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Optimization of a small molecule probe that restores e-cadherin expression

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Abstract

E-cadherin is a ubiquitous trans-membrane protein that has important functions in cellular contacts and has been shown to play a role in the epithelial mesenchymal transition. We have previously reported the use of an HTS screen to identify compounds that are capable of restoring e-cadherin in cancer cells. Here, we report the additional medicinal chemistry optimization of these molecules, resulting in new molecules that restore e-cadherin expression at low micromolar concentrations. Further, we report preliminary pharmacokinetic data on a compound, ML327, that can be used as a probe of e-cadherin restoration.

ML327

Keywords

E-cadherin; Structure activity relationship; Cancer; epithelial mesenchymal transition

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One of the hallmarks of cancer cells is escape from the epithelial phenotype to an invasive mesenchymal state.¹ As a part of the epithelial-mesenchymal transition (EMT), the expression of several proteins important to various cellular junction structures can be reduced.² This allows the malignant cell to invade surrounding tissues, as its growth is no longer dependent on normal cell-cell adhesion.

E-cadherin is a transmembrane protein that forms dimers at the cell surface and interacts with corresponding e-cadherin dimers on neighboring cells.³ Cancer cells that have undergone EMT often have reduced expression of this protein,⁴ typically as a result of changes in genetic regulation at the transcriptional level.⁵ We have previously reported the use of an immunofluorescence-based high-throughput screen (HTS) to identify molecules that restore e-cadherin expression to SW620 cells that have reduced expression of the protein (Figure 1).⁶ Exemplar molecules were found to reduce cellular invasion without affecting cellular proliferation.

Towards the goal of conducting *in vivo* investigations of the consequences of restoring e-cadherin expression with small molecules, we sought to optimize these lead compounds for potency and pharmaceutical characteristics. We have investigated additional changes to the aryl isoxazole/pyrazole core, changes in the length and composition of the mostly hydrocarbon linker, and the nature of the terminal aryl or heterocyclic moiety. All analogs were examined in the previously described In-Cell-Western (ICW) assay to evaluate the restoration of e-cadherin expression in SW620 colon carcinomas and H520 lung carcinomas.⁶ Compounds were initially tested for their ability to restore e-cadherin expression at a 10 μ M concentration, and EC₅₀ values from dose response curves were determined for selected analogs that induced high levels of e-cadherin expression.

We first examined structural changes to the aryl isoxazole/pyrazole core chemotype (Figure 2). Unfortunately, significant changes to the compounds, including changing the position of the amide bond on the isoxazole or changing the position of the amide within the tether, resulted in inactive compounds (not shown). Indeed, even relatively modest changes, such as the use of bicyclic heterocycles (**3**, **4**) and methylation of the core (**5**, **6**) or tether (**7**) produced compounds that failed to demonstrate significant restoration of e-cadherin expression in the SW620 line at 10 μ M.

Based on the narrow SAR around the heterocyclic core, we decided to concentrate a more intensive optimization effort on the evaluating changes to the amine and the tethered terminal aryl or heterocyclic moiety. To accomplish this optimization, a flexible, high throughput synthetic route that allowed the facile introduction of significant diversity was required. To improve our ability to explore changes to the tail region of the compounds, we surveyed a small group of compounds that possessed linkers with chemically tractable handles for substitution (not shown). We determined that the insertion of a additional nitrogen into the linker provided a suitable functional group for additional diversification and SAR investigation (Scheme 1).

To construct analogs with the nitrogen containing linkers, the requisite heterocyclic carboxylic acid or acid chloride (**8**) was reacted with the amine in the presence of base and

an appropriate coupling agent if needed (Scheme 1). The tethered carbamate (**9**) is then deprotected under acidic conditions and the resulting amine **10** can be used in the construction of amides using standard peptide coupling reactions or amines using either standard reductive amination conditions or S_NAr chemistry. Interestingly, propyl-based linkers consistently produced lower yields in the final step than ethyl-based linkers.

Based on the structural requirements learned from the previous study, we surveyed a wide variety of functionalized phenyl and heterocyclic units (Table 1). Compounds **11a** and **b** are isosteric with previously reported effective compounds.⁶ While the isoxazole **11a** retains potency, the pyrazole **11b** displays very sharply reduced restoration of e-cadherin. This trend is replicated with the matched pair of thiazole compounds (**11c, d**). While the isoxazole and pyrazole containing compounds were previously reported to be equipotent, this distinct difference in activity with the amide-containing linkers led us to turn our focus exclusively on isoxazole-containing compounds. Changes to the linker length and the nature of the tethered heterocycle were generally well tolerated. Although **11c** is an exception, a slight preference was typically observed with a 3 carbon versus a 2 carbon separation between the linker nitrogens (compare **11e, f**). In accordance with prior observations, 2-thiophenyl and phenyl variants of the core substitution were both well tolerated. A wide range of terminal heterocycles exhibited robust restoration of e-cadherin expression (**11a-l**). Although the SAR was more narrow, a range of phenyl rings substituted with polar functionality and/or halogens were also tolerated (**11m-p**). Success was realized by combining these two patterns of substitution, with substituted heterocycles such as **11r** consistently ranking as among the best compounds. Interestingly, we found that an additional methylene could be inserted between the amide carbonyl and the ring system (**11u-11x**) and that selected alkyl substituents (e.g. **11y**) also retained activity.

Focusing on the phenylisoxazole core with a three carbon linker, we also examined a set of non-amide-based substitutions (Table 2). These compounds were prepared using knowledge gained from the survey of amide attachments. However, while many compounds exhibited similar activities as their amide counterparts, more distinct SAR requirements were observed with this sub-series of analogs. For example, among a series of heterocyclic amines (**12a-i**), pyridyl analogs (**12 a-d**) were effective at restoring e-cadherin expression, while the efficacy of isoxazole and oxazole containing compounds (e.g. **12 e,f**) varied widely with changes in the substitution pattern. Occasionally, over-alkylation was observed in the reductive amination reactions and one such compound resulting from this over reaction, **12b**, displayed an exceptional level of activity. However, no other over-alkylated compounds possessed significant activity. An interesting pattern was also observed with 5,6-bicyclic heterocycles (**12g-i**). While simple indole and azaindole compounds were relatively inactive, an acetylated indole (**12i**) displayed robust e-cadherin restoration. A small range of aryl ring substitutions was also examined. As indicated by the hydroxylated compounds **12j-l**, a narrow SAR, distinct from that in the amide series, was observed. It appears that a 2-hydroxyl moiety is critical in this sub-series (**12l**), while meta and para hydroxyls diminish activity (**12j, k**). We conducted S_NAr reactions to place heterocyclic rings directly on the terminal nitrogen of the linker. From among the relatively small set of compounds examined, several compounds that produced high e-cadherin restoration were obtained, for

example the cyanopyridine **12m**. The azidopyrimidine **12n** was prepared using sequential S_NAr reactions of 2,4-dichoropyrimidines and represents the most efficacious compound at 10 μM we have yet observed.

Analogs that displayed significant restoration of e-cadherin expression at 10 μM were evaluated for dose response in the SW620 cell line. Most compounds displayed a full dose response, with a sigmoidal curve shape from which EC_{50} values could be calculated and Hill slopes approaching unity (Table 3). The EC_{50} of e-cadherin restoration values did not correlate perfectly with the effect observed at 10 μM . For example, **11c** and **12n**, with the highest levels of e-cadherin restoration, had EC_{50} values not distinctly better than compounds with more modest levels of e-cadherin restoration at 10 μM (e.g. **11g** and **11q**, among others). The indole-containing compounds **11v** and **11w**, along with the hydroxypyridine **11r**, possess the most potent EC_{50} values in this set.

In part due to the combination of its significant level of restoration of e-cadherin expression at 10 μM and its potent EC_{50} , **11r** was selected for additional characterization. We have demonstrated that this compound has a profound effect on the expression of e-cadherin at multiple timepoints at both the protein and mRNA level, and is capable of reversing the EMT phenotype and reducing the invasive potential of SW620 cell lines.⁷ Compound **11r** possesses an excellent *in vitro* DMPK profile, albeit with significant differences in data between species. Although the mouse clearance was well in excess of hepatic blood flow, low intrinsic clearance was noted in rat (33.1 mL/min/kg) and negligible intrinsic clearance was noted in human microsomal studies. Notably, the Cl_{int} was inversely correlated with the free fraction, which was significantly lower in human (f_u 0.01) than in rodents (f_u 0.11 in rat, 0.19 in mouse). No inhibition of P450 isozymes (3A4, 2C9, 2D6, 1A2) was noted at 30 μM . Further, **11r** demonstrates a low clearance and long half life following an IV dose in rat and suitable exposure from both IV and IP dosing to enable *in vivo* testing.

In summary, the SAR around a series of isoxazole-based compounds that are capable of restoring e-cadherin expression has been more fully evaluated. Specific requirements for superior activity were noted and the best compounds can exert this effect at low micromolar concentrations. Taken together, the data that we have collected has led us to select compound **11r** as a molecular probe of e-cadherin expression, **ML327**. This molecule is freely available from the Molecular Library Probe Center Network (MPLCN). Experiments to define the mechanism of action of this compound and to fully evaluate the ramifications of restoring e-cadherin expression *in vitro* and *in vivo* are in progress.

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Figure 1.
Previously reported molecules that restore e-cadherin expression.

Figure 2.
Chemotype changes. Data represent average fold change from two assessments of e-cadherin expression in SW620 cells at a dose of 10 μ M compound.

Scheme 1. Construction of optimized analogs.^a

^a**Reagents and conditions:** a) for X = Cl, BOC-NH-ethyl-NH₂ or BOC-NH-propyl-NH₂, Et₃N, DCM, 1-5 h; b) for X = OH, BOC-NH-ethyl-NH₂ or BOC-NH-propyl-NH₂, HATU, DIPEA, DMF, overnight; c) TFA, DCM; d) RCO²H, COMU, DIPEA, DMF, overnight; e) RCHO, PS-triacetoxyborohydride, MeOH, rt, 12h; f) ArCl, DIPEA, EtOH, reflux.

Table 1SAR of amide-linked compounds **11**

Cpd	Ar	Y	n	R	Fold change ^a
11a	Phenyl	O	2	2	2.79
11b	Phenyl	NH	2	2	1.29
11c	Phenyl	O	2	2	8.98
11d	Phenyl	NH	2	2	1.77
11e	Phenyl	O	2	2	3.05
11f	Phenyl	O	3	3	4.43
11g	2-thiophenyl	O	3	3	4.55
11h	Phenyl	O	3	3	3.70

Cpd	Ar	Y	n	R	Fold change ^a
11i	Phenyl	O	3		3.35
11j	2-thiophenyl	O	3		3.04
11k	Phenyl	O	3		2.39
11l	Phenyl	O	2		1.62
11m	Phenyl	O	3		2.43
11n	Phenyl	O	2		2.43
11o	Phenyl	O	3		2.69
11p	Phenyl	O	3		3.93

Cpd	Ar	Y	n	R	Fold change ^a
11q	2-thiophenyl	O	3		4.26
11r	Phenyl	O	3		5.23
11s	Phenyl	O	3		4.58
11t	Phenyl	O	3		4.43
11lu	Phenyl	O	3		4.20
11v	2-thiophenyl	O	3		4.59
11w	Phenyl	O	2		3.40
11x	Phenyl	O	3		4.92
11y	2-thiophenyl	O	3		4.02

^aData represent average fold change from two assessments of e-cadherin expression in SW620 cells at a dose of 10 μ M compound.

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Table 2SAR of alkyl or aryl amine-linked compounds **12**.

Cpd	R	Fold change ^a
12a		2.52
12b	 (double alkylation)	7.36
12c		3.06
12d		3.58
12e		1.50
12f		3.40

Cpd	R	Fold change ^a
12g		1.67
12h		1.44
12i		6.09
12j		1.54
12k		2.33
12l		3.59
12m		5.77

Cpd	R	Fold change ^a
12n	 <chem>[N+]#Nc1ccncc1</chem>	15.89

^aData represent average fold change from two assessments of e-cadherin expression in SW620 cells at a dose of 10 μ M compound.

Table 3Selected ICW EC₅₀ values for e-cadherin restoration.

Cpd	Fold change ^a	EC ₅₀ (μM) ^b	Cpd	Fold change ^a	EC ₅₀ (μM) ^b
1	4.36	4.7	11u	4.20	12.6
11a	2.79	8.8	11v	4.59	1.7
11c	8.98	4.8	11w	3.40	3.7
11f	4.43	7.1	11y	4.02	9.3
11g	4.55	5.0	12b	7.36	22.9
11q	4.26	5.6	12i	6.09	15.0
11r	5.23	3.1	12m	5.77	12.0
11s	4.58	5.6	12n	15.89	5.3
11t	4.43	4.6			

^a Data represent average fold change from two assessments of e-cadherin expression in SW620 cells at a dose of 10 μM compound.^b EC₅₀ values determined in triplicate from 6 doses over a 3 log unit variation in compound concentration. Individual values are within 1.5 fold of the mean value.

Table 4DMPK data for **11r**.

Cl_{int} (mL/min/h)		P450	
Mouse	180	1A2	>30 μ M
Rat	33	2C9	>30 μ M
Human	0.0 ^a	2D6	>30 μ M
Fraction Unbound		3A4	>30 μ M
0.19 _{mouse}	0.11 _{rat}	0.01 _{human}	

IV pharmacokinetics ^b ^a Less than 10% of the compound was consumed over the time course of the assay.^b Pharmacokinetics following a single 1 mg/kg dose in male Sprague- Dawley rats.