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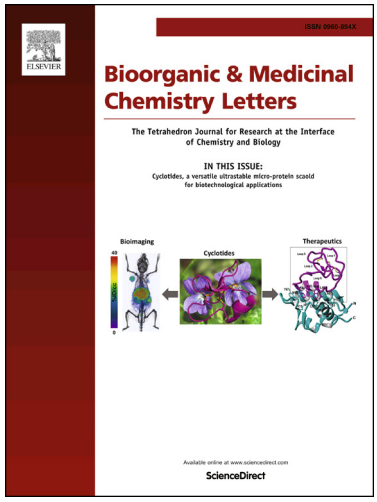

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PII: S0960-894X(17)31188-5
DOI: https://doi.org/10.1016/j.bmcl.2017.12.028
Reference: BMCL 25484

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 17 October 2017
Revised Date: 11 December 2017
Accepted Date: 13 December 2017

Please cite this article as: Krajnak, K., Dahl, R., Small molecule SUMOylation activators are novel neuroprotective agents, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: https://doi.org/10.1016/j.bmcl.2017.12.028

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Small molecule SUMOylation activators are novel neuroprotective agents

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
SUMO
Small Ubiquitin-like Modifier
Drug discovery
Small molecules
Medicinal chemistry
Neurodegeneration

ABSTRACT

Neuronal loss characterizes many of the most intractable nervous system diseases that deprive our ageing population of their quality of life. Neuroprotective pharmacological modalities are urgently needed to address this burgeoning population. Small ubiquitin-like modifier (SUMO) conjugation has been established as an endogenous neuroprotective response, and we have discovered several classes of small molecules that enhance SUMO conjugation. Herein we describe the hit to lead campaign that enabled the discovery of 3 diverse classes of drug-like SUMOylation activators. Optimized compounds were ultimately validated in cell-based models of neuronal loss and provide a foundation for establishing systemically active SUMO activators to treat degenerative diseases such as Parkinson's disease, Alzheimer's disease, and stroke.

Neuron death plays a central role in the pathogenesis of multiple diseases that currently have no available therapeutics.[1] Consequently, neuroprotection is a major goal of neuroscience drug discovery research, specifically relevant to diseases involving neuronal insult and loss. Finding small molecules that can prevent or alleviate neuron loss is critical to addressing these significant neuropathies. While neuroprotection has the potential to be an effective treatment for stroke, CNS trauma, Alzheimer's disease (AD), and Parkinson's disease (PD), there are no approved therapies that slow or halt neuron loss. The collective economic and social burden of these diseases is enormous. Currently, CNS trauma and related maladies including stroke have estimated costs of $106 billion annually in the US.[2] AD is the most frequent form of dementia in ageing affecting over 5 million Americans with annual economic burdens in the US exceeding $180 billion annually. PD affects about 1% of the population aged 60 and over, and costs over $6 billion a year.[3] All of these disorders are marked by neuronal insult and subsequent death. Thus, there is an urgent need for therapies with neuroprotective effects. Protein conjugation with small ubiquitin-like modifier (SUMO) proteins has been established by numerous studies as an endogenous neuroprotective mechanism in neurons,[4] and the small molecule SUMOylation enhancers that are presented herein serve to validate a novel strategy to treat these important disorders and deliver valuable candidate molecules for further drug development.

SUMO is a small protein that is covalently attached to target proteins via an enzymatic cascade that is analogous to the ubiquitin pathway (Figure 1). This includes an E1 enzyme (SUMO activating enzyme, SAE), an E2 enzyme (Ubc9), and, sometimes, various E3 ligases (many).[5] A major function of this conjugation is to modulate protein-protein interactions. A global increase in SUMOylation levels mark the response to neuronal insult caused by CNS trauma and neurodegenerative pathogenesis.[6] SUMO conjugation has been established as an endogenous neuroprotective strategy in multiple models of cellular stress that mimic a neurodegenerative environment. Cells exposed to stress conditions such as hypothermia and hypoxia show increased SUMO conjugation.[7] Also, SUMOylation is activated in hibernation as a neuroprotective response to decreased bloodflow and nutrient availability.[8] Massive increases in SUMO and SUMOylation machinery are seen in animal models of ischemia.[9] Remarkably, overexpression of SUMO components leads to a reduction in cell loss from stressors such as oxygen and glucose deprivation (OGD) and ischemic conditions, while knock down of SUMO in a cellular model made cells more susceptible to OGD-induced cell death.[7] Increased SUMO conjugation has also been seen concurrently with increased ubiquitination in neuronal injury as focal cerebral ischemia increased both protein ubiquitination and SUMOylation in various protein aggregates in the CNS.[10] Finally, the combination of ubiquitination and SUMOylation has been shown to play a protective effect in the brains of transgenic mice following ischemic damage.[11]

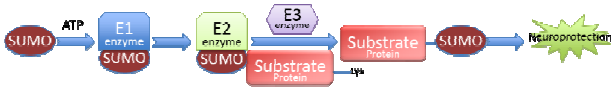


Figure 1. SUMOylation is an enzymatic process consisting of E1 (SAE), E2 (Ubc9), and, sometimes, an E3 ligase. Our activators increase the amount of SUMO conjugation, which has been shown to be endogenously protective.

While inhibition of the various SUMO pathway components to reduce protein SUMOylation has been targeted as a strategy for various diseases including cancer, viral infection, and cystic fibrosis,[12,13] relatively few reports of pharmacological activation of SUMO conjugation are currently available. One such report describes development of a high-throughput assay to find activators of SUMO conjugation via the inhibition of microRNAs 182 and 183.[14] Reported activators were shown to be neuroprotective in an in vitro model of ischemia. Another recent report describes a SUMOylation activator for the substrate protein Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA).[15] This increase in SUMO conjugation leads to the subsequent activation of SERCA and improved muscle contraction for applications in heart failure.

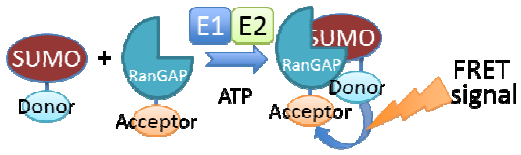


Figure 2. Components of the high-throughput HTRF assay to discover activators of SUMOylation. Increase in the FRET signal over baseline indicated potential activators which were validated in orthogonal assays that directly measured protein sumoylation.

We discovered several classes of SUMO activators via a high-throughput homogeneous time-resolved fluorescence (HTRF) assay. SUMO is covalently attached to target proteins through a concerted process that is catalyzed by three enzymes, known generally as the activation enzyme (E1), conjugation enzyme (E2), and ligase (E3).[16-21] Our HTRF assay contained SUMO-E1 (SAE), SUMO-E2 (UBC9), GST-SUMO and His-RanGap, a known SUMO substrate, as well as the respective fluorescent antibodies (Figure 2).[22] The assay was run in 1536-well plate format and the selected activators were chosen based on their ability to increase the FRET signal over a baseline level. All active series were subsequently validated in 2 orthogonal assays to confirm their ability to increase SUMOylation in HEK-293 cells. The assays included YFP-SUMO1 accumulation and SUMOylation of the known SUMO substrate Sp1 above endogenous levels. Following hit confirmation and validation, we selected 3 hit series based on several criteria. First, we wanted classes that showed nascent SAR in the primary screen rather than single hit classes (i.e. "one-hit wonders"). As can be seen in Figure 3, the classes chosen for follow up all had multiple analogues showing activity. Second, we used drug-like physicochemical filters as a means to rank order our compound classes and select leads showing acceptable physical properties in regards to lipophilicity (LogP < 5), polar surface area (PSA < 100), and size, as assessed by molecular weight (MW < 500).

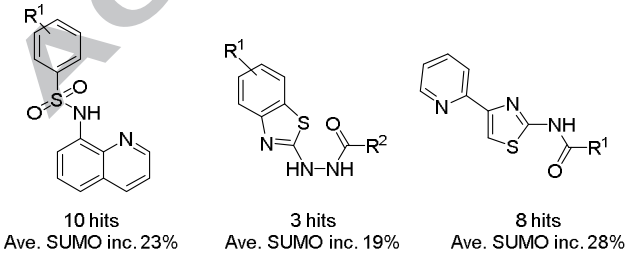
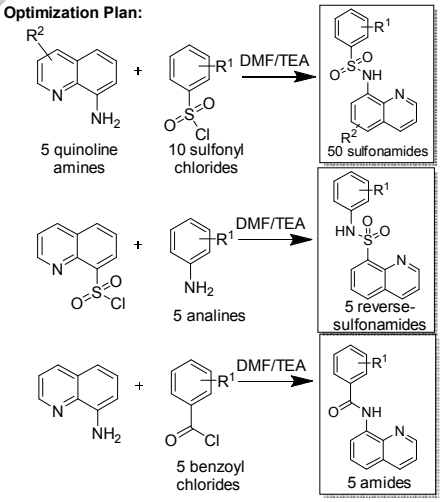


Figure 3. The 3 classes of SUMOylation activators chosen for follow-up from HTS. Compounds were chosen based on nascent SAR and drug-likeness. Shown are the average percentages of increase in the SUMOylation signal over baseline across all representatives of each scaffold.

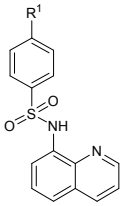
Due to our requirement for nascent SAR present in the screening hits, we were able to implement a parallel synthesis strategy of enriched, focused libraries to rapidly optimize the SUMOylation activity of the 3 hit classes. Using the bioactivity information present from the primary screen, we envisioned synthesizing 50 to 100 analogues of each scaffold to answer important SAR questions and generate lead compounds to progress to cell-based neuroprotection studies. Chemistries were chosen to minimize optimization time with final products rapidly isolated using preparative HPLC-MS.

Chemical Series 1: Quinolines. The optimization plan, synthesis, and resultant SAR for our quinoline series of SUMO activators are depicted in Scheme 1. We synthesized 3 sub-series in this class, including sulfonamides, reverse-sulfonamides, and amides using commercially available building blocks and standard reaction conditions. Briefly, acid chlorides or sulfonyl chlorides were reacted with amines, 1:1, in *N,N*-dimethylformamide (DMF) with 1.5 equivalents of triethylamine (TEA) at room temperature for 2-4 hours. As can be seen in the table, while a variety of functionality is tolerated at the *para* position of the sulfonyl aromatic portion, it should be noted that substitution at the *ortho* and *meta* positions resulted in a significant loss of activity. The bis-sulfonamide **1** gave the best activation and was progressed to cellular neuroprotection studies. Effects of substitution on the quinoline moiety were detrimental, and all actives were devoid of substitution at this moiety. In addition, replacing the existing sulfonamide with a reverse-sulfonamide or an amide, which has a subtle difference in geometry with the planar amide as opposed to the tetrahedral

Optimization Plan:



Results:

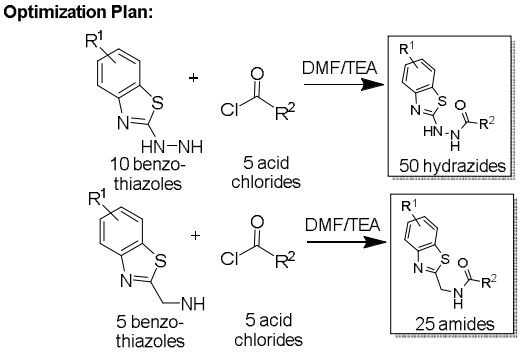


Cmpd	R^1	logP	% SUMO Inc.
1		2.1	43 ±4.6
2	CH_3	3.0	29 ±3.6
3		1.6	28 ±3.0
4	H	2.5	25 ±2.6

Scheme 1. The sulfonamide hit class was followed up via synthesis of 60 derivatives from 3 subclasses. The results of this SAR leaded the lead compound **1** which increases SUMOylation by 43%. Data are represented as mean ± SD.

sulfonamide, resulted in a loss of activity.

Chemical Series 2: Benzothiazoles. The optimization plan, synthesis, and resultant SAR for our benzothiazole series of SUMO activators are depicted in Scheme 2. There were 2 sub-series synthesized, the hydrazides and amides, using commercially available building blocks and standard reaction conditions including reaction of a benzoyl chloride and either hydrazine or amine, 1:1, in DMF with 1.2 equivalents of TEA at room temperature for 4 hours. This afforded 50 hydrazides and 25 amides, with the top activators shown in the table. As can be seen in the table, a limited number of substitutions were tolerated on the benzothiazole portion, mainly methyl groups or methoxy, with halogens or electronic withdrawing groups being inactive. The most active derivatives of this class. Compound **5** was progressed to neuroprotection in cell-based assays. It is notable that all of the compounds of the amide sub-series were inactive, thus there is a requirement for the hydrazide moiety.

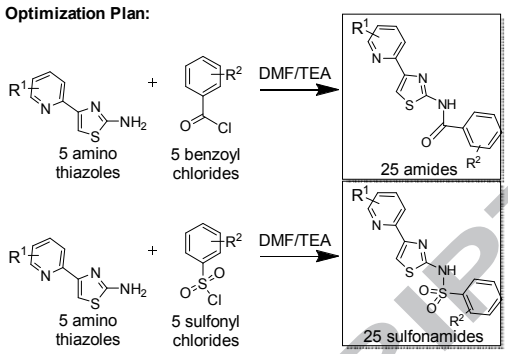


Results:

Cmpd	R^1	R^2	logP	% SUMO Inc.
5	4,5-CH_3		3.4	38 ±2.6
6	4,6-CH_3		3.4	33 ±1.1
7	4-OMe		2.4	17 ±3.1

Scheme 2. The benzothiazole hit class was followed up via synthesis of 75 derivatives in 2 subclasses. The results of this SAR leaded the lead compound **5** which increases SUMOylation by 38%. Data are represented as mean ± SD.

Chemical Series 3: Aminothiazoles. The optimization plan, synthesis, and results for the aminothiazole series of SUMO activators is depicted in Scheme 3. There were 2 sub-series synthesized that consisted of amides and sulfonamides. Again, we used commercially available building blocks and standard reaction conditions including reaction of a benzoyl chloride or sulfonyl chloride and thiazole amine, 1:1, in DMF with 1.2 equivalents of TEA at room temperature for 15 hours. We generated 50 diverse aminothiazole derivatives, with the top activators shown in the table. All top actives had the unsubstituted *ortho*-pyridyl derivative, and no substitution was tolerated on the ring. Replacement with unsubstituted phenyl produced a weakly active derivative. Optimization of the other site showed that the sulfonamide derivatives were all inactive. Amongst the benzoyl substituents, hydrophobic and heteroaromatic groups were tolerated. The preferred compound **8**



Results:

Cmpd	R^1	logP	% SUMO Inc.
8		2.6	53 ±3.6
9		3.4	37 ±2.6
10		3.2	25 ±3.0
11		2.6	20 ±2.7

Scheme 3. The aminothiazole hit class was followed up via synthesis of 50 amides and sulfonamides. All sulfonamides were inactive. The results of this SAR leaded the lead compound **8** which increases SUMOylation by 53%. Data are represented as mean ± SD.

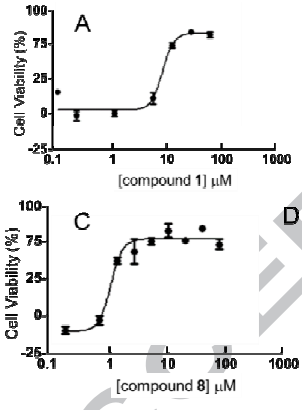
was progressed to cell-based neuroprotection assays.

Representative compounds **1**, **5**, and **8** were tested in dose response assays in a neuronal cell line using CSM14.1 cells. Additionally, all reported compounds **1-11** were tested in Buffalo green monkey kidney (BGMK) cells. In both assays, the compounds were tested for their ability to rescue cells from conditions of stress that are associated with stroke and neurodegenerative pathophysiology. This served as a validated in vitro assessment of these compounds to serve as neuroprotective drug candidates for further development. Endoplasmic reticulum (ER) stress has been established as a major cause of neuronal loss in stroke, ischemia, and neurodegeneration.[23-25] Thus, we used a known ER stress-causing agent thapsigargin (TG) to model the compromised state in vitro. In the absence of our SUMOylation activators, TG caused the complete death of both cell lines.

To assess the ability of the compounds to rescue neurons from ER stress-induced cell death, the rat striatal neuroprogenitor cell line CSM14.1 was used.[26] Briefly, cells were pre-treated for 2 hours with compound or buffer followed by injury with TG, the known inducer of ER stress. Measurement of cellular ATP content was used as a surrogate marker of cell viability. Controls consisted of TG with no added compound as the negative control (100% cell death) and DMSO without TG as the positive control (100% cell viability). The known anti-ER stress compound Salubrinal was used as a positive control as well. The results of the cell viability assays with our SUMOylation enhancers are shown in Figure 4. As can be seen, there is a clear dose-response rescue with the added compounds, and some compounds rescued

the cells to nearly 100% at higher concentrations. All EC_{50} values were in the single-digit micromolar range (1.2-9.8μM).

To assess an orthogonal cell line and further validate the ability of SUMOylation activators to rescue varied cell types, the compounds were also profiled for cytoprotection in BGMK cells.[27] Briefly, cultured BGMK cells were pretreated with SUMOylation enhancers **1-11** for 2 hours. Treated cultures were then stressed with 150 nM of thapsigargin to mimic the effects of activating the unfolded protein response and initiate the ER stress cascade that eventually leads to apoptosis. The cytoprotective effect of each compound was assessed using CCK-8 colorimetric readings to measure cell viability. Representative SUMOylation activator compounds show significant protection against thapsigargin-induced cell death (Figure 4D). As a positive control, Salubrinal was tested and rescued the injured cells to 47.22% viability. It is also noted that the level of cell rescue does not directly correlate to the sumoylation activation seen in the biochemical screening assay. In the cellular assay we are also measuring the ability of compounds to reach their biological target in addition to measuring potential for sumoylation increase. Thus, any discrepancy is most likely due to the physical properties of the respective compound classes affecting their ability to dissolve in the aqueous cellular assay buffer and subsequently cross cellular membranes. In order to accomplish this, the compounds must have a balanced physicochemical profile. Analysis of the average cell rescue ability for the compounds reported herein shows that the quinoline-sulfonamides and benzothiazoles are relatively comparable at about 50% of cell viability, whereas the aminothiazole class has an average cell rescue ability of about 66% viability. Therefore, it can be argued that the aminothiazoles have a better balance of physical properties that enable improved solubility and ability to penetrate cell membranes and can be described as being more drug-like.



D

Cmpd	BGMK % Viability (10 μM)	Cmpd	BGMK % Viability (10 μM)
1	62.38 ± 2.3**	7	38.39 ± 9.1
2	51.95 ± 0.9**	8	81.43 ± 1.7*
3	53.67 ± 2.4**	9	83.29 ± 1.2***
4	35.23 ± 4.1	10	45.29 ± 1.0**
5	54.92 ± 8.3***	11	54.88 ± 9.8*
6	60.28 ± 5.2*	Sal†	47.22 ± 4.1*

Figure 4. Neuroprotective and cytoprotective assessment of SUMO activators. Lead compounds were tested in cell-based assays to assess their ability to rescue cells from stress-induced cell death seen in stroke and neurodegeneration. Cells were pre-treated with compound followed by the ER stress inducer thapsigargin and cell viability was measured. Panels A-C show dose-response in the rat striatal neuroprogenitor cell line CSM14.1. Panel D shows the ability of compounds **1-11** to preserve viability in injured BGMK cells. Data are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001; † Sal = Salubrinal (positive control).

The cell rescue results support the growing evidence that increases in protein sumoylation are involved in inhibition of cell death via the major apoptotic pathways, establishing global sumoylation as a key determinant of cell life and death. For example, upregulation of SUMO1 in INS-1 832/13 cells was shown to protect against IL-1β-induced cell death via reduction of caspase 3 cleavage.[28] Furthermore, increased sumoylation decreased cytochrome c release via a reduction in outer mitochondrial membrane binding and subsequent fission.[29] Finally, pharmacologically increasing sumoylation levels in SHSY5Y cells protected against oxygen/glucose deprivation-induced cell death.[30] These reports indicate that sumoylation levels are intimately linked with apoptosis.

Our novel approach of treating CNS trauma and neurodegeneration by enhancing neuroprotection via pharmacological activation of SUMOylation provides a new avenue of intervention for urgently needed medications. There have been few reports of drug-like small molecules that increase SUMOylation. We have validated a novel target for neuronal loss both biologically in that SUMOylation has been shown to be neuroprotective, and chemically in that we are able to modulate this process with small molecules. The ability of these molecules to rescue degenerative cells provides hope for the development of therapeutics.

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22. In 1536-well white plates, 2μl of assay buffer was dispensed into columns 1 and 2, and 2μl of Mixture 1 (37.5 nM E1 and 100 nM His-RanGap-1) in assay buffer (50mM Tris-HCl pH 7.4, 0.3mM DTT, 10 mM $MgCl_2$, 0.005% Tween-20), was dispensed into columns 3-48. Using a HighRes biosolutions pintool, 70nl of

2mM compounds in DMSO was dispensed to columns 5-48 and 70nl of DMSO was dispensed to columns 1-4. Using the Thermo Scientific MultiDrop Combi, 2 µL of Mixture 2 (20 mM ATP, 12.5 nM E2 and 30 nM GST-SUMO in buffer) was dispensed to all wells and incubated for 30 min. at room temperature followed by 1µl of 500 mM KF. Plates were read on a BMG Labtech PheraStar in a HTRF mode (Ex: 337 nm; Em: 620/665 nm). The ratio of fluorescence 665 over 620 was utilized as a readout of the assay.

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26. CSM14.1 cells were recovered from cultures by trypsinization and plated in 96-well plates and incubated overnight at 32 °C. 5 µl of test compound was added to achieve a final concentration of 15 µg/ml. Salubrinal and DMSO without compound were used as positive and negative controls, respectively. After 2 h of preincubation, TG-containing DMEM was added to give a final concentration of 15 µM and incubated for an additional 20 h. Cell viability was assessed by a cellular ATP content assay (ATPlite, PerkinElmer Life Sciences).
27. BGMKs were subcultured at 1.2 x 10^4 cells/ml. Wells treated with compound were done so for 2hr. TG at a concentration of 0.15 µM was then added and incubated for 24hrs. After treatment, all cells were washed and fed fresh DMEM complete media and CCK-8 development vehicle, incubated for 2 hr, and absorbance was measured.
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