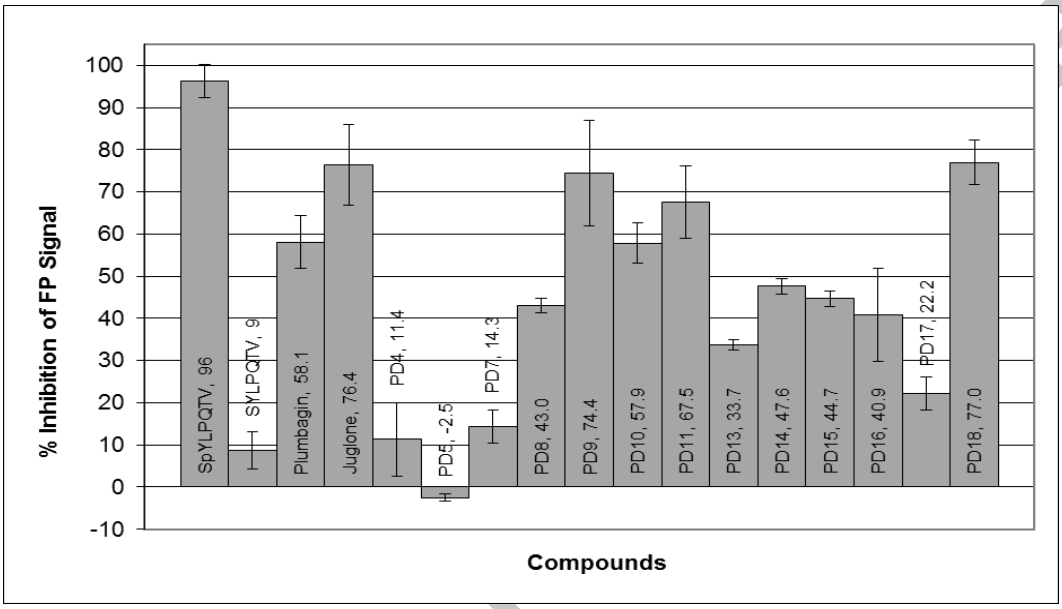


Figure 7. Percent inhibition of the fluorescence polarization signal in the presence of the indicated compounds (500 µM), SpYLPQTV (100 µM), or SYLPQTV (100 µM)

3. Conclusion

In summary, we have synthesized a series of 1,4-naphthoquinone analogues with improved antiprofilerative activity. PD9, PD10, PD11, PD13, PD14 and PD15 were found to be the most potent compound with an IC_{50} value of 1-3 µM in all cancer cell lines. The FP assay demonstrates STAT3 binding and inhibiton of STAT3 dimerization as a probable mechanism of action for their anticancer activity. PD9 and PD18 were found to be potent STAT3 dimerization inhibitors. Several other substituted anilines can be explored further in quest for more potent compounds.

4. Experimental

General Aspects: ^{1}H, ^{13}CNMR, COSY, HMBC, HSQC were obtained on Bruker 300 MHz FT-NMR. Chemical shifts (δ) are reported in ppm from an internal TMS standard. Coupling constants are reported in Hz. High resolution-Electron Spray Ionization (HR-ESI) mass spectra were obtained on a Micromass LCT spectrometer. Melting points are uncorrected and were obtained on Hoover capillary melting point.

Chemicals and solvents were obtained from Sigma Aldrich. Unless specified otherwise, all reactions were carried out under nitrogen atmosphere. Silica gel 60 (0.063-0.200mm) from Dynamic Adsorbents was used for gravity column chromatography. Reagent grade solvents were used for column chromatography and were obtained from VWR. Precoated aluminium backed TLC plates with silica gel 60 F254 from Dynamic Adsorbents were used for TLC. General compound visualization for TLC was achieved by UV light and I2 (adsorbed on silica gel in a container). Yields are unoptimized. Plumbagin, Juglone, Naphthoquinone and Menadione were obtained commercially from Sigma Aldrich. LLL12.1 was synthesized using the reported method in literature.[19]

4.1 General synthetic procedure for **PD1-PD5**, **PD13-PD15**: The appropriate naphthoquinone (1.58 mmol) was dissolved in 8 ml of EtOH followed by slow addition of aniline (1.18 mmol) at room temperature. The reaction was stirred overnight. The reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was collected, dried over sodium sulphate, concentrated under reduced pressure and purified using silica gel column chromatography in 30-45% EtOAc/Hexane to afford the pure compounds.

4.1.1. 2-(phenylamino)naphthalene-1,4-dione (PD1): Dark red solid, yield 32%; mp 189-190 ^{O}C (Lit 190-191 ^{O}C);[20] ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (t, *J*=7.9 Hz, 2H), 7.84-7.66 (m, 2H), 7.59-7.54 (m, 1H), 7.45 (t, *J*=8.4 Hz, 2H), 7.29-7.25 (m, 5H), 6.45 (s, 1H). MS (HR-ESI) M+Na calculated 272.0687; Observed 272.0650.

4.1.2. 2-((4-methoxyphenyl)amino)naphthalene-1,4-dione (**PD2)**: Dark red solid, yield 34%; mp 156-158 ^{O}C (Lit 154-158 ^{O}C);[21] ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15-8.09 (m, 1H), 7.77 (t, *J*=7.6 Hz, 1H), 7.68 (t, *J*=8.2 Hz, 1H), 7.46 (s, 1H), 7.21 (d, *J*=8.9 Hz, 2H), 6.97 (d, *J*=8.9 Hz, 2H), 6.25 (s, 1H), 3.86 (s, 3H). MS (HR-ESI) M+Na calculated 302.0792; Observed 302.0740.

4.1.3. 8-hydroxy-2-(phenylamino)naphthalene-1,4-dione (**PD3)**: Dark red solid, yield 30%; mp 218-220 ^{O}C (Lit 228 ^{O}C);[22] ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.58 (s, 1H), 7.65-7.45 (m, 6H), 7.30-7.21 (m, 4H), 6.40 (s, 1H). MS (HR-ESI) M+Na calculated 288.0637; Observed 288.0570.

4.1.4. 8-hydroxy-2-((4-methoxyphenyl)amino)naphthalene-1,4-dione (**PD4)**: Dark red solid, yield 28%; mp 206-208 ^{O}C; ^{1}H NMR (300 MHz, DMSO-d6) δ 11.55 (s, 1H), 9.21 (s, 1H), 7.73 (t, *J*=7.8 Hz, 1H), 7.45 (d, *J*=7.3 Hz, 1H), 7.32-7.23 (m, 3H), 7.02 (d, *J*=8.6 Hz, 2H), 5.85 (s, 1H), 3.78 (s, 3H). MS (HR-ESI) M+Na calculated 318.0742; Observed 318.0694.

4.1.5. 8-hydroxy-2-((3-methoxyphenyl)amino)naphthalene-1,4-dione (PD5): Dark red solid, yield 27%; mp 174-176 ^{O}C; NMR (400 MHz, DMSO-d6) δ 11.53 (s, 1H), 9.21 (s, 1H), 7.73 (t, *J*=8.2 Hz, 1H), 7.45 (d, *J*=7.6 Hz, 1H), 7.35 (t, *J*=7.7 Hz, 1H), 7.25 (d, *J*=7.6 Hz, 1H), 6.97-6.95 (m, 2H), 6.83-6.80 (m, 1H), 6.08 (s, 1H), 3.77 (s, 3H). ^{13}C NMR (400 MHz) δ 186.35, 182.77, 161.31, 160.75, 146.87, 140.00, 138.40, 133.72, 130.95, 123.13, 118.46, 116.53, 115.11, 111.71, 110.44, 103.55, 56.07. MS (HR-ESI) M+Na calculated 318.0742; Observed 318.0703.

4.1.6. 7-((3-methoxyphenyl)amino)-5,8-dioxo-5,8-dihydronaphthalene-1-sulfonamide (PD13): Reddish brown solid, yield 20%; mp 202-204 ^{O}C; ^{1}H NMR (400 MHz, DMSO-d6) δ 9.49 (s, 1H), 8.38 (d, *J*=8.6 Hz, 1H), 8.27 (d, *J*=8.5 Hz, 1H), 8.04 (t, *J*=8.1 Hz, 1H), 7.37 (d, *J*=5.0 Hz, 3H), 6.99-6.97 (m, 2H), 6.82 (d, *J*=7.6 Hz, 1H), 6.17 (s, 1H), 3.77 (s, 3H). ^{13}C NMR (400 MHz, DMSO-d6) δ 181.86, 181.57, 160.76, 147.97, 143.50, 139.94, 135.69, 135.65, 134.35, 133.08, 130.23, 128.47, 116.51, 111.85, 110.46, 102.20, 56.08. MS (HR-ESI) M+Na calculated 381.0521; Observed 381.0435.

4.1.7. 5,8-dioxo-7-(phenylamino)-5,8-dihydronaphthalene-1-sulfonamide (PD14): Reddish brown solid, yield 17%; mp 237-239 ^{O}C; ^{1}H NMR (300 MHz, DMSO-d6) δ 9.55 (s, 1H), 8.38 (d, *J*=8.5 Hz, 1H), 8.28 (d, *J*=8.0 Hz, 1H), 8.05 (t, *J*=8.2 Hz, 1H), 7.56-7.37 (m, 5H), 7.33-7.20 (m, 1H), 6.12 (s, 1H). MS (HR-ESI) M+Na calculated 351.0415; Observed 351.0475.

4.1.8. *7*-((4-methoxyphenyl)amino)-5,8-dioxo-5,8-dihydronaphthalene-1-sulfonamide (PD15): Dark purple solid, yield 18%; mp >250 ^{O}C; ^{1}H NMR (300 MHz, DMSO-d6) δ 9.72 (s, 1H), 8.41 (d, *J*=6.2 Hz, 1H), 8.12-8.00 (m, 2H), 7.34 (d, *J*=9.1 Hz, 2H), 7.05 (d, *J*=7.9 Hz, 2H), 5.98 (s, 1H), 3.79 (s, 3H). MS (HR-ESI) M+Na calculated 381.0521; Observed 381.0518.

4.2. General synthetic procedure for **PD6, PD7, PD16** and **PD17-** The appropriate naphthoquinone (1.26 mmol) was dissolved in 3 ml of glacial acetic acid followed by addition of copper acetate (0.126 mmol) at room temperature. Then appropriate aniline (1.26 mmol) wad added to the reaction mixture and the reaction was heated to 60 ^{O}C in air for 4 hours. The reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was collected, dried over sodium sulphate, concentrated under reduced pressure and purified using silica gel column chromatography in 15-25% EtOAc/Hexane to afford the pure compounds.

4.2.1. 2-((4-methoxyphenyl)amino)-3-methylnaphthalene-1,4-dione (PD6): Red solid, yield 41%; mp 162-164 ^{O}C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18-8.02 (m, 2H), 7.80-7.62 (m, 2H), 7.35 (s, 1H), 6.95 (d,

J=8.7 Hz, 2H), 6.8 (d, *J*=8.7 Hz, 1H), 3.85 (s, 3H), 1.85 (s, 3H). MS (HR-ESI) M+Na calculated 316.0950; Observed 316.0910.

4.2.2. 2-((3-methoxyphenyl)amino)-3-methylnaphthalene-1,4-dione (**PD7)**: Red solid, yield 39%; mp 153-155 ^{O}C; ^{1}H NMR (300 MHz, DMSO-d6) δ 8.64 (s, 1H), 8.01 (t, *J*=7.0 Hz, 2H), 7.88-7.73 (m, 2H), 7.19 (t, *J*=7.6 Hz, 1H), 6.69-6.52 (m, 3H), 3.72 (s, 3H), 1.67 (s, 3H). MS (HR-ESI) M+Na calculated 316.0950; Observed 316.0900.

4.2.3. 5-hydroxy-3-((4-methoxyphenyl)amino)-2-methylnaphthalene-1,4-dione (PD16): Dark purple solid, yield 30%; mp 145-147 ^{O}C; ^{1}H NMR (300 MHz, DMSO-d6) δ 11.57 (s, 1H), 8.62 (s, 1H), 7.70 (*J*=7.9 Hz, 1H), 7.52 (d, *J*=7.9 Hz, 1H), 7.21 (d, *J*=7.9 Hz, 1H), 7.00 (d, *J*=8.7 Hz, 2H), 6.89 (d, *J*=8.7 Hz, 2H), 3.73 (s, 3H), 1.54 (s, 3H). MS (HR-ESI) M+Na calculated 332.0899; Observed 332.0855.

4.2.4. 5-hydroxy-3-((3-methoxyphenyl)amino)-2-methylnaphthalene-1,4-dione (PD17): Dark red color, yield 28%; mp 157-159 ^{O}C; (300 MHz, $CDCl_3$) δ 11.62 (s, 1H), 7.70-7.59 (m, 2H), 7.33 (s, 1H), 7.22 (dd, *J* = 12.4, 8.1 Hz, 2H), 6.69 (d, *J* = 8.3 Hz, 1H), 6.59 (d, *J* = 8.0 Hz, 1H), 6.53 (s, 1H), 3.82 (s, 3H), 1.80 (s, 3H).MS (HR-ESI) M+Na calculated 332.0899; Observed 332.0810.

4.3. General Synthetic procedure for **PD8-PD12** and **PD18-** The appropriate naphthoquinone (2.32 mmol) was dissolved in a mixture of 40 ml MeOH (0.058 M) and 32 ml of 2-propanol (0.072 M) at room temperature. 2-Mercaptoethanol (0.154 ml, 2.20 mmol) was added to the reaction mixture and stirred for 24 hours. The reaction mixture was then poured in 0.019 M of water and extracted with ether. The ether layer was washed with 10% Copper sulfate solution twice and then with water again. The organic layer was collected, dried over sodium sulphate, concentrated under reduced pressure and purified using silica gel column chromatography in 80-100% EtOAc/Hexane to afford the pure compounds.

4.3.1. 2-((2-hydroxyethyl)thio)-3-methylnaphthalene-1,4-dione (PD8): Yellow solid, yield 27%; mp 80-82 ^{O}C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07-7.99 (m, 2H), 7.72-7.63 (m, 2H), 3.80 (t, *J*=5.7 Hz, 2H), 3.34 (t, *J*=5.7 Hz, 2H), 2.78 (bs, 1H), 2.36 (s, 3H). MS (HR-ESI) M+Na calculated 271.0405; Observed 271.0373.

4.3.2. 5-hydroxy-2-((2-hydroxyethyl)thio)naphthalene-1,4-dione (PD9): Yellowish red solid, yield 16%; mp 148-150 ^{O}C; NMR (400 MHz, DMSO-d6) δ 12.13 (s, 1H), 7.76-7.68 (m, 1H) 7.56 (d, *J*=7.4 Hz, 1H), 7.36 (d, *J*=8.7 Hz, 1H), 6.82 (s, 1H), 5.15 (t, *J*=5.6 Hz, 1H), 3.70 (d, *J*=6.0 Hz, 2H), 3.08 (t, *J*=6.0

Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 187.42, 182.00, 161.14, 156.52, 136.88, 132.23, 127.58, 125.55, 120.06,115.16, 59.15, 33.95. MS (HR-ESI) M+Na calculated 273.0198; Observed 273.0164.

4.3.3. 8-hydroxy-2-((2-hydroxyethyl)thio)naphthalene-1,4-dione (PD10): Red solid, yield 15%; mp 135-137 ^{O}C; ^{1}H NMR (400 MHz, DMSO-d6) δ 11.46 (s, 1H), 7.73 (d, *J*=7.8 Hz, 1H), 7.54 (d, *J*=5.3 Hz, 1H), 7.30 (d, *J*=6.8 Hz, 1H), 6.79 (s, 1H), 5.15 (s, 1H), 3.77-3.66 (m, 2H), 3.12-3.00 (m, 2H). MS (HR-ESI) M+Na calculated 273.0198; Observed 273.0149.

4.3.4. 6-((2-hydroxyethyl)thio)-5,8-dioxo-5,8-dihydronaphthalene-1-sulfonamide (PD11): Yellow solid, yield 28%; mp 203-205 ^{O}C; ^{1}H NMR (400 MHz, DMSO-d6) δ 8.44 (d, *J*=7.9 Hz, 1H), 8.29 (d, *J*=8.8 Hz, 1H), 8.05 (t, *J*=7.9 Hz, 1H), 7.38 (s 2H), 6.90 (s, 1H), 3.71 (d, *J*=6.1 Hz, 2H), 3.10 (d, *J*=6.1 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 181.75, 181.45, 153.89, 143.49, 134.75, 134.74, 134.49, 131.27, 129.96, 129.13, 59.27, 33.66. MS (HR-ESI) M+Na calculated 335.9976; Observed 335.9949.

4.3.5. 6,7-bis((2-hydroxyethyl)thio)-5,8-dioxo-5,8-dihydronaphthalene-1-sulfonamide (PD12): 2 eq of mercaptoethanol used. Yellow solid, yield 22%; mp 162-164 ^{O}C; ^{1}H NMR (300 MHz, DMSO-d6) δ 8.28 (d, *J*=8.3 Hz, 1H), 8.17 (d, *J*=8.2 Hz, 1H), 7.95 (s, 1H), 7.43 (s, 2H), 4.89 (dt, *J*=16.2, 5.1 Hz, 2H), 3.59 (d, *J*=5.6 Hz, 4H), 3.23 (dt, *J* =13.4, 6.8 Hz, 4H). MS (HR-ESI) M+Na calculated 411.9959; Observed 411.9915.

4.3.6. 5-hydroxy-3-((2-hydroxyethyl)thio)-2-methylnaphthalene-1,4-dione (PD18): Yellow solid, yield 18%; mp 87-90 ^{O}C; ^{1}H NMR (300 MHz, DMSO-d6) δ 11.74 (s, 1H), 7.70 (t, *J*=8.0 Hz, 1H), 7.51 (d, *J*=7.2 Hz, 1H), 7.30 (d, *J*=8.4 Hz, 1H), 4.90 (t, *J*=5.2 Hz, 1H), 3.59 (q, *J*=5.3 Hz, 2H), 3.22 (t, *J*=6.1 Hz, 2H), 2.26 (s, 3H). MS (HR-ESI) M+Na calculated 287.0354; Observed 287.0293.

4.4. Biological activity

4.4.1 Cell Lines and Culture

Prostate (DU-145), breast (MDA-MB-231) and colon (HT-29) cancer cell lines were obtained from American Type Culture Collection. HT-29 and DU-145 cells were cultured in complete RPMI 1640 (Gibco, Invitrogen, Grand Island, NY) + 10% FBS (GIBCO), 2mM L-glutamine (GIBCO) and 1% gentamicin (GIBCO). MDA-MB-231 cells were cultured in DMEM F-12 + 10% FBS, 2mM L-glutamine and 1% gentamicin.

4.4.2 Anitproliferative assay

Cells were cultured at 37 ^{O}C (5% CO_2). Cells were plated in 96-well plates at a cell density of 2,500 cells per well in 100 µL of media and were allowed to attach overnight. On the following day a step by step serial dilution of the compounds was performed, and compounds were added to the cells (to bring the total to 200 µl per well). The treated cells were then incubated for 72 hours. Cell viability was determined by using the Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation assay (MTS). IC_{50} values were calculated with SoftMax Pro. Each compound was run at least twice in triplets.

4.4.3 Fluorescent polarization assay

4.4.3.1. Materials and Methods

STAT3 protein (>90% purity) was purchased from Abcam (Cambridge, MA). The fluorescent peptide with the amino acid sequence 5-carboxyfluorescein-SpYLPQTV (5-FAM-SpYLPQTV) (95.1% purity) was prepared by Genscript (Piscataway, NJ). The unlabeled peptide with the amino acid sequence SpYLPQTV (98.7% purity) was prepared by Genscript (Piscataway, NJ). The unlabeled peptide with the amino acid sequence SYLPQTV (98.4% purity) was prepared by Genscript (Piscataway, NJ). The fluorescent peptide buffer conditions were 10 mM HEPES, 50 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% DMSO, pH 7.6.

4.4.3.2. Fluorescence polarization assay development and optimization

The STAT3 FP assay was developed similarly to what has been previously reported.[32,36] Buffer conditions, similar to those of Schust and colleagues were applied in this assay.[35] The fluorescent peptide and unlabeled peptide used in this assay are identical to those used by Hao and colleagues. The assay was performed in black 384-well microplates (Perkin Elmer, Waltham, MA) in total volumes of 25 µL per well. Measurements were taken with a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA). The FP values were recorded using an excitation filter at 480 nm and an emission filter at 530 nm. The final concentration of fluorescent peptide used for binding experiments was 10 nM; which is identical to what has been previously reported.[32-35,37] To evaluate inhibitors, the compounds (diluted in buffer from stock solutions) were incubated at room temperature at the indicated concentrations with STAT3 protein (final concentration of 150 nM) in buffer for at least 60 minutes. The fluorescence probe was then added and the mixtures were allowed to incubate for at least 30 minutes prior to readings being taken. This method is similar to what has been previously reported.[34-36] In evaluating compounds the experiments were performed at least twice in triplicate. To determine the IC_{50} values, compounds were tested at multiple concentrations (500 to 2 µM) using a 1:2 serial dilution. SoftMax Pro version 5.4.4 (Molecular

Devices, Sunnyvale, CA) was used to determine IC_{50} values via a standard 4-parameter logistic fit. K_i values were determined using the following equation: $K_i = IC_{50} / (1 + ([STAT3] / K_d)$. The unlabeled peptide SpYLPQTV was used as a positive control while the peptide SYLPQTV was used as a negative control.

5. References

1. Huang, S. T.; Kuo, H. S.; Hsiao, C. L.; Lin, Y. L. *Bioorg. Med. Chem.* **2002,** *10*, 1947.
2. Tandon, V. K.; Chhor, R. B.; Singh, R. V.; Rai, S.; Yadav, D. B. *Bioorg. Med. Chem. Lett.* **2004,** *14*, 1079.
3. Sasaki, K.; Abe, H.; Yoshizaki, F. *Biol. Pharm. Bull.* **2002,** *25*, 669.
4. Tandon, V. K.; Singh, R. V.; Rai, S.; Chhor, R. B.; Khan, Z. K. *Boll. Chim. Farm.* **2002,** *141*, 304.
5. Ilina, T. V.; Semenova, E. A.; Pronyaeva, T. R.; Pokrovskii, A. G.; Nechepurenko, I. V.; Shults, E. E.; Andreeva, O. I.; Kochetkov, S. N.; Tolstikov, G. A. *Dokl. Biochem. Biophys.* **2002,** *382*, 56.
6. Da silva, A. J.; Buarque, C. D.; Brito, F. V.; Aurelian, L.; Macedo, L. F.; Malkas, L. H.; Hickey, R. J.; Lopes, D. V.; Noel, F.; Murakami, Y. L.; Silva, N. M.; Melo, P. A.; Caruso, R. R.; Castro, N. G.; Costa, P. R. *Bioorg. Med. Chem.* **2002,** *10*, 2731.
7. Checker, R.; Sharma, D.; Sandur, S. K.; Khanam, S.; Poduval, T. B. *Int. J. Immunopharmacol* **2009,** *9*, 949.
8. Huang, L. J.; Chang, F. C.; Lee, K. H.; Wang, J. P.; Teng, C. M.; Kuo, S. C. *Bioorg. Med. Chem.* **1998,** *6*, 2261.
9. Ding, Y.; Chen, Z. J.; Liu, S.; Che, D.; Vetter, M.; Chang, C. H. *J. Pharm. Pharmacol.* **2005,** *57*, 111.
10. Seshadri, P.; Rajaram, A.; Rajaram, R. *Free Radical Bio. Med.* **2011,** *51*, 2090.
11. Kayashima, T.; Mori, M.; Yoshida, H.; Mizushina, Y.; Matsubara, K. *Cancer Lett.* **2009,** *278*, 34.
12. Lee H. J.; Lee H. J.; Song G. Y.; Li. G.; Lee J. H.; Lu. J.; Kim S. H. *Int. J. Cancer* **2007,** *120*, 2481.
13. Liu, L.; Li, W.; Koike, K.; Zhang, S.; Nikaido, T. *Chem. Pharm. Bull.* **2004,** *52*, 566.
14. Hazra, B.; Sarkar, R.; Bhattacharyya, S.; Ghosh, P. K.; Chel, G.; Dinda, B. *Phytother. Res.* **2002,** *16*, 133.
15. Hafeez, B. B.; Jamal, M. S.; Fischer, J. W.; Mustafa, A.; Verma, A. K. *Int. J. Cancer* **2012,** *131*, 2175.
16. Dandawate, P.; Khan, E.; Padhye, S.; Gaba, H.; Sinha, S.; Deshpande, J.; Venkateswara Swamy, K.; Khetmalas, M.; Ahmad, A.; Sarkar, F. H. *Bioorg. Med. Chem. Lett.* **2012,** *22*, 3104.
17. Sugie, S.; Okamoto, K.; Rahman, K. M.; Tanaka, T.; Kawai, K.; Yamahara, J.; Mori, H. *Cancer Lett.* **1998,** *127*, 177.
18. Lamson, D. W.; Plaza, S. M. *Altern. Med. Rev.* **2003,** 8, 303.
19. Lin, L.; Hutzen, B.; Li, P. K.; Ball, S.; Zuo, M.; DeAngelis, S.; Foust, E.; Sobo, M.; Friedman, L.; Bhasin, D.; Cen, L.; Li, C.; Lin, J. *Neoplasia* **2010,** *12*, 39.
20. Blackhall A.; Thomson A. H. *J. Chem. Soc.* **1953**, 1138.
21. Agarwal N. L.; Schafer W. *J. Org. Chem*. **1980**, *45*, 5139.
22. Thomson, R. H. *J. Org. Chem*. **1948**, *13*, 870-78.
23. Koller A.; Rudi A.; Gravalos M. G.; Kashman Y. *Molecules* **2001**, *6*, 300.
24. Kallmayer H. J. *Arch. Pharm.* **1979**, *312*, 230
25. Chen C.; Liu Y. Z.; Shia K. S.; Tseng H. Y. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2729.
26. Ham S. W.; Lee S. *Bull. Korean Chem. Soc.* **2004**, *25*, 1297
27. Benites, J.; Valderrama, J. A.; Bettega, K.; Pedrosa, R. C.; Calderon, P. B.; Verrax, J. *Eur. J. Med. Chem.* **2010,** *45*, 6052.

28. Lisboa, C. S.; Santos, V. G.; Vaz, B. G.; de Lucas, N. C.; Eberlin, M. N.; Garden, S. J. *J Org Chem.* **2011,** 76, 5264-73.
29. Rothman, F. G. *J. Org. Chem.* **1958,** *23*, 1049-51.
30. Thomson, R. H. *J. Org. Chem*. **1951,** *16*, 1082-90.
31. Thomson, R. H. *J. Org. Chem*. **1948,** *13*, 870-78.
32. Schust, J.; Berg, T. *Anal. Biochem.* **2004,** *330*, 114.
33. Zhang, X.; Yue, P.; Fletcher, S.; Zhao, W.; Gunning, P. T.; Turkson, J. *Biochem. Pharmacol.* **2010,** *79*, 1398.
34. Page, B. D.; Fletcher, S.; Yue, P.; Li, Z.; Zhang, X.; Sharmeen, S.; Datti, A.; Wrana, J. L.; Trudel, S.; Schimmer, A. D.; Turkson, J.; Gunning, P. T. *Bioorg. Med. Chem. Lett.* **2011,** *21*, 5605.
35. Schust, J.; Sperl, B.; Hollis, A.; Mayer, T. U.; Berg, T. *Chem. Biol.* **2006,** *13*, 1235.
36. Hao, W.; Hu, Y.; Niu, C.; Huang, X.; Chang, C. P.; Gibbons, J.; Xu, J. *Bioorg. Med. Chem. Lett.* **2008,** *18*, 4988.
37. Shahani, V. M.; Yue, P.; Fletcher, S.; Sharmeen, S.; Sukhai, M. A.; Luu, D. P.; Zhang, X.; Sun, H.; Zhao, W.; Schimmer, A. D.; Turkson, J.; Gunning, P. T. *Bioorg. Med. Chem.* **2011,** *19*, 1823.

Graphical Abstract

Anticancer activity and SAR studies of Substituted 1,4-Naphthoquinones

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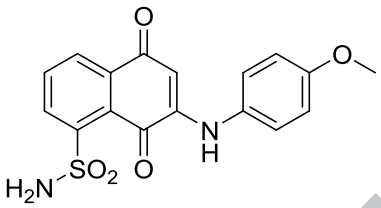

PD15

IC_{50} = 1.05 μM