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Cinnamides as selective small-molecule inhibitors of a cellular model of breast cancer stem cells

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ABSTRACT

A high-throughput screen (HTS) was conducted against stably propagated cancer stem cell (CSC)-enriched populations using a library of 300,718 compounds from the National Institutes of Health (NIH) Molecular Libraries Small Molecule Repository (MLSMR). A cinnamide analog displayed greater than 20-fold selective inhibition of the breast CSC-like cell line (HMLE_sh_Ecad) over the isogenic control cell line (HMLE_sh_eGFP). Herein, we report structure–activity relationships of this class of cinnamides for selective lethality towards CSC-enriched populations.

There is a growing consensus that many cancers, including breast cancer, contain a small population of cancer stem-like cells (CSCs) that are responsible for the initiation and propagation of cancers.[1] CSCs appear to be resistant to many current treatments and may be the basis for all too frequent relapse.[2] Since conventional anti-cancer drugs do not specifically target CSCs, there exists a need to find small molecules that are selectively toxic to CSCs. Cell viability assays conducted directly on the bulk population of tumor cells have not identified compounds selectively lethal to CSCs since the latter constitute a small portion of the total population of cancer cells.[3] In theory, a phenotypic high-throughput screen (HTS) conducted directly on CSCs with libraries of small molecules would lead to useful starting points for developing therapeutics against CSCs. Unfortunately, even after significant efforts, isolation, characterization and culturing of CSCs is not yet practical.[4]

Recently, an in vitro assay measuring cell viability using surrogate CSC-like populations that can be maintained with high purity has been developed.[3,5] The assay used a stable cell line that was induced to undergo epithelial-to-mesenchymal transition (EMT), through the introduction of a short hairpin RNA targeting the *CDH1* gene, as a method to stably propagate CSC-enriched populations. The availability of essentially isogenic control cell line (HMLE_sh_GFP), which is a mammary epithelial cell line which has not gone through EMT, for the secondary validation assay of the primary screen, minimized the probability of finding promiscuous compounds that are not selective for CSCs but rather target genetic differences between screened cell lines.

Compounds identified using this strategy were shown to selectively eradicate CSCs in heterogeneous cancer cell populations that were not experimentally induced into EMT.[5]

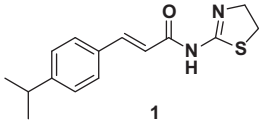

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Figure 1. Prioritized cinnamide scaffold identified from HTS screen.

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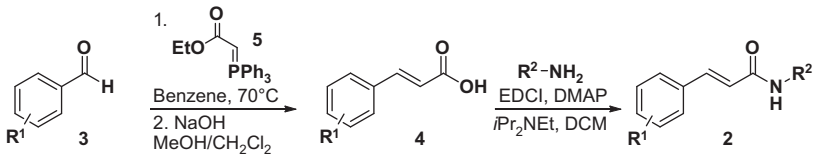
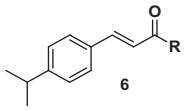


Scheme 1. General reaction scheme of analog synthesis.

Table 1
Various amide core modifications



Analog	R	EC_{50}[a] (μM)		Selectivity[c]
		HMLE_sh_eGFP	HMLE_shEcad (control)	
1		2.35	50.55	21.5
6a		Inactive[b]	Inactive	NA
6b		21.10	Inactive	21.10 versus inactive
6c		13.33	27.00	2.03
6d		3.55	7.26	2.05
6e		13.60	9.97	0.73
6f		51.60	Inactive	51.60 versus inactive
6g		4.65	5.97	1.28
6h		3.44	3.28	0.95
6i		59.65	123.3	2.07
6j		Inactive	Inactive	NA
6k		Inactive	Inactive	NA

[a] Average of at least three runs.
[b] Inactive = No inhibition at highest dose (20 μM).
[c] Selectivity = EC_{50} (HMLE_sh_eGFP)/EC_{50} (HMLE_shEcad).

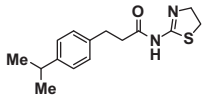
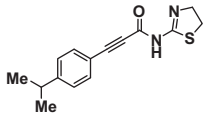
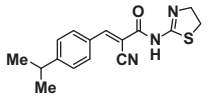
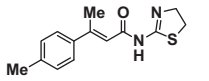
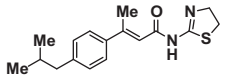
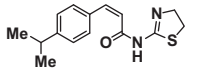
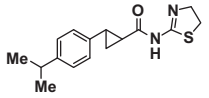
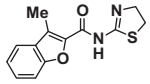
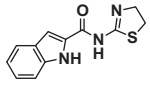
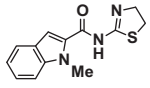
Herein, we report the identification of cinnamides as a new class of small molecules that can selectively kill breast CSC-like cells. These compounds should facilitate future studies of CSCs.

A high-throughput screen (HTS) of >300,000 compounds from the NIH MLPCN collection was performed using the assay developed by Gupta, Lander and co-workers and all data was subsequently submitted to PubChem (AID 2127).[6] A cinnamide derivative **1** was one scaffold identified as selectively cytotoxic to CSC-like cells and prioritized for further optimization (Fig. 1). The structure–activity relationships of cinnamide analog **1** is the subject of this communication.[7]

Analogs of **2** were synthesized either from aldehydes **3** or commercially available cinnamic acids **4**, as illustrated in Scheme 1. Treatment of **3** with Wittig reagent **5** followed by hydrolysis provided cinnamic acids **4** which upon coupling with various amines afforded the cinnamide analogs **2**.

Results from cellular assays for the series are described in the tables below. As can be seen in Table 1 none of the modifications of the amide core on the eastern portion of the molecule was tolerated. The free acid **6a** was found to be inactive and methylation of the amide **6b** results in a 10-fold decrease in potency. Introduction of a carbonyl functionality in the thiazoline core **6c** was not tolerated. Replacement of the thiazoline with the corresponding thiazole **6d** or other five membered heteroaromatics **6e–g** resulted in a significant drop in potency and selectivity. The analog with a larger aromatic group **6h** resulted in a drop in potency and selectivity. Alkyl amides **6i–k** demonstrated poor or no potency in cell based assays.

Table 2
Various olefin modifications

Analog	Structure	EC_{50}[a] (μM)		Selectivity[c]
		HMLE_shEcad	HMLE_sh_eGFP (control)	
7		9.91	Inactive[b]	9.91 versus inactive
8		Inactive	Inactive	NA
9		Inactive	Inactive	NA
10		Inactive	Inactive	NA
11		Inactive	Inactive	NA
12		Inactive	Inactive	NA
13		Inactive	Inactive	NA
14		Inactive	Inactive	NA
15		Inactive	Inactive	NA
16		Inactive	Inactive	NA

[a] Average of at least three runs.
[b] Inactive = No inhibition at highest dose (20 μM).
[c] Selectivity = EC_{50} (HMLE_sh_eGFP)/EC_{50} (HMLE_shEcad).

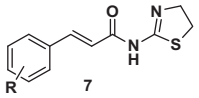
The effect of modification of the olefin is illustrated in Table 2. As can be seen, modification of the olefin of the cinnamide core resulted in a severe loss of activity. Changes in oxidation state (compounds **7** and **8**) and substitution at the α- or β-position (compounds **9**, **10**, and **11**) failed to result in compounds with activity. The *cis*-olefin **12** and cyclopropyl derivatives **13** were found to be poorly active. Replacement of the cinnamic amide moiety with different heteroaromatics such as indoles and benzofurans did not result in compounds with useful activity against the HMLE_shEcad cell line (compounds **14**–**16**).

In addition to the unsubstituted phenyl **17a**, we studied the effects of mono-substitution of chloro, methyl, methoxy, and cyano at the *ortho*, *meta*, and *para* positions of the phenyl ring and these results are summarized in Table 3. Removal of substituents from the aromatic ring, **17a**, resulted in a complete loss of activity. Substitution at the *ortho*-position or the *meta*-position of the phenyl ring with several different substituents resulted in mostly inactive compounds, while substitution at the *para*-position provided compounds with moderate activity.

The cellular data from analogs with a di- or tri-substituted phenyl ring on the western portion of the molecule is described in Table 4. 2,4-Dimethoxy substitution **18a** resulted in a decrease in potency (EC_{50} = 9.91 μM), but complete selectivity over the control cell line and a significant increase in PBS solubility (30.8 μM). Other substitutions with methoxy groups resulted in compounds with no activity **18b–e**. Disubstitution with chloro-groups provided only inactive compounds **18f–h**. Compounds with 2,4-disubstitution that contain either a larger alkoxy group **18i** or *i*-propyl group **18j** at the 4-position were synthesized in an attempt to improve the potency of the 2,4-dimethoxy compound while taking advantage of its selectivity and solubility; however, these compounds resulted in a loss of potency.

Based on the SAR describe above, we further investigated thiazoline cinnamides with substitutions at the *para*-position and these results are presented in Table 5. Two compounds that retained activity and full inhibition are the 4-ethyl analog **19d** and the 4-*i*-butyl compound **19f**. The ethyl analog **19d** resulted in a slight increase in potency, good improvement in selectivity and clear enhancement in solubility (11.5 μM for **19d** vs <1 μM for the hit compound **1**). The 4-*i*-butyl compound **19f** resulted in a significant increase in potency with a small loss of selectivity. Larger substituents provided potent compounds, such as, 4-phenyl substitution **19g**, which is slightly more potent and selective than **1**; however, it was observed to be a partial inhibitor possibly due to low solu-

Table 3
SAR of monosubstituted phenyl cinnamides



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Analog	R	EC_{50}[a]s (μM)		Selectivity[c]
		HMLE_shEcad	HMLE_sh_eGFP (control)	
17a	H	Inactive[b]	Inactive	NA
17b	2-Cl	35.0	Inactive	35.0 versus inactive
17c	3-Cl	14.95	21.5	1.44
17d	4-Cl	13.60	9.97	0.73
17e	2-Me	Inactive	Inactive	NA
17f	3-Me	Inactive	Inactive	NA
17g	4-Me	16.00	Inactive	16.00 versus inactive
17h	2-OMe	Inactive	93.3	NA
17i	3-OMe	62.15	96.20	1.55
17j	4-OMe	Inactive	Inactive	NA
17k	2-CN	Inactive	Inactive	NA
17l	3-CN	Inactive	Inactive	NA
17m	4-CN	26.93	Inactive	26.93 versus inactive

[a] Average of at least three runs.
[b] Inactive = no inhibition at highest dose (20 μM).
[c] Selectivity = EC_{50} (HMLE_sh_eGFP)/EC_{50} (HMLE_shEcad).

bility. In an effort to overcome the low solubility, related pyridine analog **19h** was synthesized. Although full inhibition was observed with **19h**, loss of selectivity prohibits further advancement.

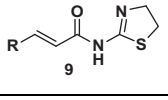
Based on the SAR described above, the 4-ethyl substituted analog **19d** was designated as a probe with the NIH Molecular Libraries Program (ML243) due to its optimal potency, excellent selectivity, and solubility profile.

The solubility of **19d** was experimentally determined to be 11.5 μM in phosphate-buffered saline (PBS, pH 7.4, 23 °C) solution (Table 6). Plasma protein binding (PPB) was determined to be 96.6% bound in human plasma. The probe is stable in human plasma, with approximately 97.1% remaining after a 5-h incubation period. The compound was found to be stable in glutathione (GSH) with 82% remaining after 48 h. The stability of **19d** in PBS (0.1% DMSO) was measured. More than 75% was present after 48 h of incubation.

Compound **19d** was screened against a panel of 68 different targets that are commonly used in drug discovery for lead profiling. The assays were done by Ricerca Biosciences, LLC and include targets from various areas such as, GPCRs, ion channels, and transporters. Compound **19d** was only active in one of the 68 assays, where **19d** blocked a radio-labeled ligand to the Adenosine A_{2A} Receptor by 51% at 10 μM.[8] As the assay used to identify this scaffold was phenotypic, the activity against Adenosine A_{2A} must be considered as these compounds are used in efforts to identify a potential target.

The current work evaluated over 300,000 compounds of the NIH-MLPCN collection to identify agents capable of selectively inhibiting a cell-line model of breast cancer stem cells. After triage through a series of counter screens and an orthogonal assay, the 4-*i*-propyl cinnamide **1** was selected for further SAR investigation. As a result of these synthetic studies, compound **19d** emerged as a potent and selective inhibitor. Compound **19d** inhibited CSC-like cells with an IC_{50} of 2.00 μM and shows 32-fold selectivity against the control cell line. This compound has been registered with NIH Molecular Libraries Program (ML243) and is available upon request. Studies are currently underway to determine the ability of this compound to inhibit other breast cancer stem cell-like cell lines along with tumor cell lines with naturally high populations of cancer stems cells. Studies are also underway in combination with other scaffolds identified in this screen[7] utilizing gene expression analysis to understand the mode of action of these compounds and to identify potential targets for selectivity toward cancer stem cells.

Table 4
SAR of di- and tri-substituted phenyl cinnamides



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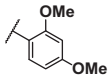
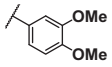
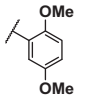
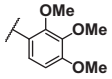
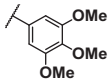
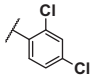
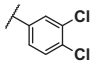
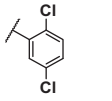
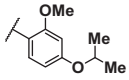
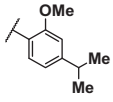
Analog	R	EC_{50}[a] (μM)		Selectivity[c]
		HMLE_shEcad	HMLE_sh_eGFP (control)	
18a		9.91	Inactive[b]	9.91 versus inactive
18b		Inactive	Inactive	NA
18c		Inactive	Inactive	NA
18d		Inactive	Inactive	NA
18e		Inactive	Inactive	NA
18f		Inactive	Inactive	NA
18g		Inactive	Inactive	NA
18h		Inactive	Inactive	NA
18i		Inactive	Inactive	NA
18j		28.43	Inactive	28.43 versus inactive

[a] Average of at least three runs.
[b] Inactive = no inhibition at highest dose (20 μM).
[c] Selectivity = EC_{50} (HMLE_sh_eGFP)/EC_{50} (HMLE_shEcad).

Acknowledgments

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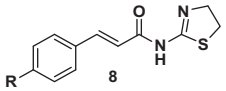
Supplementary data

Supplementary data (experimental protocols for cellular assays and for the preparation of compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.025.

Table 5
Further SAR of *para*-substituted phenyl cinnamides



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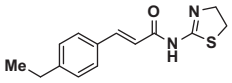
Analog	R	EC_{50}[a] (μM)		Selectivity[c]
		HMLE_shEcad	HMLE_sh_eGFP (control)	
1	*i*-Pr	2.35	50.55	21.5
19a	F	Inactive[b]	Inactive	NA
19b	CF_3	Inactive	Inactive	NA
19c	NMe_2	27.4	Inactive	27.4 versus inactive
19d	Et	2.00	63.98	32.0
19e	*t*-Bu	Inactive	20.55	NA
19f	*i*-Bu	0.478	2.17	4.54
19g	Ph	0.65	34.6	53.1
19h		3.85	5.65	1.47

[a] Average of at least three runs.
[b] Inactive = no inhibition at highest dose (20 μM).
[c] Selectivity = EC_{50} (HMLE_sh_eGFP)/EC_{50} (HMLE_shEcad).

Table 6
Physicochemical characterization of ML243



19d (ML243)

PBS solubility	PPB (human)	Plasma stability (human)	PBS stability (48 h)	GSH stability (48 h)	Lead profiling screen
11.5 μM	96.6%	97.1%	75%	82%	1/68 Targets[a]

[a] See Supplementary data for further details.

References and notes

1. Nguyen, L. V.; Vanner, R.; Dirks, P.; Eaves, C. J. *Nat. Rev. Cancer* **2012**, *12*, 133. and references therein.
2. (a) Bao, S.; Wu, Q.; McLendon, R. E.; Hao, Y.; Shi, Q.; Hjelmeland, A. B.; Dewhirst, M. W.; Bigner, D. D.; Rich, J. N. *Nature* **2006**, *444*, 756; (b) Diehn, M.; Cho, R. W.; Lobo, N. A.; Kalisky, T.; Dorie, M. J.; Kulp, A. N.; Qian, D.; Lam, J. S.; Ailles, L. E.; Wong, M. *Nature* **2009**, *458*, 780; (c) Diehn, M.; Clarke, M. F. *J. Natl. Cancer Inst.* **2006**, *98*, 1755; (d) Eyler, C. E.; Rich, J. N. *J. Clin. Oncol.* **2008**, *17*, 2839; (e) Li, X.; Lewis, M. T.; Huang, J.; Gutierrez, C.; Osborne, C. K.; Wu, M. F.; Hilsenbeck, S. G.; Pavlick, A.; Zhang, X.; Chamness, G. C. *J. Natl. Cancer Inst.* **2008**, *100*, 672.
3. Mani, S. A.; Guo, W.; Liao, M. J.; Eaton, E. N.; Ayyanan, A.; Zhou, A. Y.; Brooks, M.; Reinhard, F.; Zhang, C. C.; Shipitsin, M.; Campbell, L. L.; Polyak, K.; Brisken, C.; Yang, J.; Weinberg, R. A. *Cell* **2008**, *133*, 704.
4. Dontu, G.; Abdallah, W. M.; Foley, J. M.; Jackson, K. W.; Clarke, M. F.; Kawamura, M. J.; Wicha, M. S. *Genes Dev.* **2003**, *17*, 1253.
5. Gupta, P. B.; Onder, T. T.; Jiang, G.; Tao, K.; Kuperwasser, C.; Weinberg, R. A.; Lander, E. S. *Cell* **2009**, *138*, 645.
6. (a) Carmody, L. C.; Germain, A. R.; VerPlank, L.; Nag, P. P.; Munoz, B.; Perez, J. R.; Palmer, M. A. *J. Biomol. Screen.* **2012**, *17*, 1204; (b) All data for this project can be found at: http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=2721.
7. Germain, A. R.; Carmody, L. C.; Morgan, B.; Fernandez, C.; Forbeck, E.; Lewis, T. A.; Nag, P. P.; Ting, A.; Verplank, L.; Feng, F.; Perez, J. R.; Dandapani, S.; Palmer, M.; Lander, E. S.; Gupta, P. B.; Schreiber, S. L.; Munoz, B. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3571.
8. See Supplementary data.