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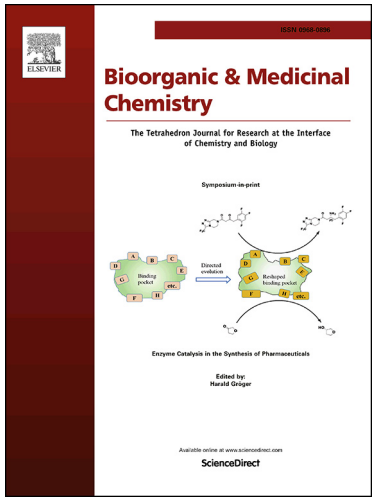

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Synthesis and SAR of Novel Capsazepine Analogs with Significant Anti-Cancer Effects in Multiple Cancer Types

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Abstract

We previously demonstrated that capsazepine (CPZ), a synthetic transient receptor potential Vanilloid subtype 1 (TRPV1) antagonist, has significant anti-cancer effects *in vivo*. The purpose of this study was to develop more potent analogs based upon CPZ pharmacophore and structure-activity relationships (SAR) across analogs. We generated 30 novel compounds and screened for their anti-proliferative effects in cultured HeLa cervical cancer cells. Cell viability assays identified multiple compounds with IC_{50s} < 15 μM and one compound, **29** with an IC_{50} < 5 μM; six fold more potent than CPZ. We validated the anti-proliferative efficacy of two lead compounds, **17** and **29**, *in vivo* using HeLa-derived xenografts in athymic nude mice. Both analogs significantly reduced tumor volumes by day 8 compared to control treated animals ($p<0.001$) with no observable adverse effects. Calcium imaging determined that compound **17** activates TRPV1 whereas **29** neither activates nor inhibits TRPV1; indicating a unique mechanism-of-action that does not involve TRPV1 signaling. Cell viability assays using a panel of additional tumor types including oral squamous cell carcinoma, non-small cell lung cancer (NSCLC), breast cancer, and prostate cancer cell lines (HSC-3, H460, MDA-231, and PC-3 respectively) demonstrated that both lead compounds were efficacious against every cancer type tested. Compounds **29** displayed IC_{50s} of 1-2.5 μM in HSC-3and PC-3cells. Thus, we propose that these novel CPZ analogs may serve as efficacious therapeutic agents against multiple tumor types that warrant further development for clinical application.

Introduction

Our previous studies demonstrate that capsazepine (CPZ), a synthetic transient receptor potential Vanilloid-1 (TRPV1) channel antagonist, has significant anti-tumor effects against oral squamous cell carcinoma (OSCC) via a secondary mechanism-of-action that is independent of TRPV1 interactions.[1] The TRPV1 agonist, capsaicin, is shown to inhibit cellular respiration by virtue of its structural similarity to coenzyme Q (CoQ) and poor redox potential resulting in competitive inhibition of electron transport, production of reactive oxygen species (ROS), and induction of apoptosis.[2] Given that CPZ is structurally similar to capsaicin (Figure 1), we postulated that its anti-cancer effects may also be due to disruption of mitochondrial function. Indeed, we confirmed that CPZ induced high ROS levels and apoptosis *in vitro*.[1] In addition, we demonstrated the efficacy of CPZ to treat OSCC in mouse xenograft models, which displayed significant reductions in tumor growth and increased apoptosis compared to control treated tumors.[1]

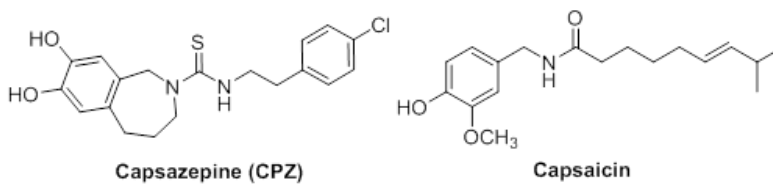


Figure 1. Structure of Capsazepine and Capsaicin

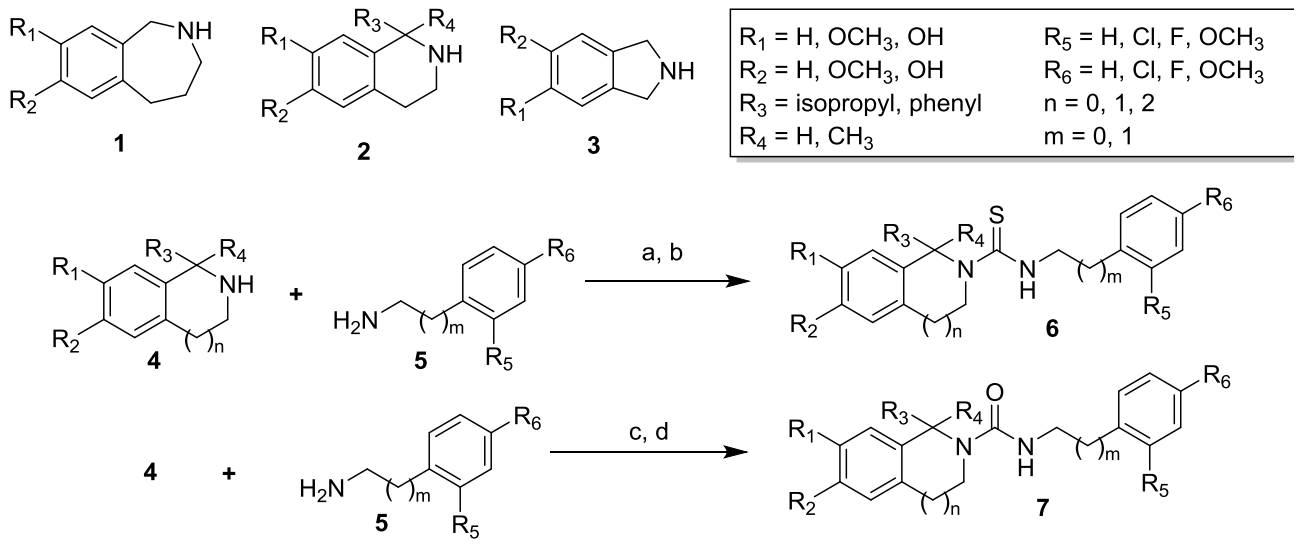
Based upon these findings, we endeavored to generate potent CPZ analogues that yield even greater anti-proliferative effects than the parent compound. To this end, our synthesis and SAR strategy was focused on manipulating both the electronic and steric nature of CPZ, while maintaining good "drug-like" physiochemical properties.[3, 4] There are several reports on the synthesis of CPZ-like analogs, however, the anti-proliferative effects of CPZ analogs and corresponding SAR has not been communicated to date.[5-12] Since our preliminary studies suggested that CPZ provides anti-proliferative activity independent of TRPV1 [1], the known

TRPV1 SAR from the literature on CPZ structural analogs would not likely be a good guide to identify more potent anti-proliferative analogs. Here we describe the synthesis and development of CPZ analogs with significant anti-proliferative effects *in vitro* and *in vivo*. We first screened our compounds based upon their anti-proliferative effects against cultured HeLa cells. Anti-tumor efficacy of lead compounds was validated in HeLa-derived mouse xenograft models. Effects on TRPV1 activity were evaluated by calcium imaging. Lastly, we evaluated the anti-proliferative effects, *in vitro*, against a panel of cancer types (HSC-3, H460, MDA-231, and PC-3) demonstrating their potential to treat multiple types of solid tumors.

Results and Discussion

Synthesis

The synthesis shown in Scheme 1 was used to access the desired CPZ analogs. Various analogs of the secondary amines, represented by compounds **1**, **2**, and **3** were prepared according to literature precedent. Analogs of compound **1** were prepared from the corresponding tetralones.[10] Derivatives of compound **2** were prepared from the corresponding phenethyl amines via a Pictet-Spengler process under thermal or microwave conditions.[13] Analogs of the 5,6-di-



1 2 3 4 5 6 7

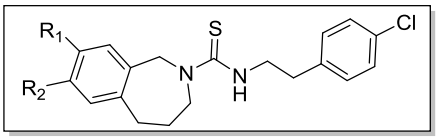
Reagents and Conditions: a) Amine **5**, Thiocarbonyldiimidazole, THF; b) Amine **4**, TEA, DMF, RT, 5-95% yield; c) Amine **5**, Cl_3CCOCl, THF; d) Amine **4**, DBU, DMSO, 80 ^oC, 5-41% yield.

Scheme 1. General route for the synthesis of thioureas 6 and ureas 7

substited isoindoline **3**, were prepared from the corresponding xylene derivatives via benzylic bromination and ring closure.[14] The structural analogs of amines **1**, **2** and **3**, represented by the general structure **4** could then be taken forward to the corresponding thiourea **6** and urea **7**. Treatment of amine **5** with thiocarbonyl diimidazole, followed by treatment of amine **4** produced the corresponding thiourea **6** in moderate to good yield.[15] Alternatively, the amine **5** could be reacted with trichloroacetyl chloride, followed by amine **4** to produce the urea **7**.[16]

Structure-Activity Relationship Studies

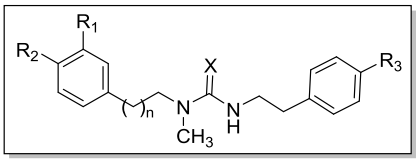
Our SAR studies focused on a systematic manipulation of the phenol substituents, ring size, thiourea and phenethyl side chain moieties. The anti-proliferative SAR on the phenol group of CPZ itself is summarized in Table 1. Removal of one of the phenol groups at either position (**8** and **9**) provided a two-fold decrease in potency relative to CPZ. Similarly, replacement of the phenols with methoxy groups (**10** and **11**) produced analogs completely devoid of anti-proliferative activity. Manipulation of the tetrahydro-1H-benzo[c]azepine ring produced some interesting results. Complete removal of the ring, as represented by the acyclic analogs **12-16** in Table 2 produced a significant reduction in potency relative to CPZ. Interestingly, similar analogs developed by Walpole *et. al.* (1994), demonstrated TRPV1 agonist activity; thus loss of the tetrahydro-1H-benzo[c]azepine ring



Compound	R_1	R_2	[1] HeLa IC_{50} (μM)	MW	log P	tPSA
CPZ	OH	OH	30	376.9	4.02	55.7
8	H	OH	65	360.9	4.72	35.5
9	OH	H	50	360.9	4.72	35.5
10	OCH_3	H	>100	374.93	4.87	24.5
11	H	OCH_3	>100	374.93	4.87	24.5

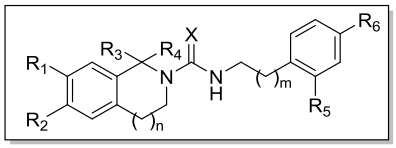
Table 1. SAR on 7-member ring analogs. [1] MTS cell viability assay of HeLa cells treated with 7-member ring analogs for 24h (n=4 per group). Positive control compound CPZ IC_{50} = 30 μM.

structure appears to be critical to CPZ analogs' anti-proliferative effects, but not to their TRPV1 activity.[3] Based on these results, we next turned our attention to 5- and 6-membered ring analogs and studied the effect on three key SAR points; 1) substitution effects on the R_3 and R_4 position of the fused ring systems, 2) thiourea, and urea derivatives, and 3) length and substitution (R_5 and R_6) of the phenethyl side chains (Table 3). These studies produced analogs with improvements in potency relative to CPZ. Installation of the unsubstituted tetrahydroisoquinoline 6-membered ring (**17**) produced significant improvement in anti-proliferative activity (<10 uM) and the corresponding urea analog (**18**) provided analogous activity. Similar to earlier observations, replacement of the phenols with methyl ethers (**19** and **20**) decreased activity substantially. Replacement of the tetrahydroisoquinoline 6-membered ring in **17** with the 5,6-isoindoline 5-membered ring (**21**) also resulted in a marked decrease in cytotoxicity. The observed anti-proliferative effects of these analogs were also found to be dependent on the nature of the phenethyl side chain and the phenyl substitution pattern (R_5 and R_6). Replacement of the para-chloro substituent (R_6) with hydrogen, fluorine, or methoxy decreased the anti-proliferative activity (**22**, **23** and **24**). Similar results were obtained with the incorporation of the 2,4-dichlorophenethyl or substituted benzyl groups, as represented by **25** and



Compound	n	R_1	R_2	R_3	X	[1]HeLa IC_{50} (μM)	MW	log P	tPSA
12	0	OH	OH	Cl	S	>100	350.86	3.93	55.73
13	1	OH	OH	Cl	S	30	364.89	4.22	55.73
14	0	OCH_3	OCH_3	Cl	O	>30	362.85	3.33	50.8
15	1	OH	OH	Cl	O	>30	348.83	3.33	72.8
16	0	OH	OH	Cl	O	30	334.8	3.04	72.8

Table 2. SAR on 7-member ring analogs. [1] MTS cell viability assay of HeLa cells treated with 7-member ring analogs for 24h (n=4 per group). Positive control compound CPZ IC_{50} = 30 μM.



Compound	n	R_1	R_2	R_3	R_4	R_5	R_6	X	m	[1]HeLa IC_{50} (μM)	MW	log P	tPSA
17	1	OH	OH	H	H	H	Cl	S	1	<10	362.87	3.97	55.73
18	1	OH	OH	H	H	H	Cl	O	1	15	346.81	3.08	72.8
19	1	OCH_3	OCH_3	H	H	H	Cl	S	1	>100	390.93	4.26	33.73
20	1	OCH_3	OCH_3	H	H	H	Cl	O	1	>100	374.87	3.37	50.8
21	0	OH	OH	H	H	H	Cl	S	1	85	348.85	3.68	55.73
22	1	OH	OH	H	H	H	OCH_3	S	1	>100	358.46	3.21	64.96
23	1	OH	OH	H	H	H	F	S	1	25	346.42	3.51	55.73
24	1	OH	OH	H	H	H	H	S	1	25	328.43	3.37	55.73
25	1	OH	OH	H	H	Cl	Cl	S	1	30	397.31	4.58	55.73
26	1	OH	OH	H	H	H	H	S	0	>100	314.4	3.08	55.73
27	1	OH	OH	phenyl	H	H	Cl	S	1	<5	438.97	5.76	55.73
28	1	OH	OH	isoPr	H	H	Cl	S	1	55	404.95	5.28	55.73
29	1	OH	OH	phenyl	H	H	Cl	S	0	<5	390.5	4.86	55.73
30	1	OH	OH	phenyl	H	H	H	S	1	25	404.53	5.15	55.73
31	1	OH	OH	isoPr	H	H	H	S	1	67	370.51	4.67	55.73
32	1	OH	OH	phenyl	H	Cl	Cl	S	1	20	473.41	6.36	55.73
33	1	OH	OH	isoPr	H	Cl	Cl	S	1	46	439.4	5.88	55.73
34	1	OH	OH	phenyl	H	H	OCH_3	S	1	24	434.55	4.99	64.96
35	1	OH	OH	phenyl	H	H	F	S	1	17	422.52	5.29	55.73
36	1	OH	OH	isoPr	H	H	F	S	1	28	388.5	4.81	55.73
37	1	OH	OH	isoPr	H	H	Cl	S	0	35	356.48	4.38	55.73

Table 3. SAR on 5- and 6-membered cyclic analogs. [1] MTS cell viability assay of HeLa cells treated with 5- and 6-membered cyclic analogs for 24h (n=4 per group). Positive control compound CPZ IC_{50} = 30 uM.

26. Modification of the R_3 and R_4 positions of the tetrahydroisoquinoline 6-membered ring produced some interesting results. Introduction of a phenyl group at the R_4 position while maintaining the para-chlorophenethyl group produced the thiourea **27**, which showed a marked increase in anti-proliferative activity; however the corresponding isopropyl derivative (**28**) was completely devoid of any appreciable activity. Maintaining the phenyl group in the R_4 position, while shorting the phenethyl chain to para-chlorobenzyl produced the thiourea **29**, which displayed potent anti-proliferative effects similar to **27**. As shown in table 3, (compounds **30** to **37**), further modifications of the R_3, R_4, R_5 and R_6 positions provided analogs with either equivalent activity or decreased activity as compared to **27** and **29**. Based on these SAR studies

and evaluation of physiochemical properties, compounds **17** and **29** were taken forward and assessed in a variety of cancer cell lines, and in a mouse xenograft model.

Compounds 17 and 29 have significant anti-tumor effects in HeLa-derived mouse xenografts. To validate the anti-cancer activities of these compounds, we generated HeLa-derived tumors in athymic nude mice. Once tumors reached 150 mm^3, mice were treated every other day with **17**, **29**, or vehicle control. Both compounds significantly reduced tumor growth by day 8 compared to control treated tumors and maintained significantly reduced tumor growth throughout the remainder of the study ($p<0.001$; Figure 2A). No significant difference in efficacy was seen between **17** and **29**. At the conclusion of the experiment, (day 14) the average tumor volumes for control, **17**, and **29** treated mice were 562 mm^3, 351 mm^3, and 283 mm^3 respectively (Figure 2B; $p<0.01$). There were no observable adverse effects on adjacent non-malignant tissues, neurological function, and respiration in mice treated with **17** and **29** and no reduction of body weight, mobility, or motor functions were noted.

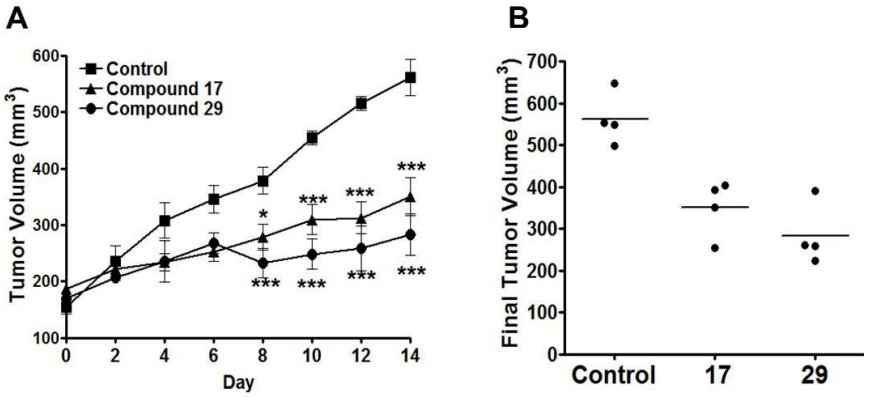


Figure 2. Anti-tumor effects of 17 and 29 in HeLa-derived mouse xenograft models. *Panel A:* Tumor volumes of HeLa-derived xenografts (n=4 per group) treated every other day with 40 μg of indicated analog or vehicle control for a period of 14 days. Significant reduction in tumor volumes is seen by day 8 and continued throughout the duration of the study (*$p<0.05$ and ***$p<0.001$). *Panel B:* Scatter plot of HeLa-derived tumors' final volumes at day 14. Median tumor volume for control tumors was 562 mm^3 whereas median tumor volume for **17**-treated tumors was 351 mm^3 and median tumor volume for **29**-treated tumors was 283 mm^3.

Compounds 17 and 29 have anti-proliferative effects against multiple cancer types in vitro that does not require TRPV1 interactions. The anti-proliferative effects of these lead compounds were tested against a panel of cancer cell lines including OSCC (HSC-3), NSCLC (H460), breast cancer (MDA-231) and prostate cancer (PC-3). Both analogs significantly reduced cell viability of each cancer type tested (Figures 3A and 3B; Table 4). Slight differences in potencies were noted for each analog depending on the cell line tested. Calcium imaging revealed that **17** is a TRPV1 agonist (Figure 3C) and elicits a concentration dependent influx of calcium ions into CHO-TRPV1 cells. In contrast, **29** failed to activate TRPV1 channels in vitro. In addition, pre-treatment of CHO-TRPV1 cells with **29** followed by capsaicin determined that **29** does not inhibit TRPV1 activation

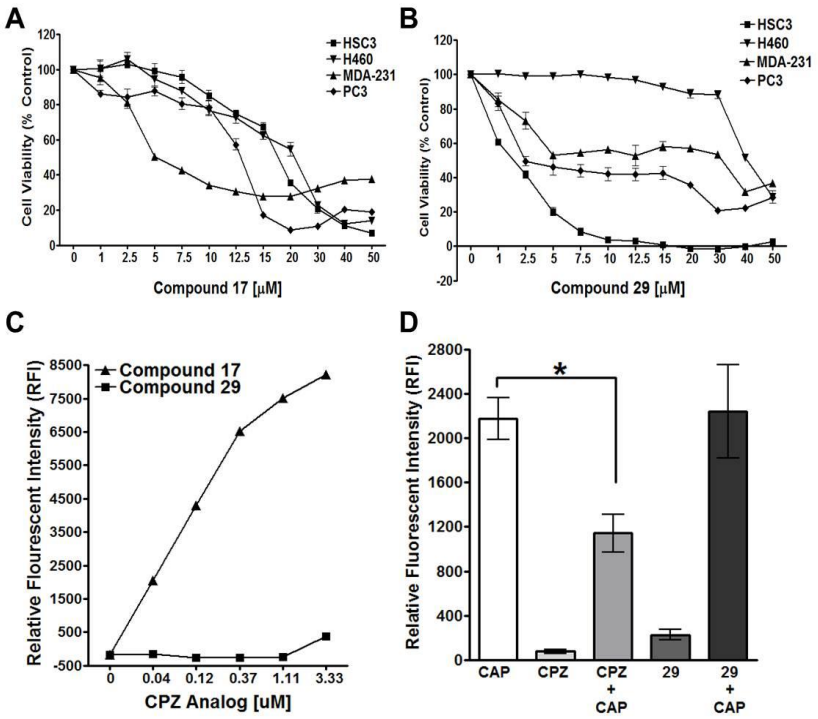


Figure 3: Effects of CPZ analogs on proliferation of multiple cancer types and TRPV1 activation *in vitro*. *Panels A and B:* MTS cell viability assays of HSC-3 (OSCC), MDA-231 (breast cancer), H460 (NSCLC) and PC-3 (prostate cancer) cell lines treated with CPZ analogs **17** and **29** for 24 h; n=4 per group. *Panel C*: Calcium imaging of CHO-TRPV1 cells treated with increasing concentrations of CPZ analogs **17** and **29**. *Panel D:* Calcium imaging of CHO-TRPV1 cells pre-treated with CPZ or **29** followed by capsaicin; *p<0.05.

Cell Line	17	29
HSC-3	20 μM	2 μM
H460	23 μM	42 μM
MDA-231	5 μM	32 μM
PC-3	13 μM	2.5 μM

Table 4. IC_{50s} of Compound 17 and 29 in cancer cell lines.

(Figure 3D). Thus, **29** is neither a TRPV1 agonist nor an antagonist, whose anti-cancer mechanism is independent of TRPV1.

Conclusion

These studies provide evidence that analogs of CPZ can be identified with potent anti-proliferative activity *in vitro* and substantial *in vivo* activity in reducing tumor growth in mouse xenograft models. SAR studies leading to the identification of **17** and **29** were focused on a three-point SAR strategy to assess the anti-proliferative activity of analogs, while providing minimal changes in physiochemical properties. Given that TRPV1 mediates thermoregulation, bronchoconstriction, and pain[16-20] and that TRPV1 interactions do not play a role in CPZ anti-cancer activity, our goal was to eliminate TRPV1 effects, while improving the anti-cancer efficacy. Calcium imaging studies confirm that **17** is a TRPV1 agonist; however **29** neither activates nor inhibits TRPV1 making it an attractive lead compound for further development. Additional SAR studies are underway to determine any other potential TRP channel activity including TRPA1, which can form heterotetromer channels with TRPV1.[21] Notably, no adverse reactions associated with TRPV1 activity were observed with either analog (erythema, ulcerations, or respiratory distress); thus CPZ analogs may provide a novel therapeutic approach for treating cancers.

Experimental Methods

Chemistry

Chemicals were purchased from established commercial suppliers, including Sigma Aldrich (St. Louis, MO), Chembridge Corporation (San Diego, CA), ChemDiv (San Diego, CA), and Specs (Hopkinton, RI), Cayman (Ann Arbor, MI) and Pfaltz & Bauer (Waterbury, CT). The

identity of all the tested compounds was confirmed by ^{1}H NMR and HPLC-MS, and the purity was ensured to be ≥ 95%.

General procedures. Unless otherwise indicated all reactions were conducted in standard commercially available glassware using standard synthetic chemistry methods and setup. All air- and moisture-sensitive reactions were performed under nitrogen atmosphere with dried solvents and glassware under anhydrous conditions. Starting materials and reagents were commercial compounds of the highest purity available and were used without purification. Solvents used for reactions were indicated as of commercial dry or extra-dry or analytical grade. Analytical thin-layer chromatograph (TLC) was carried out using silica gel 60 F_{254} TLC plates. TLC visualization was achieved with a UV lamp or by staining in an iodine chamber. Flash chromatography was done on a system using prepacked silica gel columns or using silica gel 60A (230-400 mesh) or with preparative thin-layer chromatography plates (1000 micron F_{254}), or using a Biotage Isolera One 2.2, using commercial columns that were pre-packed with Merck Kieselgel 60 (230– 400 mesh) silica gel. Solvent systems employed consisted of (EtOAc/Hex or DCM/MeOH or DCM/MeOH/Conc. NH_4OH). All moisture- and air-sensitive reactions and reagent transfers were carried out under dry nitrogen. Final compounds for biological testing are all ≥95% purity as determined by HPLC-MS and ^{1}H NMR.

NMR. ^{1}H NMR experiments were recorded on Agilent DD2 400MHz spectrometers at ambient temperature. Samples were dissolved and prepared in deuterated solvents ($CDCl_3$, CD_3OD and $DMSOd_6$) with residual solvents being used as the internal standard in all cases. All deuterated solvent peaks were corrected to the standard chemical shifts ($CDCl_3$, d_H = 7.26 ppm; CD_3OD, d_H = 3.31 ppm; DMSO-d_6, d_H = 2.50 ppm). Spectra were all manually integrated after automatic baseline correction. Chemical shifts (*d*) are given in parts per million (ppm), and coupling

constants (*J*) are given in Hertz (Hz). The proton spectra are reported as follows: d (multiplicity, coupling constant *J*, number of protons). The following abbreviations were used to explain the multiplicities: app = apparent, b = broad, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dddd = doublet of doublet of doublet of doublets, m = multiplet, s = singlet, t = triplet, ABq = AB quartet.

HPLC-MS. All samples were analyzed on Agilent 1290 series HPLC system comprised of binary pumps, degasser and UV detector, equipped with an auto-sampler that is coupled with Agilent 6150 mass spectrometer. Purity was determined via UV detection with a bandwidth of 170nm in the range from 230-400nm. The general LC parameters were as follows: Column - Zorbax Eclipse Plus C18, size 2.1 X 50 mm; Solvent A: 0.10 % formic acid in water, Solvent B: 0.00 % formic acid in acetonitrile; Flow rate – 0.7 mL/min; Gradient: 5 % B to 95 % B in 5 min and hold at 95 % B for 2 min; UV detector – channel 1 = 254 nm, channel 2 = 254 nm. Mass detector Agilent Jet Stream – Electron Ionization (AJS-ES).

General Procedures

Compounds of general structure **1**, **2** and **3** were prepared according to the referenced literature precedent.

*General procedure for the synthesis of thiourea **6***

To a stirring solution of 1,1'-thiocarbonyldiimidazole (1.2 equiv) in 5 mL of DMF at 50 ^{o}C was added a solution of TEA (1 equiv) and amine **5** (1 equiv) in DMF dropwise. The resulting solution stirred at room temperature for 1-24 hours and was monitored by TLC. The resulting crude solution of the isothiocyanate was used in the next step without purification.

To a stirring solution of amine **4** (1 equiv) and TEA (3 equiv) in DMF (2 mL) was added the crude isocyanate solution prepared above (1.2 equiv) and the resulting mixture was stirred at

room temperature for 2-4 hours. Once complete by TLC, the mixture was diluted with ethyl acetate and washed with excess water and brine. The ethyl acetate layer was collected, dried with sodium sulfate and concentrated *in vacuo*. The resulting crude material was subjected to SiO_2 flash chromatography (eluent: ethyl acetate/DCM gradient) to provide the desired products of general structure **6** in 5-95 % yield.

*General procedure for the synthesis of urea **7***

To a stirring solution of amine **5** (1 equiv) in THF (0.2 M) was add trichloroacetyl chloride (1 equiv) dropwise at room temperature. After 4 hours, the solution was concentrated *in vacuo* to afford the corresponding trichloroacetamide derivatives as a white solids that was used in the next step without purification.

To the amine **4** (1 equiv), in DMSO was added DBU (2 equiv) and the trichloroacetamide (1 equiv) at room temperature. The reaction was heated to 80 ^{o}C and monitored by TLC. After cooling to room temperature, the mixture was diluted with DCM and washed with 1M HCl, saturated $NaHCO_3$ and brine. The organic layer was concentrated *in vacuo* and the resulting crude material was subjected to SiO_2 flash chromatography (eluent: ethyl acetate/DCM gradient) to provide the desired products of general structure **7** in 5-41 % yield.

The following compounds were prepared using the general procedures described above.

N-[2-(4-chlorophenyl)ethyl]-7-hydroxy-2,3,4,5-tetrahydro-1H-2-benzazepine-2-carbothioamide (**8**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.22 – 7.17 (m, 2H), 7.11 – 7.07 (m, 2H), 7.04 (d, *J* = 8.1 Hz, 1H), 6.60 (d, *J* = 2.6 Hz, 1H), 6.48 (dd, *J* = 8.1, 2.6 Hz, 1H), 4.69 (s, 2H), 4.06 (s, 2H), 3.74 (dd, *J* = 7.8, 6.8 Hz, 2H), 2.87 – 2.77 (m, 4H), 1.76 (qt, *J* = 11.3, 5.5 Hz, 2H). ESI-MS m/z $[M + Na]^+$: 383.4

N-[2-(4-chlorophenyl)ethyl]-8-hydroxy-2,3,4,5-tetrahydro-1H-2-benzazepine-2-carbothioamide (**9**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.23 – 7.13 (m, 2H), 7.13 – 7.03 (m, 2H), 6.94 (d, *J* = 8.1 Hz, 1H), 6.80 (d, *J* = 2.6 Hz, 1H), 6.58 (dd, *J* = 8.1, 2.6 Hz, 1H), 4.80 – 4.74 (m, *J* = 3.9 Hz, 2H), 4.01 (s, 2H), 3.78 – 3.69 (m, *J* = 8.0, 6.8 Hz, 2H), 2.88 – 2.77 (m, *J* = 14.1, 7.0 Hz, 4H), 1.74 (quint, *J* = 11.2, 5.6 Hz, 2H). ESI-MS m/z $[M + H]^+$: 361.3

N-[2-(4-chlorophenyl)ethyl]-7-methoxy-2,3,4,5-tetrahydro-1H-2-benzazepine-2-carbothioamide (**10**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.23 – 7.19 (m, 2H), 7.15 (d, *J* = 8.3 Hz, 1H), 7.13 – 7.09 (m, 2H), 6.74 (d, *J* = 2.7 Hz, 1H), 6.62 (dd, *J* = 8.2, 2.7 Hz, 1H), 4.75 (s, 2H), 4.09 (s, 2H), 3.79 – 3.74 (m, 5H), 2.94 – 2.83 (m, *J* = 20.0, 10.2, 4.9 Hz, 4H), 1.79 (quint, *J* = 11.4, 5.6 Hz, 2H). ESI-MS m/z $[M + H]^+$: 375.3

N-[2-(4-chlorophenyl)ethyl]-8-methoxy-2,3,4,5-tetrahydro-1H-2-benzazepine-2-carbothioamide (**11**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.22 – 7.13 (m, 2H), 7.11 – 7.03 (m, 3H), 6.93 (d, *J* = 2.7 Hz, 1H), 6.73 (dd, *J* = 8.3, 2.7 Hz, 1H), 4.83 – 4.77 (m, *J* = 4.2 Hz, 2H), 4.08 (s, 2H), 3.81 – 3.69 (m, 5H), 2.91 – 2.81 (m, 4H), 1.78 (quint, *J* = 11.3, 5.6 Hz, 2H). ESI-MS m/z $[M+ H]^+$: 375.3

1-[2-(4-chlorophenyl)ethyl]-3-[(3,4-dihydroxyphenyl)methyl]-3-methylthiourea (**12**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.29 – 7.23 (m, 2H), 7.23 – 7.18 (m, 2H), 6.75 – 6.69 (m, *J* = 5.2 Hz, 2H), 6.56 (dd, *J* = 8.1, 2.1 Hz, 1H), 4.59 (s, 3H), 3.85 – 3.79 (m, 2H), 2.97 (s, 2H), 2.93 (t, 2H). ESI-MS m/z $[M+H]^+$: 351.2

1-[2-(4-chlorophenyl)ethyl]-3-[2-(3,4-dihydroxyphenyl)ethyl]-3-methylthiourea (**13**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.27 (m, 2H), 7.16 – 7.11 (m, 2H), 6.80 (d, *J* = 8.0 Hz 1H), 6.71 (d, *J* = 2.0 Hz, 1H), 6.60 (dd, *J* = 8.0, 2.0 Hz, 1H), 3.89 – 3.79 (m, *J* = 12.2, 4.8 Hz, 4H), 2.98 (s, 3H), 2.85 (t, *J* = 6.9 Hz, 2H), 2.78 (t, *J* = 7.2 Hz, 2H). ESI-MS m/z [M+H]: 365.3

1-[2-(4-chlorophenyl)ethyl]-3-[(3,4-dimethoxyphenyl)methyl]-3-methylurea (**14**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 – 7.20 (m, 2H), 7.09 – 7.04 (m, 2H), 6.80 (d, *J* = 8.1 Hz, 1H), 6.74 (d, *J* = 1.9 Hz, 1H), 6.70 (dd, *J* = 8.1, 2.0 Hz, 1H), 4.38 (s, 2H), 3.88 (s, 3H), 3.86 (s, 3H), 3.48 (dd, *J* = 12.1, 6.7 Hz, 2H), 2.84 (s, 3H), 2.78 (dd, *J* = 8.3, 5.3 Hz, 2H). ESI-MS m/z $[M+H]^+$: 363.3

1-[2-(4-chlorophenyl)ethyl]-3-[2-(3,4-dihydroxyphenyl)ethyl]-3-methylurea (**15**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 – 7.20 (m, 2H), 7.09 – 7.04 (m, 2H), 6.80 (d, *J* = 8.1 Hz, 1H), 6.74 (d, *J* = 1.9 Hz, 1H), 6.70 (dd, *J* = 8.1, 2.0 Hz, 1H), 4.38 (s, 2H), 3.88 (s, 3H), 3.86 (s, 3H), 3.48 (dd, *J* = 12.1, 6.7 Hz, 2H), 2.84 (s, 3H), 2.78 (dd, *J* = 8.3, 5.3 Hz, 2H). ESI-MS m/z [M+H]:363.3

1-[2-(4-chlorophenyl)ethyl]-3-[(3,4-dihydroxyphenyl)methyl]-3-methylurea (**16**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.20 – 7.15 (m, 2H), 7.12 – 7.07 (m, 2H), 6.63 (d, *J* = 8.0 Hz, 1H), 6.57 (d, *J* = 2.1 Hz, 1H), 6.42 (dd, *J* = 8.0, 2.1 Hz, 1H), 4.24 (s, 2H), 3.33 – 3.27 (m, 2H), 2.71 (d, *J* = 7.6 Hz, 2H), 2.68 (s, 3H). ESI-MS m/z $[M+H]^+$: 335.3

N-[2-(4-chlorophenyl)ethyl]-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**17**). ^{1}H NMR (400 MHz, DMSO) δ 8.73 (d, *J* = 6.8 Hz, 2H), 7.65 (t, *J* = 5.2 Hz, 1H), 7.30 (d, *J* = 8.2 Hz, 2H), 7.20 (d, *J* = 8.3 Hz, 2H), 6.50 (s, 1H), 6.46 (s, 1H), 3.82 (t, *J* = 5.8 Hz, 2H), 3.65 (dd, *J* = 14.1, 6.1 Hz, 2H), 2.60 (t, *J* = 5.7 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 180.91, 144.41, 144.16, 139.05, 131.08, 130.93, 128.66, 125.70, 124.17, 115.43, 113.41, 49.11, 46.91, 45.88, 34.58, 27.79. ESI-MS m/z $[M+H]^+$:363.2

N-[2-(4-chlorophenyl)ethyl]-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline-2-carboxamide (**18**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.23 – 7.20 (m, 2H), 7.17 – 7.13 (m, 2H),

6.53 (s, 1H), 6.51 (s, 1H), 4.32 (s, 2H), 3.50 (t, *J* = 5.9 Hz, 2H), 3.39 – 3.32 (m, 2H), 2.76 (t, 2H), 2.63 (t, *J* = 5.9 Hz, 2H). ESI-MS m/z $[M+H]^+$: 347.3

N-[2-(4-chlorophenyl)ethyl]-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**19**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 – 7.25 (m, 2H), 7.17 – 7.12 (m, 2H), 6.66 (s, 1H), 6.62 (s, 1H), 4.76 (s, 2H), 3.98 – 3.91 (m, *J* = 11.7, 5.9 Hz, 2H), 3.88 – 3.80 (m, 8H), 2.95 (t, *J* = 6.9 Hz, 2H), 2.82 (t, *J* = 5.9 Hz, 2H). ESI-MS m/z $[M+H]^+$: 391.3

N-[2-(4-chlorophenyl)ethyl]-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-2-carboxamide (**20**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 – 7.26 (m, 2H), 7.15 – 7.10 (m, 2H), 6.64 (s, 1H), 6.59 (s, 1H), 4.40 (s, 2H), 3.86 (s, 3H), 3.85 (s, 3H), 3.57 – 3.53 (m, 2H), 3.53 – 3.48 (m, 2H), 2.82 (t, *J* = 6.9 Hz, 2H), 2.76 (t, *J* = 6.0 Hz, 2H). ESI-MS m/z $[M+H]^+$: 375.3

N-[2-(4-chlorophenyl)ethyl]-5,6-dihydroxy-2,3-dihydro-1H-isoindole-2-carbothioamide (**21**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.32 – 7.22 (m, 4H), 6.70 (s, 2H), 3.85 – 3.78 (m, *J* = 8.3, 6.8 Hz, 2H), 2.95 (t, 2H). ESI-MS m/z $[M+H]^+$: 349.2

6,7-dihydroxy-N-[2-(4-methoxyphenyl)ethyl]-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**22**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.15 – 7.10 (m, 2H), 6.84 – 6.79 (m, 2H), 6.59 (s, 1H), 6.55 (s, 1H), 4.68 (s, 2H), 3.90 (t, *J* = 5.9 Hz, 2H), 3.83 – 3.77 (m, *J* = 7.5, 5.9 Hz, 2H), 3.75 (s, 3H), 2.87 (t, *J* = 8.0, 2H), 2.73 (t, *J* = 5.9 Hz, 2H). ESI-MS m/z $[M+H]^+$: 359.1

N-[2-(4-fluorophenyl)ethyl]-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**23**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.24 – 7.17 (m, 2H), 7.01 – 6.92 (m, 2H), 6.57 (s, 1H), 6.54 (s, 1H), 4.67 (s, 2H), 3.88 (t, *J* = 5.9 Hz, 2H), 3.79 (t, 2H), 2.90 (t, 2H), 2.71 (t, *J* = 5.9 Hz, 2H). ESI-MS m/z $[M+H]^+$: 347.1

6,7-dihydroxy-N-(2-phenylethyl)-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**24**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.31 – 7.13 (m, 5H), 6.59 (s, 1H), 6.55 (s, 1H), 4.69 (s, 2H), 3.90

(t, *J* = 5.9 Hz, 2H), 3.83 (t, 2H), 2.94 (t, 2H), 2.73 (t, *J* = 5.9 Hz, 2H). ESI-MS m/z $[M+H]^+$: 329.1

N-[2-(2,4-dichlorophenyl)ethyl]-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**25**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.41 (d, *J* = 2.1 Hz, 1H), 7.27 (d, 1H), 7.20 (dd, *J* = 8.0, 2.2 Hz, 1H), 6.59 (s, 1H), 6.55 (s, 1H), 4.69 (s, 2H), 3.94 – 3.83 (m, 4H), 3.10 (d, *J* = 4.0 Hz, 2H), 2.73 (t, *J* = 5.9 Hz, 2H). ESI-MS m/z $[M+H]^+$: 397.1

N-benzyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**26**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.41 (d, *J* = 2.1 Hz, 1H), 7.27 (d, 1H), 7.20 (dd, *J* = 8.0, 2.2 Hz, 1H), 6.59 (s, 1H), 6.55 (s, 1H), 4.69 (s, 2H), 3.94 – 3.83 (m, 4H), 3.10 (d, *J* = 4.0 Hz, 2H), 2.73 (t, *J* = 5.9 Hz, 2H). ESI-MS m/z $[M+H]^+$: 397.1

N-[2-(4-chlorophenyl)ethyl]-6,7-dihydroxy-1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**27**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 – 7.18 (m, 5H), 7.14 – 7.05 (m, 4H), 6.90 (s, 1H), 6.70 (s, 1H), 6.63 (s, 1H), 5.54 (s, 1H), 3.93 (q, *J* = 12.0, 8.0 Hz, 2H), 3.71 – 3.62 (m, 2H), 2.90 (td, *J* = 6.8, 2.4 Hz, 2H), 2.68 – 2.54 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 180.90, 144.99, 143.93, 142.62, 138.97, 131.09, 130.99, 128.64, 128.46, 127.64, 127.32, 125.87, 115.67, 115.38, 47.09, 40.59, 34.50, 26.92. ESI-MS m/z $[M+H]^+$: 439.1

N-[2-(4-chlorophenyl)ethyl]-6,7-dihydroxy-1-(propan-2-yl)-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**28**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (d, *J* = 7.3 Hz, 2H), 7.12 (d, *J* = 8.3 Hz, 2H), 6.55 (s, 2H), 5.51 (s, 1H), 4.00 – 3.87 (m, 2H), 3.57 – 3.47 (m, 1H), 3.00 – 2.84 (m, 3H), 2.72 (s, 1H), 2.00 – 1.91 (m, 1H), 0.96 (d, *J* = 6.8 Hz, 3H), 0.86 (d, *J* = 6.6 Hz, 3H). ESI-MS m/z $[M+H]^+$: 405.2

N-benzyl-6,7-dihydroxy-1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**29**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.22 (m, 10H), 6.76 (s, 1H), 6.67 (s, 1H), 5.77 (s, 1H), 4.90

(qd, *J* = 14.4, 4.7 Hz, 2H), 3.90 – 3.70 (m, 2H), 2.84 – 2.59 (m, 2H). ^{13}C NMR (100 MHz, Acetone) δ 183.35, 145.41, 144.28, 143.38, 140.69, 129.00, 128.96, 128.87, 128.43, 128.33, 128.31, 128.19, 127.75, 127.55, 127.53, 115.85, 115.82, 62.42, 50.06, 49.93, 43.18, 27.88. ESI-MS m/z $[M+H]^+$: 391.2

6,7-dihydroxy-1-phenyl-N-(2-phenylethyl)-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**30**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.12 (m, 10H), 6.97 (s, 1H), 6.73 (s, 1H), 6.67 (s, 1H), 5.53 (s, 1H), 5.33 (d, *J* = 6.5 Hz, 1H), 3.99 (s, 2H), 3.74 – 3.60 (m, 2H), 3.02 – 2.87 (m, 2H), 2.73 – 2.53 (m, 2H). ESI-MS m/z $[M+H]^+$: 405.2

6,7-dihydroxy-N-(2-phenylethyl)-1-(propan-2-yl)-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**31**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.27 (m, 2H), 7.23 – 7.17 (m, 2H), 6.61 (s, 1H), 6.59 (s, 1H), 5.40 (t, *J* = 2.0 Hz, 1H), 5.32 – 5.24 (m, 1H), 4.01 – 3.86 (m, 2H), 3.58 – 3.49 (m, 1H), 2.99 – 2.86 (m, 3H), 2.79 – 2.68 (m, 1H), 2.03 – 1.92 (m, 1H), 0.95 (d, *J* = 6.9 Hz, 3H), 0.86 (d, *J* = 6.6 Hz, 3H). ESI-MS m/z $[M+H]^+$: 371.2

N-[2-(2,4-dichlorophenyl)ethyl]-6,7-dihydroxy-1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**32**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, *J* = 1.8 Hz, 2H), 7.29 – 7.21 (m, 3H), 7.19 – 7.11 (m, 3H), 7.03 (s, 2H), 6.74 (s, 2H), 6.67 (s, 2H), 5.63 (s, 2H), 3.97 (ddd, *J* = 12.0, 6.8, 2.6 Hz, 2H), 3.70 (d, 1H), 3.08 (octet, *J* = 13.8, 6.8 Hz, 2H), 2.76 – 2.57 (m, 2H). ESI-MS m/z $[M+H]^+$: 473.1

N-[2-(2,4-dichlorophenyl)ethyl]-6,7-dihydroxy-1-(propan-2-yl)-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**33**). ^{1}H NMR (400 MHz, DMSO) δ 8.82 – 8.59 (m, 2H), 7.52 – 7.50 (m, 1H), 7.29 – 7.21 (m, 2H), 6.49 (s, 1H), 6.45 (s, 1H), 3.77 – 3.64 (m, 2H), 3.54 – 3.40 (m, 1H), 3.28 – 3.17 (m, 1H), 3.00 – 2.89 (m, 2H), 2.75 – 2.61 (m, 2H), 1.98 – 1.85 (m, 1H), 0.86 (d, *J* = 6.4 Hz, 7H). ESI-MS m/z $[M+H]^+$: 423.2

6,7-dihydroxy-N-[2-(4-methoxyphenyl)ethyl]-1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**34**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 – 7.20 (m, 1H), 7.11 (dd, *J* = 18.9, 7.7 Hz, 4H), 6.93 (s, 1H), 6.83 (d, *J* = 8.5 Hz, 2H), 6.71 (s, 1H), 6.66 (s, 1H), 5.52 (s, *J* = 4.0 Hz, 1H), 5.38 (d, *J* = 27.8 Hz, 2H), 3.99 – 3.88 (m, 2H), 3.80 (s, 1H), 3.74 – 3.60 (m, 2H), 2.95 – 2.82 (m, 1H), 2.73 – 2.55 (m, 1H). ESI-MS m/z $[M+H]^+$: 435.2

N-[2-(4-fluorophenyl)ethyl]-6,7-dihydroxy-1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**35**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.33 – 7.14 (m, 7H), 6.97 (t, *J* = 8.8 Hz, 2H), 6.63 (s, 2H), 3.92 – 3.77 (m, 3H), 3.53 – 3.36 (m, 2H), 2.95 (t, *J* = 7.3 Hz, 2H), 2.77 – 2.68 (m, 1H), 2.54 – 2.46 (m, 1H). ESI-MS m/z $[M+H]^+$: 423.2

N-[2-(4-fluorophenyl)ethyl]-6,7-dihydroxy-1-(propan-2-yl)-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**36**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.22 – 7.16 (m, 2H), 6.98 – 6.92 (m, 2H), 6.58 (s, 2H), 3.84 – 3.77 (m, 2H), 3.61 (quintet, *J* = 12.7, 6.3 Hz, 1H), 2.91 (dt, *J* = 8.0, 2.4 Hz, 2H), 2.82 – 2.73 (m, 1H), 2.06 – 2.00 (m, 1H), 1.01 – 0.92 (m, 6H). ESI-MS m/z $[M+H]^+$: 389.1

N-benzyl-6,7-dihydroxy-1-(propan-2-yl)-1,2,3,4-tetrahydroisoquinoline-2-carbothioamide (**37**). ^{1}H NMR (400 MHz, CD_3OD) δ 7.33 – 7.09 (m, 5H), 6.60 (d, *J* = 2.5 Hz, 2H), 4.92 (d, *J* = 15.6 Hz, 2H), 3.72 (dt, *J* = 12.6, 6.3 Hz, 1H), 2.98 – 2.78 (m, 2H), 2.12 – 2.01 (m, 1H), 1.03 (d, *J* = 6.8 Hz, 3H), 0.96 (d, *J* = 6.6 Hz, 3H). ESI-MS m/z $[M+H]^+$:357.2

Physiochemical Property Calculations

Data were archived and analyzed using the CDD Vault from Collaborative Drug Discovery (Burlingame, CA. https://www.collaborativedrug.com). Within CDD Vault, ChemAxon's JChem Base was used for structure searching, chemical database access and

management, and chemical property calculations. JChem v17.7.0, 2017, ChemAxon (http://www.chemaxon.com)

Reagents

Working stocks of CPZ, **17,** and **29** were made fresh prior to each experiment by diluting each compound in 100% EtOH to a final stock concentration of 100 mM. If needed, stocks were stored at -20 ºC for periods no longer than 48 h.

Cell Lines

HeLa (cervical cancer), MDA-231 (breast cancer), H460 (NSCLC), and PC-3 (prostate cancer) cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). HSC-3 cells were kindly provided by Dr. Brian Schmidt at New York University College of Dentistry. Cell lines were authenticated prior to use by Genetica DNA Laboratories (Burlington, NC). Cells were maintained in DMEM culture medium with high glucose (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained at 37°C in 5% CO_2. Fore calcium imaging studies we obtained CHO cells overexpressing TRPV1 (CHO-TRPV1), kindly provided by Dr. Ardem Patapoutian at the Scripps Research Institute and cultured as previously described at 37°C in 5% CO_2.[22]

MTS Cell Viability Assays

The Cell Titer 96 ® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) was used to assess cell viability in response to treatments according to manufacturer's protocol. Cells were plated and treated for 24 h with serum free DMEM containing CPZ analogs at the indicated concentrations as previously described [1]. Final EtOH concentrations were maintained at 0.1% or less. Absorbance values of test groups were compared to vehicle-treated controls (n=4).

Mouse Efficacy Studies

All studies were approved by the UT Health Institutional Animal Care and Use Committee and follow the ARRIVE guidelines. Six week-old female athymic nude mice (Envigo Laboratories, Indianapolis, IN) were used in a laminar air-flow cabinet under pathogen-free conditions. Mice were provided with a 12 h light/dark schedule at controlled temperature and humidity with food and water ad libitum and acclimated for one week prior to study initiation.

Mice were injected subcutaneously in the right flank with 6×10^6 HeLa cells in 0.1 ml of sterile PBS as previously described. [1] Two weeks post-inoculation, tumors grew to an average volume of 170 mm^3 and mice were stratified into three experimental groups (n=4 per group) which received the following treatments via intra-tumor injection: vehicle control (100 µl of 5% EtOH diluted in sterile saline) or 40 µg of **17** and **29** diluted in 100 µl sterile saline (final concentration of 5% EtOH). Treatments were repeated every day for a period of 14 days. Mice were monitored daily for tumor growth (using digital calipers), cachexia, and weight loss. Tumor volumes were calculated by the elliptical formula: 1/2(length x $width^2$). [1, 23]

Calcium Imaging

Calcium imaging was performed using FLIPR Calcium 6 Evaluation Kit (Molecular Devices, LLC, Sunnyvale, CA, USA) according to the manufacturer's protocol and as previously described.[1] CHO-TRPV1 cells ($9x10^3$) were plated in a 384 well plate, loaded with Calcium 6 dye for 2 h and the experiment run on Pherastar FS multimode plate reader (BMG Labtech, Cary, NC, USA). Dose response curves were performed followed by analysis of 1 µM of compounds **17** and **29** compared to 100 nM capsaicin (positive control) and 1µM CPZ (negative control; n=3 per group). Effects on calcium influx were measured by changes in fluorescent

intensity (535 nm). In addition, cells were pre-treated with 1 μM CPZ, **17**, or **29** followed by 100nM capsaicin to determine potential TRPV1 inhibitory effects of CPZ analogs.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism4 (San Diego, CA, USA). Calcium imaging results were analyzed by one-way ANOVA and Dunnett's multiple comparison post-tests. Tumor growth was analyzed by two-way ANOVA with repeated measures and Bonferroni's post-hoc tests. A p value less than 0.05 was considered statistically significant.

Key Words: Capsazepine, TRPV1, Structure-Activity Relationships, Solid Tumors, Cancer Therapy

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