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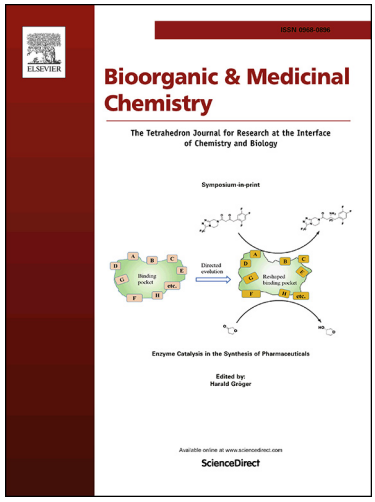

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Novel Tertiary Sulfonamides as Potent Anti-Cancer Agents

Karl J. Okolotowicz[a], Mary Dwyer[a], Daniel Ryan[a], Jiongjia Cheng[a], Emily A. Cashman[a], Stephanie Moore[a], Mark Mercola[b] and John R. Cashman[a,]*

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Keywords: anti-proliferative, Wnt inhibitors, p53 activator, apoptosis, sulfonamides, breast cancer, Doxorubicin synergy, anti-cancer

Abstract. For adult women in the United States, breast cancer is the most prevalent form of cancer. Compounds that target dysregulated signal transduction can be efficacious anti-cancer therapies. A prominent signaling pathway frequently dysregulated in breast cancer cells is the Wingless-related integration site (Wnt) pathway. The purpose of the work was to optimize a "hit" from a screening campaign. 76,000 compounds were tested in a Wnt transcription assay and revealed potent and reproducible "hit," compound **1**. Medicinal chemistry optimization of **1** led to more potent and drug-like molecules, **19**, **24** and **25** (i.e., Wnt pathway IC_{50} values = 11, 18 and 7 nM, respectively). The principal results showed compounds **19**, **24** and **25** were potent anti-proliferative agents in breast cancer cell lines, MCF-7 (i.e., IC_{50} values = 10, 7 and 4 nM, respectively) and MDA-MB 231 (i.e., IC_{50} values = 13, 13 and 16 nM, respectively). Compound **19** synergized anti-proliferation with chemotherapeutic Doxorubicin in vitro. A major conclusion was that compound **19** enhanced anti-proliferation of Doxorubicin in vitro and in a xenograft animal model of breast cancer.

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Abbreviations. Wnt, Wingless-related integration site gene, p53, tumor protein p53, Pd/C, palladium on carbon, HCl, hydrochloric acid, (ER+), estrogen receptor-positive, (ER-), estrogen receptor-negative, (PR-), progesterone receptor-negative, (HER2-), human epidermal growth factor receptor 2-negative, (DMEM), Dulbecco's Modified Eagle's cell culture medium, (FBS), fetal bovine serum

1. Introduction.

In the United States, breast cancer is the most prevalent form of cancer and second most prevalent cause of death for adult women[1, 2]. Common anti-breast cancer therapeutic modalities typically use a combination of surgery, radiation and chemotherapeutics. Currently used chemotherapies include anthracyclines and taxanes (e.g., Doxorubicin and paclitaxel) and are usually used in combination with other chemotherapeutics including fluorouracil and cyclophosphamide[3].

Therapies that target protein components of dysregulated signal transduction pathways can be efficacious anti-cancer therapies with minimal adverse effects[4]. Prominent among dysfunctional breast cancer signal transduction pathways is the Wingless-related integration site (Wnt) pathway[5]. Wnt is named after Int1, a mouse mammary proto-oncogene and Wingless (Wg), a gene from the Drosophila essential for wing development[6]. In adult women, genes for a dysfunctional Wnt pathway have been established in causal association with breast cancer[7]. Currently, targeting the Wnt pathway has been pursued for drug development, but only a few small molecules that modulate the Wnt pathway are approved for treatments, (i.e., Pyrvinium, Celecoxib and Sulindac)[8].

In addition to the Wnt pathway, dysregulation of the tumor protein p53 (p53) pathway is linked to the complexity of cancer biology[9]. Therapies that have targeted p53 have focused on restoration of functional activity of p53, but increases in endogenous p53 causes problems with cell adhesion and maintenance of tissue structure[10, 11].

Previously, a screen of 76,000 small molecules[12] was conducted for inhibition of Wnt transcription[13, 14]. On the basis of results from this screen, one selective and reproducible "hit", (i.e., compound **1**) was identified[15, 16]. In contrast to other Wnt inhibitors described in the literature that inhibit signaling at the Axin level[13, 14, 17], **1** was shown to inhibit Wnt signaling far downstream in the pathway[15]. Herein, we describe the medicinal chemistry and development of potent Wnt inhibitors derived from "hit" **1**. The compounds elaborated were evaluated for Wnt inhibition, inhibition of breast cancer cell proliferation and induction of p53 transcription *in vitro*. Compound

1 was optimized for both potency of inhibition of Wnt and for improvement in physiochemical properties. Based on the results for Wnt inhibition and inhibition of breast cancer cell proliferation, a structure-activity relationship (SAR) arose for the compounds examined. The results led to synthesis of significantly more potent Wnt inhibitors with much improved anti-cancer and physicochemical properties. From SAR optimization of **1**, several compounds emerged (i.e., **19**, **24** and **25**) that showed increased Wnt potency and improved physicochemical properties. Compounds **19**, **24** and **25** were shown to be effective inhibitors of breast cancer cell (i.e., MCF-7 and MDA-MB 231) proliferation. In the presence of Doxorubicin, compound **19** showed increased inhibition of proliferation of breast cancer cells in vitro and in in vivo xenograft studies. Compounds **19**, **24** and **25** are new, potent small molecules with potent anti-breast cancer functional activity.

2. Results and Discussion

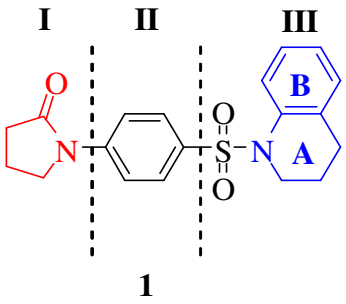
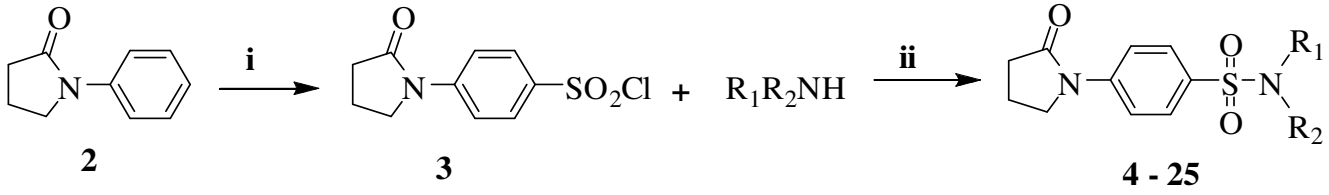


Figure 1. "Hit" **1** identified from a screen for inhibition of Wnt transcription. Regions **I**, **II** and **III** conceptually represent the regions of modification of "hit" **1** for optimization of the molecule.

2.1. Chemical Synthesis

Sulfonamides **4 – 25** were prepared using the synthetic route described in **Scheme 1**. In a typical synthesis, 1-phenylpyrrolidinone, **2**, was directly converted to the *para*-substituted sulfonyl chloride **3** using chlorosulfonic acid. Generally, the crude solid material was isolated in sufficient purity to use without further purification. Sulfonamides **4 - 25** were prepared by addition of the requisite amine to sulfonyl chloride **3**.

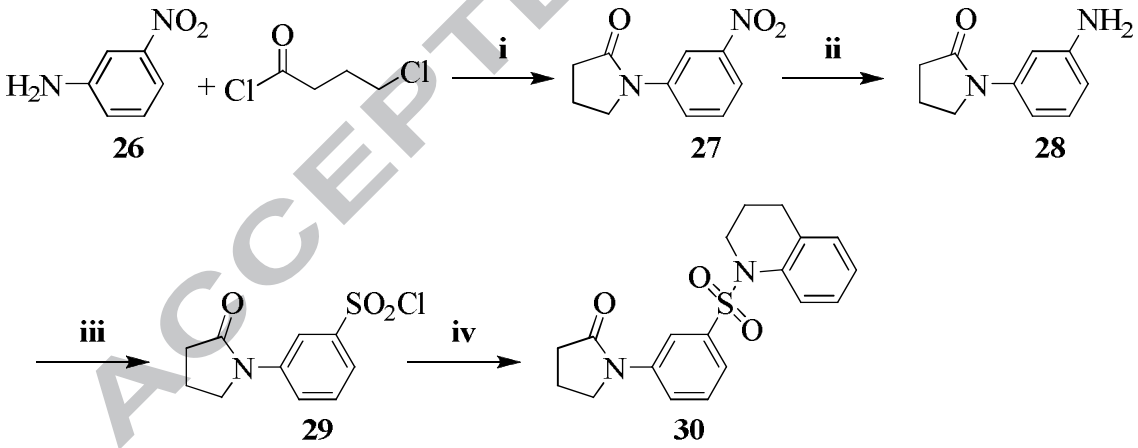
Scheme 1. General synthesis of pyrrolidinone phenylsulfonamides **4** - **25**[a].



[a]Reagents and conditions: **i)** chlorosulfonic acid, 25 °C, 15 h, **ii)** triethylamine, acetonitrile, 130 °C, 30 min.

For the synthesis of *meta*-substituted sulfonamide **30**, a modified synthesis was used that followed literature procedures[18, 19] (**Scheme 2**). 3-Nitroaniline, **26**, was converted to γ-lactam **27** by treatment with 4-chlorobutyryl chloride that was added slowly by addition funnel. In a second step, the crude reaction product was added to a freshly prepared solution of sodium ethoxide in ethanol to afford cyclization and produced a yellow-orange solid, **27**. Hydrogenation of **27** in the presence of Pd/C gave aniline **28**. Aniline **28** was converted to a diazonium salt with sodium nitrite under acidic conditions followed by addition of sulfur dioxide gas and copper (II) chloride dihydrate to afford **29**. Sulfonyl chloride **29** was combined with the requisite amine to provide the *meta*-substituted sulfonamide **30** in a similar manner as described in **Scheme 1**.

Scheme 2. Synthesis of *meta*-substituted phenylpyrrolidinone sulfonamide **30**[a].

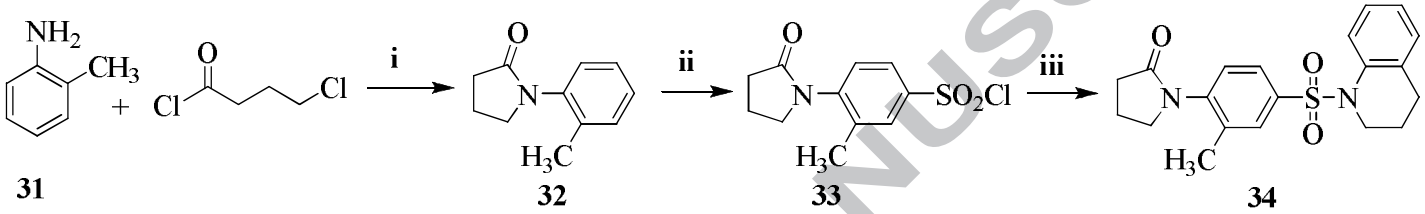


[a]Reagents and conditions: **i**) sodium phosphate dibasic, chloroform, 25 °C, 15 h.; sodium ethoxide, ethanol, 25 °C, 15 h, **ii)** H_2, Pd/C, ethyl acetate, 25 °C, 15 h., **iii**) sodium nitrite, acetic acid/HCl, acetonitrile 0 °C, 1 h.; $SO_{2(g)}$,

copper (II) chloride dihydrate, 0 - 25 °C, 15 h., **iv)** 1,2,3,4-tetrahydroquinoline, triethylamine, acetonitrile, 130 °C, 30 min.

The synthesis of aryl-substituted compounds (e.g., **34**) was done beginning with synthesis of key intermediate **32** (i.e., 2-methylphenyl pyrrolidinone) according to a literature method[19] (**Scheme 3**). Aryl-substituted compound **34** was prepared by combining the sulfonyl chloride with an amine in a similar manner as described in **Scheme 1**.

Scheme 3. Synthesis of aryl-substituted sulfonamide **34**[a].

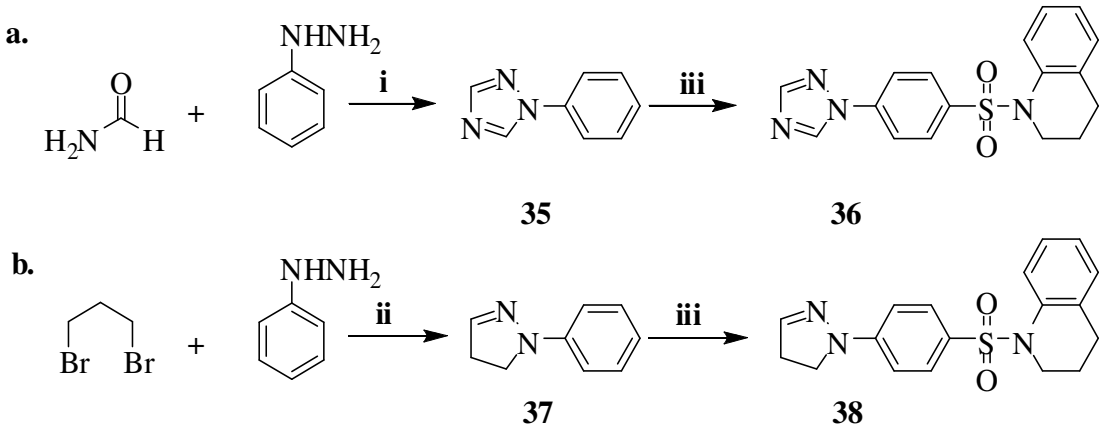


31 **32** **33** **34**

[a]Reagents and conditions: **i**) sodium phosphate dibasic, chloroform, 25 °C, 15 h.; sodium ethoxide, ethanol, 25 °C, 15 h., **ii)** chlorosulfonic acid, 25 °C, 15 h, **iii)**1,2,3,4-tetrahydroquinoline, triethylamine, acetonitrile, 130 °C, 30 min.

Analogs were synthesized to examine the effect of substituents on the heterocyclic ring of the pyrrolidine moiety shown in Region **I** (**Figure 1**). For example, to prepare 1,2,4-triazole analog **36** (**Scheme 4a**), phenylhydrazine hydrochloride and formamide were combined in the absence of solvent in a microwave vial and heated at 175 °C to afford 1-phenyl-1,2,4-triazole, **35**.[20] Intermediate **35** was directly converted to *para*-substituted sulfonyl chloride by treatment with chlorosulfonic acid. Likewise, dihydropyrazole analog **38** (**Scheme 4b**), was prepared. Phenylhydrazine and dibromopropane were combined in the absence of solvent in a microwave vial and heated at 175 °C to provide the dihydropyrazole **37** following a literature procedure.[21, 22] Intermediate **37** was converted to its *para*-substituted sulfonyl chloride by treatment with chlorosulfonic acid. Sulfonamides **36** and **38** were prepared by combining sulfonyl chlorides formed from **35** and **37** with 1,2,3,4-tetrahydroisoquinoline according to the same procedure shown in **Scheme 1**.

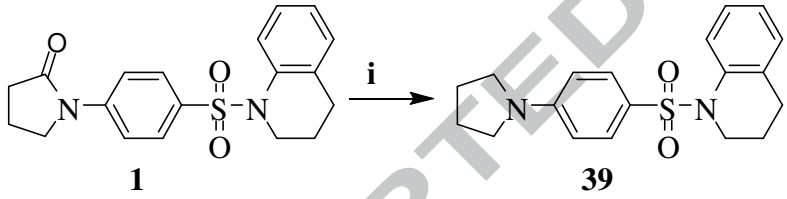
Scheme 4. Synthesis of heterocyclic sulfonamides **36** and **38**[a].



[a]Reagents and conditions: **i)**175 °C, microwave 1.5 h., **ii)**130 °C, microwave 30 min, **iii)** chlorosulfonic acid, 25 °C, 15 h.;1,2,3,4-tetrahydroquinoline, triethylamine, acetonitrile, 130 °C, 30 min.

Pyrrolidine analogs (e.g., compound **39**) were prepared by direct reduction of pyrrolidinone **1** with zinc / triethoxysilane[23] as shown in **Scheme 5**.

Scheme 5. Synthesis of phenylpyrrolidine sulfonamide **39**[a].



[a]Reagents and conditions: **i**) $Zn(OAc)_2$ (10 mol %), $HSi(OEt)_3$, THF, 70 °C, 30 h.

3. Biological studies

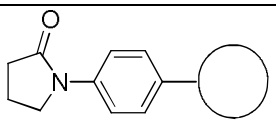
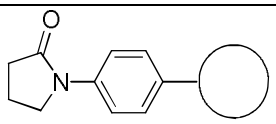
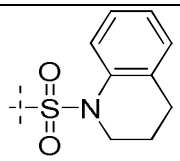
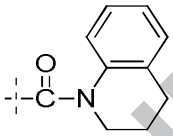
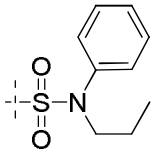
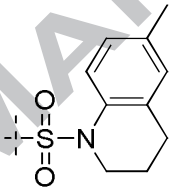
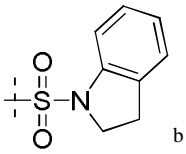
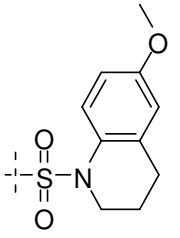
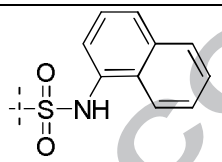
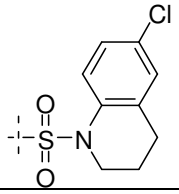
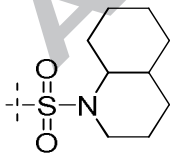
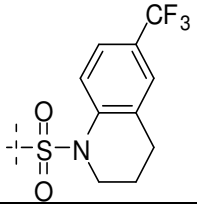
3.1. Effect of Sulfonamides on the Wnt Pathway. A diverse set of 76,000 compounds[24] were tested for inhibition of Wnt transcription in a cell-based assay in HEK293 cells, where cells were transfected with a Wnt luciferase plasmid to over-express Wnt[25]. From a screen for inhibition of Wnt, one reproducible "hit" was found (i.e., compound **1**, IC_{50} = 25 nM). Counterscreens showed none of the compounds examined inhibited luciferase. Based on the structure of "hit" **1**, synthetic analogs **4-25**, **30**, **34**, **36**, **38** and **39** were synthesized and tested for inhibition of Wnt transcription. Each compound was tested in a dose-response study (i.e., 0 – 5 μM, nine

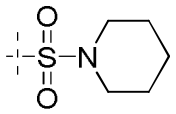
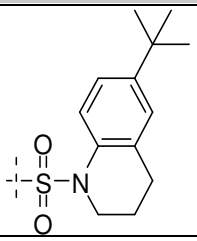
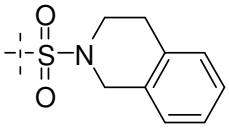
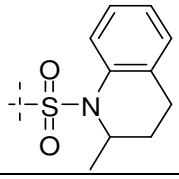
concentrations, in triplicate) to determine an IC_{50} for inhibition of Wnt transcription. To systematically determine a structure-activity relationship (SAR) for inhibition of Wnt transcription and quantify the effect of modification of **1**, the molecule was conceptually divided into three exploratory regions: (**I**) the pyrrolidinone region, (red), (**II**) the central aryl region, (black) and (**III**) the 1,2,3,4-tetrahydroquinoline region, (blue). The 1,2,3,4-tetrahydroquinoline ring (**III**) was further conceptually divided into **A** and **B** rings (**Figure 1**). Initially, a small group of approximately 20 compounds directed at structural modifications of the 1,2,3,4-tetrahydroquinoline portion of **1** (i.e., Region **III**) were synthesized and tested for inhibition of Wnt transcription (**Table 1**). The results of these studies showed that a sulfonyl-1,2,3,4-tetrahydroquinoline moiety was essential to maintain potency. For example, compared to **1**, ring-opening and ring contraction of the **A** ring of Region **III** (i.e., **4** and **5**, respectively, $IC_{50} > 5{,}000$ nM) resulted in loss of potency. Various other unsaturated ring-systems (e.g., **6,** $IC_{50} > 5{,}000$ nM) or saturated ring-systems (e.g., **7** and **8,** $IC_{50} > 5{,}000$ nM, respectively) showed that other ring systems in Region **III** of the molecule decreased potency. To observe the effect of the regio orientation of the 1,2,3,4-tetrahydroquinoline nitrogen in ring system **III**, analog **9** was prepared and tested. Compared to **1**, 1,2,3,4-tetrahydroisoquinoline analog **9** gave decreased potency (IC_{50} = 634 nM). This indicated that the position of the nitrogen atom in the 1,2,3,4-tetrahydroquinoline ring system was essential to maintain maximal potency. The data for **4** – **9**, **Table 1**, showed the 1,2,3,4-tetrahydroquinoline moiety was necessary to maintain potency. Finally, to explore the effect of the sulfonamide moiety on inhibition of Wnt transcription, the analogous amide analog of **1** was prepared (i.e., **10**). Replacement of the sulfonamide moiety with an amide moiety resulted in loss of potency (i.e., **10** had an $IC_{50} > 5{,}000$ nM). This data showed that the sulfonamide moiety of **1** was essential for the potency of the molecule.

To further examine the SAR around region **III**, substituted 1,2,3,4-tetrahydroquinolines were prepared (i.e., compounds **11 – 16**). The effect of small substituents on the aryl portion of the 1,2,3,4-tetrahydroquinoline ring of **1** on inhibition of Wnt transcription showed that both electron-withdrawing or electron-donating substituents showed similar potency (i.e., **11 – 14,** IC_{50} = 7, 6, 13, 54 nM, respectively). Large bulky groups, (e.g., *t*-butyl-substituted compound, **15**, IC_{50} = 660 nM) showed decreased potency compared to **1**. This suggested the potency

of inhibition of Wnt transcription was sensitive to steric constraints and large bulk at this position resulted in low potency. Compared to **1**, a racemic methyl group *ortho* to the sulfonamide moiety on the 1,2,3,4-tetrahydroquinoline was quite potent (i.e., **16**, IC_{50} = 11 nM). This showed *ortho* substituents were well-tolerated at this position (**Table 1**).

Table 1. Effect of substituents of Region **III** of **1** on inhibition of Wnt transcription.

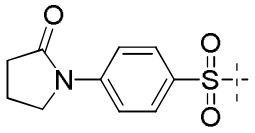
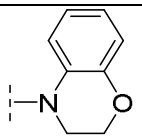
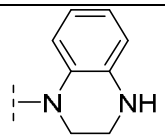
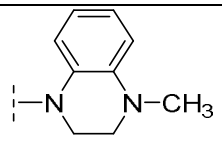
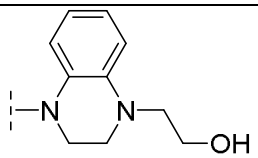
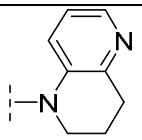
	**Compound**	IC_{50}±Std. Dev. (nM)[a]		**Compound**	IC_{50}±Std. Dev. (nM)[a]
	**1**	25 ± 3		**10**	> 5,000
	**4**	> 5,000		**11**	7 ± 2
	**5**	> 5,000		**12**	6 ± 2
	**6**	> 5,000		**13**	13 ± 2
	**7**	> 5,000		**14**	54 ± 1

	**8**	> 5,000		**15**	660 ± 2
	**9**	634 ± 4		**16**	11 ± 3

[a]IC_{50} is the mean of three determinations ± Std Dev. [b]Commercially available.

Compounds **17** and **19** (i.e., IC_{50} = 10 nM and 11 nM, respectively, **Table 2**) were more potent than **1**. Compared to **1**, compounds **17** and **19** increased the number of potential H-bond donors and increased the aqueous solubility approximately 10-fold. By comparison, the *N*-desmethyl analog, **18**, and 4-hydroxyethyl analog, **20**, showed poor potency for inhibition of Wnt transcription (**Table 2**). Compared to **1**, compound **21** with a 1,5-naphthyridine substructure showed loss of potency (i.e., IC_{50} > 5,000 nM). In summary, compared to **1**, the results from **Table 2** and **Supporting Table S1** showed that incorporation of tertiary amines into the 1,2,3,4-tetrahydroquinoline ring increased aqueous solubility of the molecule more than 10-fold . Data suggested that compared to **1**, compounds **17** and **19** possessed superior physicochemical properties. For example, (i.e., solubility (i.e., 9.5 and 10.5 µg/mL), $logD_{3.0}$/$logD_{7.0}$ (1.7/1.7 and 1.9/1.9) and Calc. tPSA (i.e., 66.9 and 60.9 Å^2) for **17** and **19**, respectively, were superior to compound **1** solubility (2.8 µg/mL), $logD_{3.0}$/$logD_{7.0}$ (2.5/2.5) and Calc. tPSA (i.e., 57.7 Å^2). A small group, (i.e., *N*-methyl), in the 4-position of the 1,2,3,4-tetrahydroquinoline, (i.e., **19**, IC_{50} = 11 nM), afforded considerable potency while improving compound solubility (**Supporting Table S1**). By contrast, 1,5-naphthyridine **21** resulted in loss of potency. However, solubility alone does not explain all the results. For example, compounds **18** and **19** have similar solubilities but vastly different potency. It may be that the nature of the substituent (H-bond donor versus H-bond acceptor) and its interaction with the biological target(s) may play an important role in the overall potency.

Table 2. Effect of modifications of the 1,2,3,4-tetrahydroquinoline moiety on inhibition of Wnt transcription.

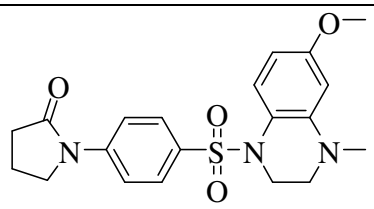
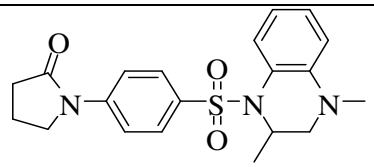
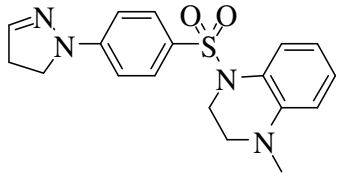
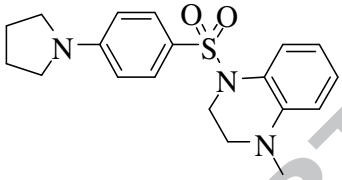
	**Compound**	IC_{50} ± Std. Dev. (nM)[a]
	**17**	10 ± 1
	**18**	>5000
	**19**	11 ± 3
	**20**	> 5000
	**21**	>5000

[a]IC_{50} is mean of three determinations ± Std. Dev.

To further explore the effects of the SAR of analogs of **1**, several analogs were prepared to study the synergistic effects of multiple substituents, for the most potent compounds listed in **Tables 1** and **2**. **Table 3** summarizes the results for **22 – 25**. The 6-methoxy substituent of **12** was combined with the 1,2,3,4-tetrahydroquinoxaline moiety of **19** to provide **22**. Compound **22** was shown to be equipotent as **1** (i.e., IC_{50} = 17 nM for **22** vs IC_{50} = 25 nM for **1**). Compound **23** (i.e., IC_{50} = 9 nM), the conceptual hybridization of **16** and **19**, showed an improvement in potency compared to **1**. The results from **22** and **23** showed C-2 substitutions maintained potency whereas C-6 substitutions of **1** afforded slightly less potency. The conceptual hybridization of **38** and **39** with **19** resulted in **24** and **25**, respectively. Compound **24** was shown to be equipotent to **1**, (i.e., IC_{50} = 18 nM for **24** vs IC_{50} = 25

nM for **1**). In contrast, **25** was shown to be equipotent to **19** (i.e., IC_{50} = 7 nM for **25** vs IC_{50} = 11 nM for **19**). These results showed that a saturated ring in Region **I** was important for potency.

Table 3. Effect of pyrrolidinone and tetrahydroquinoline functionality on inhibition of Wnt transcription.

Structure	**Compound**	IC_{50}±Std. Dev. (nM)[a]
	**22**	17 ± 2
	**23**	9 ± 2
	**24**	18 ± 2
	**25**	7 ± 1

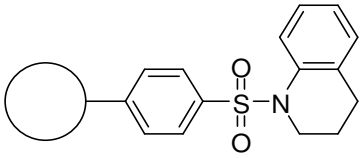
[a]IC_{50} is mean of three determinations ± Std. Dev.

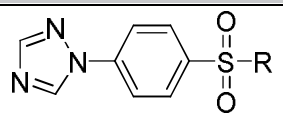
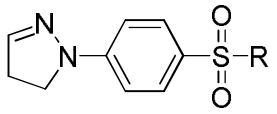
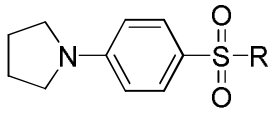
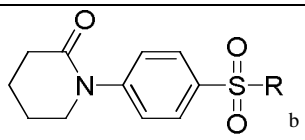
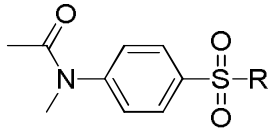
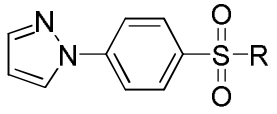
To explore the effect of substitutions of the aryl ring of Region **II,** two analogs were synthesized (i.e., **30** and **34**). Compound **30** (**Scheme 2**) was prepared to investigate the effect of regio-orientation of the aryl Region **II** on potency. The *meta*-substituted sulfonamide **30** was weakly potent (i.e., IC_{50} > 5,000 nM). We conclude that *para*- but not *meta*-orientation of the sulfonamide group was required for potency. Compound **34** (**Scheme 3**) showed very low potency (i.e., IC_{50} > 5,000 nM). The loss of potency of **34** could be due to an *ortho*-methyl group effect on Region **II**. Compared to **1**, the methyl group of **34** may "lock" the molecule in a suboptimal conformation for binding to its biological target due to potential steric interactions between the *ortho*-methyl group and the carbonyl

of the pyrrolidinone ring. In summary, *para*-substituted sulfonamides were required for potency and aryl *ortho*-substituents abolished potency. Accordingly, synthesis and testing of other aryl-substituted compounds was not extensively pursued.

Based on the potency of **1**, **4** and **7**, the 1,2,3,4-tetrahydroquinoline ring was used as a starting point to investigate modification of the pyrrolidinone ring system of Region **I** (**Table 4**). Replacement of the pyrrolidinone ring with a 1,2,4-triazole (i.e., **36**) resulted in loss of potency (i.e., $IC_{50} > 5{,}000$ nM). The partially reduced pyrazole, (i.e., dihydropyrazole **38**, $IC_{50} = 15$ nM) was a potent inhibitor of Wnt transcription. Further work showed chemical reduction of the carbonyl of pyrrolidinone **1** to afford the pyrrolidine **39** maintained potency (i.e., $IC_{50} = 20$ nM). Together, this data showed the carbonyl of the pyrrolidinone ring was not essential for Wnt inhibition potency. The apparent potency of **38** and **39** prompted the preparation of additional ring-opened and ring-closed nitrogen-containing compounds of Region I to examine the effect of heterocycle structure in Region **I** on inhibition of Wnt transcription. Ring expansion of the 5-membered lactam **1** to a 6-membered lactam (**40**) resulted in loss of potency (i.e., **40** = $IC_{50} > 5{,}000$ nM). Synthesis and testing of the ring-opened tertiary amide analog **41** (i.e., $IC_{50} > 5{,}000$ nM) resulted in loss of potency. The results from **40** and **41** showed the necessity of the 5-membered ring system for inhibition of Wnt transcription. Replacement of the pyrrolidine ring in **39** with a pyrazole ring (i.e., **42**) afforded a compound with a significant decrease in potency (i.e., $IC_{50} = 1{,}650$ nM). These results showed that, compared to **1**, introduction of more conjugated systems, (i.e., **36** and **42**), resulted in loss of potency, whereas compounds with saturated or partially saturated ring systems, (i.e., **38** and **39**), showed equal or greater potency. In contrast, ring- expanded or ring-opened analogs in Region **I** of **1** (i.e., **40** and **41**, respectively) resulted in loss of potency (**Table 4**).

Table 4. Effect of modifications of Region **I** on inhibition of Wnt transcription.

	**Compound**	IC_{50}±Std. Dev. (nM)[a]

	**36**	> 5000
	**38**	14 ± 2
	**39**	20 ± 2
 [b]	**40**	> 5000
	**41**	> 5000
	**42**	1650 ± 3

[a]IC_{50} is mean of three determinations ± Standard Deviation (Std. Dev.). [b]Commercially available.

Synthesis and testing of structural analogs centered around Regions **I** – **III** showed: 1) for Region **I**, 5-membered aliphatic heterocycles afforded considerable potency, 2) for Region **II**, a *para*-substitution pattern was essential for potency and 3) for Region **III**, a 1,2,3,4-tetrahydroquinoline moiety gave significant potency. In addition to pharmacological properties, modulation of physicochemical properties were conducted to improve the overall properties of the most potent compounds. For example, the solubility of **1** was increased by incorporation of a tertiary amine HCl salt as in compound **19** and this resulted in increased potency (i.e., IC_{50} = 11 nM, **Table 2**). In addition, compared to **1**, the **A** ring of Region **III** of the 1,2,3,4,-tetrahydroquinoline nucleus permitted small substitutions (e.g., **16**, IC_{50} = 11 nM, **Table 1**) provided that the ring system of the 1,2,3,4-tetrahydroquinoline remained intact.

3.1.1. SAR Summary. In summary, a systematic SAR was developed around analogs of **1** to examine inhibition of Wnt transcription in a cell-based assay. The structural features for potent inhibition of Wnt transcription included: 1) a 5-membered aliphatic heterocycle for Region **I,** 2) a *para*-substituted arylsulfonamide for Region **II** and 3) a 1,2,3,4-tetrahydroquinoline or a 4-methyl-1,2,3,4-tetrahydroquinoxaline ring with small substituents at the 4- or 6-positions of Region **III**. We elected to examine highly potent Wnt inhibitors (i.e., **19**, **24** and **25**) in further in vitro and in vivo breast cancer studies.

3.2. Effects of sulfonamides on Proliferation of Breast Cancer cell lines.

Dysfunction of the Wnt pathway has been linked to proliferation of several types of cancers including colon[26], pancreatic[27] and breast cancer[6, 7,24]. Two human breast cancer cell lines with a dysfunctional Wnt pathway were used for cell-based proliferation studies (i.e., MCF-7 and MDA-MB 231)[28, 29]. MCF-7 breast cancer cells are estrogen receptor-positive (ER+) and human epidermal growth factor receptor 2-negative (HER2-). In contrast, MDA-MB 231 breast cancer cells are ER- and HER2-[30]. MCF-7 cells are one of the most commonly used cell lines in breast cancer research[31] because of its hormone sensitivity due to expression of the estrogen receptor[32]. MDA MB-231 cells are an example of a triple negative cell line[33] where cells are ER- and progesterone receptor (PR-) and HER2-. Wnt proteins are known to be expressed in human breast cancer tissue[34, 35] and for adult women, dysregulated Wnt pathways have been associated with breast cancer[7]. Based on the effect of sulfonamides on Wnt transcription inhibition described above, a group of 11 sulfonamides ranging from very potent (i.e., IC_{50} = 11 nM) to less potent (i.e., IC_{50} > 5000 nM) were tested for their effect on cell proliferation in both MDA-MB 231 and MCF-7 breast cancer cell lines. The results showed sulfonamides with potent inhibition of Wnt transcription (i.e., **19**, **24**, **25** with IC_{50} = 11, 18, 7 nM, respectively), were potent anti-proliferation agents against the MCF-7 (i.e., IC_{50} = 10, 7, 4 nM, respectively, **Table 5**). In good agreement, in MDA-MB 231 cells, potent inhibition of Wnt transcription was also directly correlated to potent inhibition of proliferation of MDA-MB 231 breast cancer cell lines (i.e., **19**, **24**, **25** with IC_{50} = 13, 13, 16 nM, respectively, **Table 5**).

Table 5. Effect of Sulfonamides on Wnt Inhibition and Inhibition of Proliferation of Breast Cancer Cells *In Vitro*.

Compound	IC_{50} ± Std. Dev. (nM)[a]			Compound	IC_{50} ± Std. Dev. (nM)[a]		
	Wnt Inhibition	MDA-MB 231	MCF-7		Wnt Inhibition	MDA-MB 231	MCF-7
1	25 ± 3	15 ± 2	17 ± 2	**12**	6 ± 2	20 ± 2	25 ± 1
19	11 ± 3	13 ± 2	10 ± 1	**22**	17 ± 2	14 ± 2	14 ± 2
25	7 ± 1	16 ± 2	4 ± 1	**4**	> 5000	> 5000	> 5000
24	18 ± 2	13 ± 2	7 ± 2	**15**	660 ± 2	608 ± 2	318 ± 3
11	7 ± 2	17 ± 2	15 ± 1	**36**	> 5000	> 5000	> 5000

[a]IC_{50} is mean of three determinations ± Std. Dev. [b]Wnt inhibition described in Methods. [c]MDA-MB 231 and MCF-7 data refer to IC_{50} values for inhibition of proliferation.

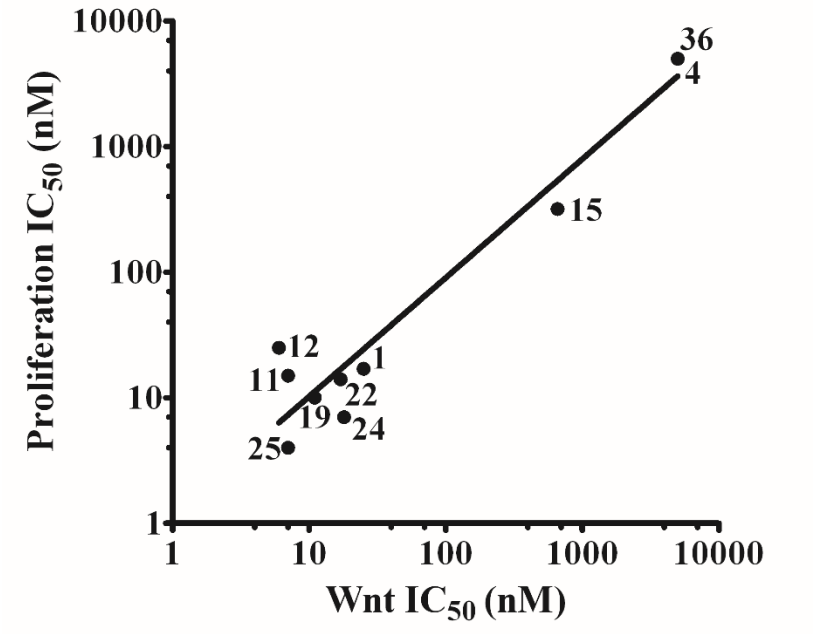


Figure 2. Plot of log of IC_{50} of inhibition of proliferation of MCF-7 breast cancer cells versus the log of the IC_{50} values for inhibition of Wnt transcription in HEK 293 cells for compounds **1**, **4**, **11**, **12**, **15**, **19**, **22**, **24**, **25** and **36**. Correlation coefficient, $r^2 = 0.93$.

A plot of IC_{50} values of the compounds in **Table 5** for inhibition of MCF-7 cell proliferation versus IC_{50} values for inhibition of Wnt transcription (**Figure 2**) showed a large, positive correlation (i.e., $r^2 = 0.93$). Similarly, a plot of the IC_{50} values for inhibition of MDA-MB 231 cell proliferation versus inhibition of Wnt transcription (**Supporting Figure S1**) also showed a large, positive correlation (i.e., $r^2 = 0.97$). Thus, for the sulfonamides examined, the more potent the degree of inhibition of Wnt transcription, the more potent the inhibition of breast cancer cell proliferation.

3.3. Effect of Sulfonamides on p53 Transcription in HEK-293 cells.

p53 is a unique protein that plays a role in several physiological processes including cell metabolism[36], stem cell maintenance[37] and cell adhesion[38]. In cancer cells, p53 is often inactivated and this results in cancer cells unable to conduct apoptotic function[39, 40]. In cancer cells, a decrease in p53 function has been linked to increased Wnt functional activity[41]. As a consequence of increased Wnt functional activity and a decrease in p53 in cancer cells, a decrease in apoptosis occurs. Conversely, activation or stabilization of p53 helps a cancer cell regulate the physiological processes controlled by p53[42] and increases apoptotic function. To test the function of sulfonamides described herein on p53 transcription, sulfonamides listed in **Table 5** were examined for their effects on p53 transcriptional activity using a p53 transcriptional reporter assay[43]. A plot of IC_{50} values of the compounds listed in **Table 5** for inhibition of MCF-7 cell proliferation versus the IC_{50} values for activation of p53 (**Supporting Table S2, Figure S2**) showed a large, positive correlation (i.e., $r^2 = 0.61$). This is in good agreement with inhibition of MCF-7 and MDA-MB 231 cell proliferation described above (**Table 5**). Western blot analysis of MCF-7 cell lysates treated with **19** showed a significant increase in total p53 protein with an EC_{50} = 18 nM (**Supporting Figure S3**). This result showed that the sulfonamides described herein that are potent inhibitors of cancer cell proliferation are also potent activators of the p53 pathway. Also, the data showed that the more potent inhibitors of Wnt transcription were also more potent activators of p53. That potent Wnt inhibitors and p53 activators (i.e., **19, 24** and **25**) also potently induced Caspase-Glo-3/7 (**Supporting Table S3**) showed that these compounds promote apoptosis in breast cancer cells.

3.4. Synergistic effects of 19 and Doxorubicin on the proliferation of MCF-7 and MDA-MB-231 cells.

Combination drug treatments have shown promising results as cancer treatments[44]. One of the most common chemotherapeutics for treatment of breast cancer is Doxorubicin[3] and is usually given in combination with other chemotherapeutics, including paclitaxel or 5-fluorouracil[45]. Based on the overall potent anti-proliferative effects of **19** on MCF-7 and MDA-MB 231 cells (i.e., IC_{50} = 10 nM and 13 nM, respectively, **Table 5**), **19** was examined in the presence of Doxorubicin to inhibit MCF-7 or MDA-MB 231 cell proliferation. The results from these studies showed **19** and Doxorubicin had a synergistic effect on the inhibition of proliferation of either MCF-7 or MDA-MB 231 breast cancer cells. Compared to Doxorubicin alone, inhibition of proliferation for Doxorubicin in the presence of **19** was 2-3-fold greater ($p < 0.05$) in both MCF-7 and MDA-MB-231 cells (**Supporting Figure S4**). In the presence of Doxorubicin, **19** synergized inhibition of proliferation of MCF-7 and MDA-MB-231 cells and showed a low "combination index" value (CI < 1) by Chou-Talalay synergism analysis[46] (**Supporting Table S4**). Compared to treatments in MCF-7 cells, drug combination studies done in MDA-MB-231 cells showed greater synergism (**Supporting Table S4, ED_{90}**).

3.5. In vivo effects of 19 in the presence of absence of Doxorubicin on breast cancer xenografts.

Pharmaceutical studies showed **19** was suitable for further studies. For example, viability studies in mammalian cells showed that **19** did not cause any detectable cytotoxicity at the concentrations examined[15]. Compound **19** did not cause any detectable inhibition of hERG up to 50 μM. No detectable off-target inhibition of kinases (CEREP panel) was observed[15].

As a prelude to efficacy studies of **19**, in vitro and in vivo DMPK studies were conducted[15]. Incubations with human or rat liver microsomes (+ NADPH) showed **19** was metabolically stable for > 60 mins as judged by HPLC. The only minor metabolite observed was the *N*-demethylation metabolite, **18**, that was not potent (**Table 2**). No apparent inhibition of human cytochrome P-450 (i.e., testosterone hydroxylase) by **19** (50 μM) was observed in vitro[15]. Pharmacokinetic studies in rats showed **19** possessed acceptable bioavailability (i.e., 15-20%)[15]. Accordingly, in vivo efficacy in xenograft studies with mice were done.

Human breast tumors were implanted subcutaneously (1 million MDA-MB-231 cells) in female *nu/nu* mice and assessed by daily caliper measurements. After the tumors were established (on day 35, average size,

75.5 ± 12 mm^3), test compounds were administered by i.p. injection. The design of the xenograft study was to compare treatment of mice in four groups (i.e., vehicle, Doxorubicin, **19**, and **19** plus Doxorubicin, n = 9) at the end of 16 days of treatment. Compared to vehicle-treated animals, Doxorubicin (5 mg/kg/week) decreased human breast cancer tumor volume by 84.3%, **19** (20 mg/kg, 16 consecutive days) decreased breast cancer volume by 91.5% and **19** (20 mg/kg, 16 consecutive days) plus Doxorubicin (5 mg/kg/week) decreased breast cancer volume 96.2%. On day 51, animals treated with **19** plus Doxorubicin decreased tumor volume on average to 24.7 ± 5.9 mm^3, a 6.2.-fold decrease in tumor volume compared to the volume of tumor on day 35 (i.e., 154.3 ± 37 mm^3) ($P < 0.01$). In contrast, vehicle-treated animals grew tumors to an average volume of 653 ± 192 mm^3 or a 4.2-fold increase in tumor volume at day 51 compared to tumor volume of vehicle-treated animals on day 35 (**Figure 3B**). Thus, after tumors were established, treatment with **19** and Doxorubicin for 16 days dramatically decreased tumor volume compared to vehicle-treated mice. Compared to vehicle-treated animals, at the end of the 16-day study, weights of excised tumors for animals treated with **19** plus Doxorubicin were significantly lower (i.e., 93.7% lower) ($P < 0.01$) (**Figure 3C**).

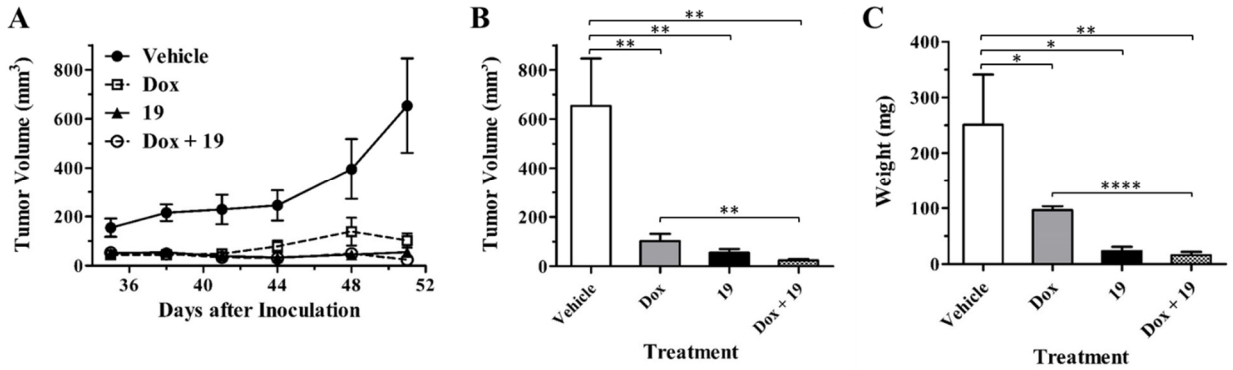


Figure 3. Effect of Doxorubicin, compound **19** or Doxorubicin plus **19** on MDA-MB-231 xenograft tumor growth in *nu/nu* mice. **A.** The effect of vehicle (solid circle, n=7), 5 mg/kg/week Doxorubicin (Dox) (open square, n=8), 20 mg/kg/day **19** (solid triangle, n=6) or **19** (20 mg/kg/day) plus Doxorubicin (5 mg/kg/week) (open circle, n=5) on tumor volume of subcutaneous (back) MDA-MB-231 breast cancer xenografts in mice. Treatment was administered for 16 days starting on day 35 by intraperitoneal injection. **B.** Tumor volume and **C.** tumor weights at day 51 from the same animals as **A**. White bar, vehicle, n=7; grey bar, Doxorubicin, n=8; black bar, **19**, n=6

and hatched bar, Doxorubicin plus **19**, n=5. Data are mean ± s.e.m. in **A**, **B** and **C**. The *P*-value was estimated by Student's *t*-test test in **B** and **C** (*P< 0.05, **P< 0.01, ****P< 0.0001).

At the end of the study, serum concentrations of **19** from **19**-treated animals were determined to be ~ 0.2 µg/ml by HPLC (see **Supporting Information**) and confirmed by LCMS. Tumors excised from animals upon termination of the study (i.e., Day 51) and analyzed for amounts of p53, phospho Ser15-p53 protein and percent PARP cleavage by Western blot analysis showed, compared to vehicle-treated animals, animals treated with **19** plus Doxorubicin had approximately 1.5- and 3.0-fold greater p53 and phospho Ser15-p53 protein, respectively, and 30.0-fold greater PARP cleavage (P < 0.05) (**Supporting Figure S5**).

4. Conclusion. From a screen of 76,000 compounds for inhibition of Wnt transcription, a selective and potent "hit" compound **1** was observed. From over 120 medicinal chemical analogues of compound **1**, several lead compounds were developed. For example, compared to **1,** compounds **19**, **24** or **25** had improved potency for Wnt inhibition and improved potency of inhibition of breast cancer cell proliferation and increased aqueous solubility. Compounds **19**, **24** or **25** were shown to have potent anti-proliferative effects in two breast cancer cell lines with dysregulated Wnt signaling. A large positive correlation between inhibition of Wnt transcription and breast cancer cell proliferation in both MCF-7 and MDA-MB 231 breast cancer cell lines was observed. This indicated that for the compounds examined, Wnt transcription inhibition and inhibition of breast cancer cell proliferation go hand in hand. In addition, an increase in p53 transcriptional functional activity also correlated to inhibition of Wnt transcription and anti-proliferation effects. The results suggested that compounds **19**, **24** or **25** inhibit Wnt and induce apoptosis in breast cancer cell lines. Finally, in an *in vitro* study, **19** was observed to synergize with breast cancer chemotherapy drug Doxorubicin used at a relatively low dose to afford improved cancer cell anti-proliferative effects. In vivo, compared to Doxorubicin alone, **19** and Doxorubicin markedly decreased breast cancer tumor size and weight in xenografts. It may be that using non-toxic **19** in conjunction with lower doses of chemotherapeutics could afford a combination treatment with overall lower toxicity. Thus, from this study,

compounds **19**, **24**, and **25** emerged as potent inhibitors of Wnt transcription and anti-breast cancer compounds with excellent physiochemical properties.

5. Experimental Section

5.1. General. Reagents, starting materials and solvents were purchased in the highest purity available from commercial suppliers and used as received. Compounds **26**, **31**, **41**, **43** and **44** were commercially available from Sigma-Aldrich (St. Louis, MO). Chemical names were created with ChemDraw Ultra version 11 (PerkinElmer, Waltham, MA). Microwave reactions were conducted using a Biotage Initiator microwave synthesizer (Biotage, Uppsala, Sweden). Microwave reactions were run under ambient pressure and variable power to reach the indicated temperature. Reaction products were purified, when necessary, using an Isco Combiflash R_f flash chromatography system (Teledyne-Isco, Lincoln, NE) with the solvent systems indicated. Nuclear magnetic resonance (NMR) data were recorded on a Varian Mercury 300 MHz Spectrometer (Agilent, Santa Clara, CA) using TMS as an internal standard and $CDCl_3$ as a solvent except where indicated. Electrospray ionization (ESI) mass spectral data was obtained using an Agilent 1100 LC/MS (Agilent, Santa Clara, CA). Final test compounds had a purity greater than 95% on the basis LCMS analysis using UV-Vis detection at 275 nM and 220 nM. Compounds were prepared as hydrochloride salts unless otherwise noted. Hydrochloride salts were prepared by dissolution of the appropriate compound in a minimum amount of dichloromethane and addition of excess 2 M HCl in ether. The solvent was evaporated and hydrochlorides were used directly for evaluation. Aqueous solubilities were determined by preparing a DMSO stock solution of the desired compound at 10 mM and then diluting the stock solution in distilled water to the desired final concentration or until turbidity was observed. Dulbecco's Modified Eagle's cell culture medium (DMEM) was purchased from Lonza (Allendale, NJ) and fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). The reagents used in the transfection assay, OptiMEM and lipofectamine 2000, as well as the phosphate buffered saline (PBS) and SYBR Green used in cell proliferation assays were purchased from Life Technologies (Carlsbad, CA). The Dual Luciferase Assay Kit to measure the Wnt and p53 transcriptional activity and the Caspase-Glo 3/7 assay kit to measure the cell apoptosis were purchased from Promega

(Fitchburg, WI). Fluorescence was determined using a Tecan SPECTRAFluor Plus plate reader (Tecan, San Jose, CA). Luminescence was recorded on a Wallac Victor plate reader (PerkinElmer Inc., Waltham, MA).

4-(2-Oxopyrrolidin-1-yl)benzene-1-sulfonyl chloride, ***3***. Two g of phenylpyrrolidinone was added to 20 mL of chlorosulfonic acid. The mixture was stirred overnight at room temperature. The mixture was stopped by slow addition to ice. The aqueous mixture was extracted with 4 volumes of dichloromethane. The organic layers were combined, dried over Na_2SO_4 and concentrated *in vacuo* to give an off white solid. The sulfonyl chloride was used without further purification, (88 % yield). ^{1}H NMR ($CDCl_3$): 2.19 – 2.30 (m, 2H), 2.69 (t, J = 8.0 Hz, 2H), 3.93 (t, J = 7.2 Hz, 2H), 7.91 (d, J = 9.4 Hz, 2H), 8.01 (d, J = 7.4 Hz, 2H).

General synthesis of pyrrolidinone sulfonamides. To a microwave vial was added 30 mg of the sulfonyl chloride, 1.5 eq. of the appropriate amine, 1.2 eq. of triethylamine, and 300 µL of acetonitrile. The vial was sealed and heated at 130 °C for 30 min. The mixture was diluted with dichloromethane and the organic phase was washed 2 times with water and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Crude sulfonamide products were purified either by recrystallization from ethanol or by flash chromatography 0 – 75 % EtOAc/Hexanes.

1-(4-(3,4-Dihydroquinolin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***1***. ESI/MS: calculated $C_{19}H_{20}N_2O_3S$ *m/z* = 356.4, found *m/z* = 357.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.60-1.70 (m, 2H), 2.12-2.22 (m, 2H), 2.44 (t, J = 6.6 Hz, 2H), 2.63 (t, J = 6.6 Hz, 2H), 3.78-3.86 (m, 4H), 6.97-7.19 (m, 2H), 7.54 (d, J = 9.1 Hz, 2H), 7.69 (d, J = 9.1 Hz, 2H), 7.77 (d, J = 8.8 Hz, 2H).

1-(4-(6-Methyl-3,4-dihydroquinolin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***4***. ESI/MS: calculated $C_{20}H_{22}N_2O_3S$ *m/z* = 370.1, found *m/z* = 371.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.56-1.65 (m, 2H), 2.14-2.21 (m, 2H), 2.29 (s, 3H), 2.39 (t, J = 6.6 Hz, 2H), 2.64 (t, J = 6.6 Hz, 2H), 3.76-3.88 (m, 4H), 6.79 (bs, 1H), 6.98 (d, J = 9.1 Hz, 1H), 7.55 (d, J = 9.1 Hz, 2H), 7.65-7.72 (m, 4H).

1-(4-(Octahydroquinolin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***5***. ESI/MS: calculated $C_{19}H_{26}N_2O_3S$ *m/z* = 362.2, found *m/z* = 363.0 [M+1]. ^{1}H NMR ($CDCl_3$): 0.87-1.77 (m, 14H), 2.14-2.24 (m, 2H), 2.37-2.49 (m, 1H), 2.65 (t, J = 6.6 Hz, 2H), 3.89 (t, J = 6.6 Hz, 2H), 4.04-4.11 (m, 1H), 7.72-7.82 (m, 4H).

1-(4-(Piperidin-1-ylsulfonyl)phenyl)pyrrolidin-2-one, ***6***. ESI/MS: calculated $C_{15}H_{20}N_2O_3S$ *m/z* = 308.1, found *m/z* = 309.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.36-1.44 (m, 2H), 1.60-1.67 (m, 4H), 2.16-2.26 (m, 2H), 2.66 (t, J = 6.6 Hz, 2H), 2.96 (t, J = 6.6 Hz, 2H), 3.90 (t, J = 6.6 Hz, 2H), 7.70-7.82 (m, 4H).

4-(2-Oxopyrrolidin-1-yl)-N-phenyl-N-propylbenzenesulfonamide, ***7***. ESI/MS: calculated $C_{19}H_{22}N_2O_3S$ *m/z* = 358.1, found *m/z* = 359.0 [M+1]. ^{1}H NMR ($CDCl_3$): 0.9 (t, J = 7.4 Hz, 3H), 1.38 – 1.48 (m, 2H), 2.16 - 2.27 (m, 2H), 2.67 (t, J = 8.4 Hz 2H), 3.47 – 3.52 (m, 2H), 3.86 (t, J = 6.9 Hz, 2 H), 7.02 – 7.06 (m, 2H), 7.28 – 7.32 (m, 2H), 7.56 (d, J = 9.0 Hz, 2 H), 7.74 (d, J = 9.0 Hz, 2 H).

N-(Naphthalen-1-yl)-4-(2-oxopyrrolidin-1-yl)benzenesulfonamide, ***8***. ESI/MS: calculated $C_{20}H_{18}N_2O_3S$ *m/z* = 366.1, found *m/z* = 367.0 [M+1].^{1}H NMR ($CDCl_3$): 2.10-2.21 (m, 2H), 2.62 (t, J = 6.6 Hz, 2H), 3.80 (t, J = 6.6 Hz, 2H), 7.08 (bs, NH), 7.32-7.47 (m, 3H), 7.63-7.92 (m, 8H).

1-(4-(1,2,3,4-Tetrahydroquinoline-1-carbonyl)phenyl)pyrrolidin-2-one, ***9***. ESI/MS: calculated $C_{20}H_{20}N_2O_2$ *m/z* = 320.2, found *m/z* = 321.1 [M+1]. ^{1}H NMR ($CDCl_3$) 2.07 (quintet, *J* = 6.3 Hz, 2H),2.19 (quintet, *J* = 7.7 Hz, 2H), 2.64 (t, *J* = 8.3 Hz, 2H), 2.86 (t, *J* = 6.3 Hz, 2H), 3.86 (t, *J* = 6.8 Hz, 2H), 3.92 (t, *J* = 6.3 Hz, 2H), 6.71 (m, 1H), 6.88 (m, 1H), 7.00 (m, 1H), 7.16 (m, 1H), 7.39 (m, 2H), 7.58 m, 2H).

1-(4-(3,4-Dihydroisoquinolin-2(1H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***11***. ESI/MS: calculated $C_{19}H_{20}N_2O_3S$ *m/z* = 356.1, found *m/z* = 357.0 [M+1]. ^{1}H NMR ($CDCl_3$): 2.16 – 2.27 (m, 2H), 2.66 (t, J = 8.0 Hz 2H), 2.90 (t, J = 6.0 Hz 2H), 3.36 (t, J = 6.0 Hz 2H), 3.90 (t, J = 7.0 Hz 2H), 4.25 (s, 2 H), 7.00 – 7.15 (m, 4H) 7.82 (s, 4H).

1-(4-(2-Methyl-3,4-dihydroquinolin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***12***. ESI/MS: calculated $C_{20}H_{22}N_2O_3S$ *m/z* = 370.1, found *m/z* = 371.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.3 (d, J = 6.6 Hz, 3 H), 1.26 – 1.40 (m, 2H), 1.77 – 1.88 (m, 2H), 2.13 – 2.23 (m, 2H), 2.35 – 2.45 (m, 2H), 2.64 (t, J = 8.3 Hz 2H), 3.82 – 3.87 (m, 2 H), 4.32 – 4.42 (m, 1 H), 6.95 – 6.97 (m, 1H), 7.07 – 7.12 (m, 1H), 7.19 – 7.25 (m, 1H), 7.46 (d, J = 9.0 Hz, 2 H), 7.66 (d, J = 9.0 Hz, 2 H), 7.72 – 7.77 (m, 1H).

1-(4-(6-Chloro-3,4-dihydroquinolin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***13***. ESI/MS: calculated $C_{19}H_{19}ClN_2O_3S$ *m/z* = 390.1, found *m/z* = 391.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.51 – 1.66 (m, 2H), 2.15 – 2.25 (m,

2H), 2.43 (t, J = 6.1 Hz 2H), 3.77 – 3.81 (m, 2H), 3.86 (t, J = 8.3 Hz 2H), 3.82 – 3.87 (m, 2 H), 6.97 – 6.99 (m, 1H), 7.12 – 7.16 (m, 1H), 7.56 (d, J = 9.0 Hz, 2 H), 7.73 (d, J = 9.0 Hz, 2 H), 7.74 – 7.77 (m, 1H).

1-(4-(6-Methoxy-3,4-dihydroquinolin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***14***. ESI/MS: calculated $C_{20}H_{22}N_2O_4S$ *m/z* = 386.1, found *m/z* = 387.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.53 – 1.62 (m, 2H), 2.14 – 2.25 (m, 2H), 2.35 (t, J = 6.9 Hz 2H), 2.64 (t, J = 8.5 Hz 2H), 3.75 – 3.78 (m, 2H), 3.78 (s, 3 H), 3.86 (t, J = 7.2 Hz 2H), 6.50 – 6.51 (m, 1H), 6.73 – 6.77 (m, 1H), 7.52 (d, J = 9.1 Hz, 2 H), 7.67 – 7.72 (m, 3H).

1-(4-(6-(Trifluoromethyl)-3,4-dihydroquinolin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***15***. ESI/MS: calculated $C_{20}H_{19}F_3N_2O_3S$ *m/z* = 424.1, found *m/z* = 425.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.67 – 1.75 (m, 2H), 2.13 – 2.25 (m, 2H), 2.56 – 2.67 (m, 4H), 3.83 – 3.89 (m, 4H), 7.38 – 7.42 (m, 1H), 7.61 (d, J = 8.8 Hz, 2H), 7.75 (d, J = 8.8 Hz, 2 H), 7.90 – 7.93 (m, 1H), 8.00 – 8.04 (m, 1H).

Racemic 1-(4-(6-tert-Butyl-3,4-dihydroquinolin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***16***. ESI/MS: calculated $C_{23}H_{28}N_2O_3S$ *m/z* = 412.2, found *m/z*= 413.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.30 (s, 9H), 1.59 – 1.68 (m, 2H), 2.14 – 2.24 (m, 2H), 2.48 (t, J = 6.6, 2H), 2.64 (t, J = 8.3, 2H), 3.77 – 3.80 (m, 2H), 3.86 (t, J = 6.9, 2H), 6.98 (d, J = 2.5 Hz, 1H), 7.19 (dd, J = 2.5 Hz and 8.8 Hz, 1H), 7.59 (d, J = 9.1 Hz, 2H), 7.67 (d, J = 8.8 Hz, 1 H), 7.70 (d, J = 9.1 Hz, 2 H).

1-(4-(2H-Benzo[b][1,4]oxazin-4(3H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***17***. ESI/MS: calculated $C_{18}H_{18}N_2O_4S$ *m/z* = 357.1, found *m/z* = 358.0 [M+1]. ^{1}H NMR ($CDCl_3$): 2.16 – 2.21 (m, 2 H), 2.64 (t, J = 8.3 Hz, 2H), 2.93 – 2.96 (m, 2H), 3.79 – 3.87 (m, 4H), 6.43 – 6.46 (m, 1H), 6.66 – 6.71 (m, 1H), 6.94 – 6.99 (m, 1H), 7.54 (d, J = 8.8 Hz, 2H), 7.64 – 7.68 (m, 1 H), 7.70 (d, J = 9.0 Hz, 2 H).

1-(4-(4-Methyl-3,4-dihydroquinoxalin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***18***. ESI/MS: calculated $C_{19}H_{21}N_3O_3S$ *m/z* = 371.1, found *m/z* = 372.0 [M+1]. ^{1}H NMR ($CDCl_3$): 2.14 – 2.24 (m, 2 H), 2.64 (t, J = 8.5 Hz, 2H), 2.66 (s, 3H), 2.88 (t, J = 5.5 Hz, 2H), 3.83 – 3.89 (m, 4H), 6.55 – 6.58 (m, 1H), 6.65 – 6.71 (m, 1H), 6.55 – 6.58 (m, 1H), 7.50 (d, J = 9.0 Hz, 2H), 7.59 – 7.62 (m, 1 H), 7.69 (d, J = 9.0 Hz, 2 H).

1-(4-(4-Methyl-3,4-dihydroquinoxalin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***19***. ESI/MS: calculated $C_{19}H_{21}N_3O_3S$ *m/z* = 371.1, found *m/z* = 372.0 [M+1]. ^{1}H NMR ($CDCl_3$): 2.14 – 2.24 (m, 2 H), 2.64 (t, J = 8.5 Hz,

2H), 2.66 (s, 3H), 2.88 (t, J = 5.5 Hz, 2H), 3.83 – 3.89 (m, 4H), 6.55 – 6.58 (m, 1H), 6.65 – 6.71 (m, 1H), 6.55 – 6.58 (m, 1H), 7.50 (d, J = 9.0 Hz, 2H), 7.59 – 7.62 (m, 1 H), 7.69 (d, J = 9.0 Hz, 2 H).

1-(4-(4-(2-Hydroxyethyl)-3,4-dihydroquinoxalin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***20***. ESI/MS: calculated $C_{20}H_{23}N_3O_4S$ *m/z* = 401.1, found *m/z* = 402.0 [M+1]. ^{1}H NMR ($CDCl_3$): 2.15 – 2.25 (m, 2H), 2.63 (t, J = 8.3, 2H), 3.00 (t, J = 5.1, 2H), 3.26 (t, J = 5.1, 2H), 3.46 – 3.50 (m, 2H), 3.80 – 3.88 (m, 4H), 6.58 (d, J = 8.2, 1H), 6.68 (t, J = 7.7, 1H), 7.05 – 7.10 (m, 1H), 7.44 – 7.51 (m, 4H), 7.62 (d, J = 8.0 Hz, 1H).

1-(4-(3,4-Dihydro-1,5-naphthyridin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***21***. ESI/MS: calculated $C_{18}H_{19}N_3O_3S$ *m/z* = 357.1, found *m/z* = 358.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.65 – 1.74 (m, 2H), 2.15 – 2.24 (m, 2H), 2.62 – 2.73 (m, 4H), 3.79 – 3.88 (m, 4H), 7.15 (dd, J = 4.7 Hz and 8.3 Hz, 1H), 7.57 (d, J = 9.4 Hz,2H), 7.74 (d, J = 9.1 Hz, 2H), 8.16 (dd, J = 1.4 Hz and 8.3 Hz, 1H), 8.30 (dd, J = 1.4 Hz and 4.7 Hz, 1H).

1-(4-(6-Methoxy-4-methyl-3,4-dihydroquinoxalin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***22***. ESI/MS: calculated $C_{20}H_{23}N_3O_4S$ *m/z* = 401.1 found *m/z* = 402.0 [M+1]. ^{1}H NMR ($CDCl_3$): 2.14 – 2.25 (m, 2H), 2.62 (s, 3H), 2.63 – 2.67 (m, 2H), 2.81 – 2.85 (m, 2H), 3.80 (s, 3H), 3.83 – 3.89 (m, 4H), 6.13 (d, J = 2.8 Hz, 1H), 6.30 (dd, J = 2.8 Hz and 8.8 Hz, 1H), 7.48 (d, J = 9.4 Hz, 2H), 7.52 (d, J = 8.8 Hz, 1H), 7.69 (d, J = 9.1 Hz, 2H).

Racemic 1-(4-(2,4-Dimethyl-3,4-dihydroquinoxalin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***23***. ESI/MS: calculated $C_{20}H_{23}N_3O_3S$ *m/z* = 385.2 found *m/z* = 386.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.15 (d, J = 6.9 Hz, 3H), 2.12 – 2.23 (m, 2H), 2.63 – 2.65 (m, 2H), 2.68 (s, 3H), 2.69 – 2.75 (m, 2H), 3.83 (t, J = 7.2 Hz, 2H), 4.41 – 4.51 (m, 1H), 6.56 (d, J = 8.3 Hz, 1H), 6.67 – 6.73 (m, 1H), 7.05 – 7.11 (m, 1H), 7.43 (d, J = 8.9 Hz, 2H), 7.63 – 7.67 (m, 1H), 7.66 (d, J = 8.9 Hz, 2H).

1-(3-(3,4-Dihydroquinolin-1(2H)-ylsulfonyl)phenyl)pyrrolidin-2-one, ***30***. ESI/MS: calculated $C_{19}H_{20}N_2O_3S$ *m/z* = 356.1 found *m/z* = 357.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.64 – 1.72 (m, 2H), 2.09 – 2.19 (m, 2H), 2.44 (t, J = 6.9, 2H), 2.60 (t, J = 8.0, 2H), 3.65 (t, J = 6.9, 2H), 3.81 – 3.85 (m, 2H), 6.98 – 7.09 (m, 2H), 7.16 – 7.21 (m, 1H), 7.34 – 7.42 (m, 2H), 7.49 (s, 1H), 7.78 (d, J = 8.3 Hz, 1H), 8.11 – 8.14 (m, 1H).

1-(4-(3,4-Dihydroquinolin-1(2H)-ylsulfonyl)-2-methylphenyl)pyrrolidin-2-one, ***34***. ESI/MS: calculated $C_{20}H_{22}N_2O_3S$ *m/z* = 370.1 found *m/z* = 371.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.64 – 1.69 (m, 2H), 2.18 – 2.23 (m, 2H),

2.26 (s, 3H), 2.45 (t, J = 6.6 Hz, 2H), 2.52 (t, J = 8.2 Hz, 2H), 3.61 (t, J = 7.1 Hz, 2H), 3.77 – 3.79 (m, 2H), 7.00 – 7.02 (m, 1H), 7.07 – 7.10 (m, 1H), 7.18 – 7.21 (m, 1H), 7.28 (d, J = 8.3 Hz, 1H), 7.36 – 7.40 (m, 2H), 7.78 (d, J = 8.2, 1H).

1-Phenyl-1H-1,2,4-triazole, ***35****.* To a 15 mL microwave vial 2 g (13.8 mmol) of phenyl hydrazine hydrochloride was added to 10 mL of formamide. The vial was sealed and heated at 175°C for 1.5 h in a microwave reactor. The mixture was poured into water and extracted 3 times with ethyl acetate. The organic layers were combined and washed 3 times with water and once with brine. The organic phase was dried over sodium sulfate and concentrated *in vacuo*. The crude material was purified by flash chromatography 0 – 50 % EtOAc/Hexanes and a yellow oil was isolated that solidified upon standing, 1.2 g. ESI/MS: calculated C_8H_7N *m/z* = 145.1 found *m/z* = 146.0 [M+1]. ^{1}H NMR ($CDCl_3$): 7.37 – 7.42 (m, 1H), 7.47 – 7.53 (m, 2H), 7.66 (d, J = 8.3 Hz, 2H), 8.10 (s, 1H), 8.54 (s, 1H).

1-(4-(1H-1,2,4-Triazol-1-yl)phenylsulfonyl)-1,2,3,4-tetrahydroquinoline, ***36****.* To 10 mL of chlorosulfonic acid at 0 °C was added 1 g (6.9 mmol) of 1-phenyl-1H-1,2,4-triazole, **35**. The mixture was stirred overnight and then cooled to 0 °C. The solution was slowly added to ice and extracted 3 three times with dichloromethane. The organic layers were combined, dried over sodium sulfate and concentrated to dryness *in vacuo* to give 1 g of a tan solid. The crude sulfonyl chloride was used directly without further purification. To a microwave vial was added 25 mg (0.095 mmol) of the sulfonyl chloride, 15 mg (0.11 mmol) of 1,2,3,4-tetrahydroquinoline, 14 mg (0.014 mmol) of triethylamine and 300 µL of acetonitrile. The vial was sealed and heated at 130 °C for 30 min. The mixture was diluted with dichloromethane and the organic phase was washed 2 times with water and brine. The organic layer was dried over sodium sulfate and concentrated *in vacuo*. The crude sulfonamide was purified by flash chromatography 0 – 75 % EtOAc/Hexanes. ESI/MS: calculated $C_{17}H_{16}N_4O_2S$ *m/z* = 340.1 found *m/z* = 341.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.63 – 1.71 (m, 2H), 2.43 – 2.48 (m, 2H), 3.83 – 3.87 (m, 2H), 6.99 – 7.01 (m, 1H), 7.07 – 7.12 (m, 1H), 7.18 – 7.23 (m, 1H), 7.69 – 7.81 (m, 5H), 8.11 (s, 1H), 8.60 (1H).

1-Phenyl-4,5-dihydro-1H-pyrazole, ***37****.* 1.4 g (10 mmol) of phenylhydrazine hydrochloride, 1 mL (10 mmol) 1,3-dibromopropane, 1.4 g (10 mmol) potassium carbonate and 10 mL water were added to a 15 mL microwave vial

and heated at 130 °C for 30 min. After cooling, the mixture was extracted 4 times with ethyl acetate. The organic layers were combined, dried over sodium sulfate and concentrated *in vacuo* to give a yellow residue. The crude mixture was purified by flash chromatography 0 – 25 % EtOAc/Hexanes and 300 mg of a yellow solid was isolated. ESI/MS: calculated $C_9H_{10}N_2$ *m/z* = 146.1 found *m/z* = 147.0 [M+1]. ^{1}H NMR ($CDCl_3$): 2.93 (dt, J = 10.5 Hz and 1.7 Hz, 2H), 3.67 (t, J = 10.5 Hz, 2H), 6.82 – 6.88 (m, 2H), 7.03 (d, J = 7.7 Hz, 2H), 7.24 – 7.31 (m, 2H).

1-(4-(4,5-Dihydro-1H-pyrazol-1-yl)phenylsulfonyl)-1,2,3,4-tetrahydroquinoline, ***38***. To 1.8 mL (27 mmol) of chlorosulfonic acid at 0 °C was added 200 mg (1.4 mmol) of **37**. The mixture was stirred overnight and then cooled to 0 °C. The solution was slowly added to ice and extracted 3 three times with dichloromethane. The organic layers were combined, dried over sodium sulfate and concentrated to dryness *in vacuo* to give 150 mg of an orange solid. The sulfonyl chloride was used without any further purification. To a microwave vial was added 50 mg (0.20 mmol) of the sulfonyl chloride, 30 mg (0.25 mmol) of 1,2,3,4-tetrahydroquinoline, 30 mg (0.3 mmol) of triethylamine and 300 µL of acetonitrile. The vial was sealed and heated at 130 °C for 30 min. After cooling, the mixture was diluted with dichloromethane and the organic layer was washed 2 times with water and brine. The organic layer was dried over sodium sulfate and concentrated *in vacuo*. The crude sulfonamide was purified by flash chromatography 0 – 75 % EtOAc/Hexanes to give 30 mg of a yellow solid. ESI/MS: calculated $C_{17}H_{16}N_4O_2S$ *m/z* = 341.1 found *m/z* = 342.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.62 – 1.68 (m, 2H), 2.45 – 2.48 (m, 2H), 2.97 – 3.02 (m, 2H), 3.66 – 3.71 (m, 2H), 3.78 – 3.80 (m, 2H), 6.90 – 6.92 (m, 3H), 6.98 – 6.99 (m, 1H), 7.03 – 7.06 (m, 1H), 7.16 – 7.19 (m, 1H), 7.46 (d, J = 9.3 Hz, 2H), 7.80 (d, J = 8.2 Hz, 1H).

1-(4-(Pyrrolidin-1-yl)phenylsulfonyl)-1,2,3,4-tetrahydroquinoline, ***39***. To a microwave vial was added 5 mg (0.028 mmol) of zinc acetate. The vial was sealed and purged / filled three times with vacuum then argon. A solution of 100 mg (0.28 mmol) of **1** and 137 mg (0.84 mmol) of triethoxysilane in 750 µL of tetrahydrofuran was added. The mixture was heated at 80 °C for 15 h. To the mixture was added 1 M sodium hydroxide and stirred at ambient temperature for 2 h. The aqueous fraction was extracted 3 times with ethyl acetate, the organic phase dried over sodium sulfate and concentrated. The crude mixture was purified by flash chromatography, 0 – 15 % EtOAc/Hexanes to give 70 mg of a white solid. ESI/MS: calculated $C_{19}H_{22}N_2O_2S$ *m/z* = 342.1 found *m/z* =

343.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.61 – 1.70 (m, 2H), 2.00 – 2.05 (m, 4H), 2.47 – 2.51 (m, 2H), 3.27 – 3.42 (m, 4H), 3.76 – 3.80 (m, 2H), 6.41 (d, J = 9.1 Hz, 2H), 6.95 – 7.06 (m, 2H), 7.13 – 7.19 (m, 1H), 7.39 (d, J = 9.1 Hz, 2H), 7.80 (d, J = 8.3 Hz, 1H).

1-(4-(1H-pyrazol-1-yl)phenylsulfonyl)-1,2,3,4-tetrahydroquinoline, ***42***. ESI/MS: calculated $C_{18}H_{17}N_3O_2S$ *m/z* = 339.1 found *m/z* = 340.0 [M+1]. ^{1}H NMR ($CDCl_3$): 1.62 – 1.71 (m, 2H), 2.45 (t, J = 6.6, 2H), 3.82 – 3.86 (m, 2H), 6.50 – 6.51 (m, 1H), 6.99 (d, J = 7.4 Hz, 1H), 7.09 (t, J = 7.4 Hz, 1H), 7.20 (t, J = 7.4 Hz, 1H), 7.65 (d, J = 8.8 Hz, 2H), 7.73 (d, J = 8.8 Hz, 2H), 7.73 – 7.74 (m, 1H), 7.81 (d, J = 8.3, 1H), 7.94 (d, J = 2.8, 1H).

5.2. Biology. *Effect of Sulfonamides on Wnt Transcriptional Activity*. A Super(8x)TOPflash (STF) and a Wnt3A-expression vector to activate the Wnt pathway were co-transfected with a TK-driven Renilla luciferase plasmid (Rluc) (for transfection efficiency normalization) into HEK-293 cells (culture media: DMEM, 10% FBS). After incubation at 37° C, 5% CO_2 for 8 h, cells were counted and seeded on a 96-well plate at a density of 25,000 cells per well with 135 µL media. The cells were treated with sulfonamides at a final concentration of 1.6, 8, 40, 200, 1000, 5000 nM (1.6 nM to 5 µM) or DMSO (vehicle control) for 20-22 h. Each condition was run in triplicate. After administration of each compound, media was thoroughly removed and 50 µL of passive cell lysis buffer from a Dual Luciferase Assay Kit (Promega) was added. Luminescent signal was measured sequentially by adding Firefly and Renilla luciferase substrate that was proportional to luciferases activity. Data was processed by normalizing (dividing) the Firefly luciferase signal by the Renilla luciferase signal. The average and standard deviation of relative Firefly luciferase luminescent signal for compound-treated cells (relative to DMSO treated cells) for each concentration (sample ran in triplicate) was calculated and then analyzed by nonlinear regression (GraphPad Prism, San Diego, CA) for IC_{50} determination of Wnt transcriptional activity.

5.2.1. *Effect of Sulfonamides on p53 Transcriptional Activity*. HEK-293 cells were transiently transfected with a p53-Luciferase (p53-Luc) vector driven by a (13x) p53-binding consensus-firefly luciferase response element, together with a TK-driven Renilla luciferase plasmid as an internal control to normalize the luminescence signal for transfection efficiency[13]. The detailed protocol was similar to the Wnt transcriptional assay but the final serum

concentration was kept at 2.5% to minimize basal reporter activity of p53-Luc. Each condition was run in triplicate. The transfected cells were treated with sulfonamides or DMSO for 20-22 h. Each condition was done in triplicate. After administration of each compound, media was thoroughly removed and 50 µL of passive cell lysis buffer in the Dual Luciferase Assay Kit (Promega) was added. Luminescent signal was measured sequentially by adding Firefly and Renilla luciferase substrate reagent. Data was processed by normalizing (dividing) the Firefly luciferase signal by the Renilla luciferase signal. The average and standard deviation of relative Firefly luciferase luminescent signal for compound-treated cells (relative to DMSO-treated cells) for each concentration (sample ran in triplicate) was calculated and then analyzed by nonlinear regression (GraphPad Prism) for IC_{50} determination of p53 transcriptional activity.

5.2.2. *Effect of sulfonamides on breast cancer cell proliferation.* Cells were plated at a density of 500 cells per well in eight 96-well plates with DMEM and 10% FBS culture media. After cells were attached to the plate (i.e., 24 h at 37 °C 5% CO_2), compounds to be tested were added to cells that afforded a concentration of 1.6 nM to 5 µM or DMSO (vehicle control). Seven plates were incubated at 37° C, 5% CO_2 and every 24 h one plate was harvested and stored at -20 °C for 7 days. Following harvest of all the plates (i.e., for 7 days), SYBR Green (1X stock by diluting 10,000 X original stock in PBS) (Life Technologies) was added to the cell lysate. Cellular DNA was detected by measuring the fluorescence (Ex_{495} nm, Em_{535} nm). The average ± standard deviation of relative fluorescence for compound treated cells (relative to DMSO treated cells) for each concentration (sample ran in triplicate) was calculated and analyzed by a nonlinear regression (GraphPad Prism) for each IC_{50} determination.

5.2.3. *Effect of sulfonamides on cell apoptosis.* Cells were plated at a density of 25,000 cells per well in 96-well plates (culture media: DMEM, 10% FBS). Following 24 h incubation after plating, the cells were treated with sulfonamides at a final concentration of 1.6 nM to 5 µM or DMSO (vehicle control) for 24 h. At the end of 24 h, apoptosis was measured using a Caspase-Glo 3/7 reaction system (Promega) monitoring the activity of Caspase 3/7 according to the manufacturer's protocol. Luminescent signal was measured by adding Caspase-Glo 3/7 reagent to each well. The average and standard deviation of relative luminescence for compound-treated cells

(relative to DMSO treated cells) for each concentration (sample ran in triplicate) was calculated and then analyzed by a nonlinear regression (GraphPad Prism) for IC_{50} determination.

5.2.4. *Synergistic effects of **19** and Doxorubicin on proliferation of MCF-7 and MDA-MB-231 cells.* MCF-7 and MDA-MB-231 cells (ATCC, Manassas, Virginia) were plated at a density of 2,000 cells per well in 96-well plates (culture media: DMEM, 10% FBS) and incubated for 24 h at 37 °C 5% CO_2. After 24 h, **19** was added to cells that afforded a concentration of 1.6 nM to 2 µM in the presence or absence of Doxorubicin (Sigma Aldrich, St. Louis, MO) (1.6 nM to 2 µM in MCF-7 cells and 4 nM to 5 µM in MDA-MB-231 cells) or DMSO as vehicle control and incubated for 72 h at 37 °C 5% CO_2. After 72 h, SYBR Green (1X stock by diluting 10,000 X original stock in PBS) (Life Technologies) was added to the cell lysate. Cellular DNA was detected by measuring the fluorescence (Ex_{495} nm, Em_{535} nm). The average and standard deviation of relative fluorescence for compound-treated cells (relative to DMSO-treated cells) for each concentration (samples run in triplicate) was calculated and analyzed by nonlinear regression (GraphPad Prism) for each IC_{50} determination and Chou-Talalay analysis[46].

6. Supporting Information Available: Synthesis and ^{1}H NMR of compounds **27** – **29**, **32**, **33** and synthesis and ^{1}H NMR of 1,2,3,4-tetrahydroquinoxalines used for synthesis of **22** and **23** are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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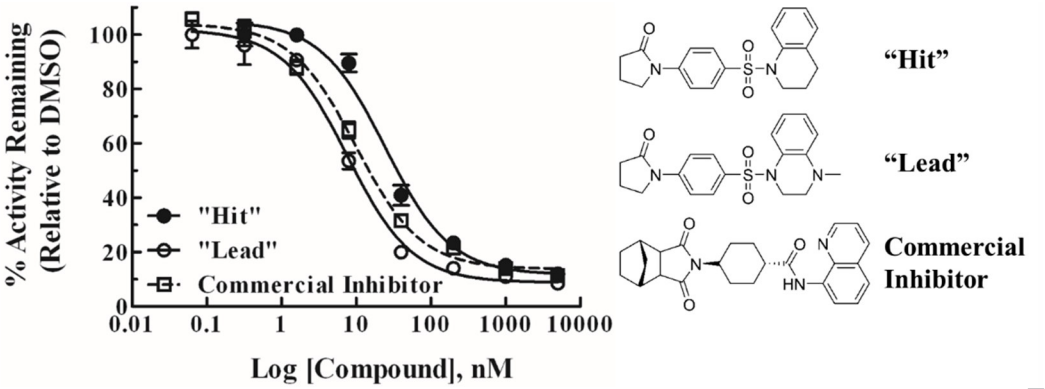

Highlights

- 76,000 compounds were tested for inhibition of a Wnt transcription assay.
- One “hit” compound was found to be reproducibly potent at Wnt inhibition.
- The “hit” was optimized by medicinal chemistry and “lead” **19** was examined.
- Compound **19** potently inhibited human breast cancer cell lines.
- **19** synergized Doxorubicin to inhibit cancer proliferation *in vitro* and *in vivo*.