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Synthesis and Evaluation of Ginkgolic Acid Derivatives as SUMOylation Inhibitors

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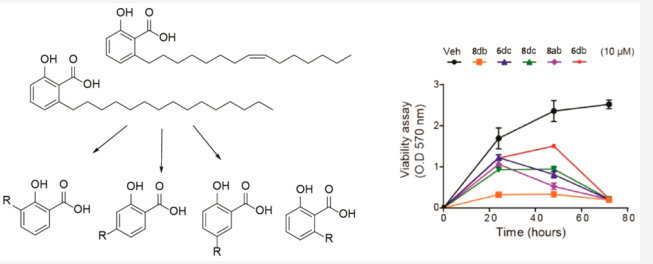
ABSTRACT: SUMOylation has emerged as an important post-translational modification that has been shown to modulate protein activity associated with various signaling pathways, and consequently, it has emerged as an important therapeutic target. While several natural products have been shown to inhibit enzymes involved in the SUMOylation process, there has been little progress toward the development of more selective and potent SUMOylation inhibitors. Ginkgolic acid was one of the first natural products discovered to inhibit the SUMO E1 enzyme. Despite its use to mechanistically investigate the SUMOylation process, ginkgolic acid also modulates other pathways as well. In this Letter, preliminary structure−activity relationships for ginkgolic acid as a SUMOylation inhibitor are presented.



KEYWORDS: *SUMO, SAE inhibitors, cancer, autophagy, apoptosis*

Post-translational modifications are critical to cellular processes that regulate protein function and, to date, more than 450 unique modifications have been discovered.[1] SUMOylation, the post-translational conjugation of the small ubiquitin-like modifier (SUMO) to a protein, is an emerging area of study due to the wide array of cellular processes that it controls.[2] Protein SUMOylation was first identified in 1996[3] and has been shown to be associated with DNA damage repair, immunological response, protein stability, nuclear-cytosolic transport, cell cycle progression, and apoptosis to name a few.[4] Thus, it is not surprising that dysregulation of SUMOylation is associated with many forms of cancer and neurodegenerative disorders.[5]

The SUMOylation pathway is mechanistically very similar to the process of ubiquitinylation. Initially, the E1 enzyme forms a thioester bond with the C-terminus of the SUMO peptide. Next, the activated SUMO is transferred to the E2 enzyme, which is responsible for conjugation and transesterification, resulting in a new thioester bond. The E3 ligases can then associate with the loaded E2 enzyme, which catalyze the transfer of SUMO to the ε-amino group on a specific lysine within the substrate.[6] This enzymatic pathway is also reversible as sentrin specific proteases (SENPs) are responsible for cleaving the bond between the substrate and SUMO (Figure 1).[7]

There are four SUMO isoforms: SUMO1−SUMO4. SUMO2, and SUMO3 share 97% sequence homology and are often referred to as SUMO2/3, as they have not yet been functionally distinguished.[8] Their sequence is only 50% similar to that of SUMO1, whereas SUMO4 is the least similar and the least studied of the isoforms. While SUMO 1−3 are ubiquitously expressed, SUMO4 mRNA is only present in the kidneys, spleen, and lymph nodes.[2] While there are significant differences in their sequence homology, they all exhibit a similar three-dimensional structure.

The SUMOylation pathway is upregulated in several cancers and has emerged as a potential target for the development of small molecule inhibitors.[6] In fact, several natural product inhibitors of SUMOylation have been identified including ginkgolic acid (**1**) and its structural analogue, anacardic acid (**2**).[9,10] While there are many structurally related ginkgolic acids, the C15:1 derivative **1** and the fully saturated analogue **2** were the analogues first reported as SUMOylation inhibitors. Ginkgolic acid exhibits anticancer activity and inhibits the migration of several different cancer cell lines.[11−13] RAC1 and NEMO are proteins that control cellular migration, and because SUMOylation modulates both of these proteins, it is not surprising that SUMOylation inhibitors manifest anti-metastatic activity.[14,15] In fact, recent studies have demonstrated that the antimigratory and anticancer activities manifested by ginkgolic acid are linked to inhibition of RAC1 and NEMO SUMOylation.[16,17]

Despite the increased attention that SUMOylation has received in recent years and ginkgolic acid's widespread use as

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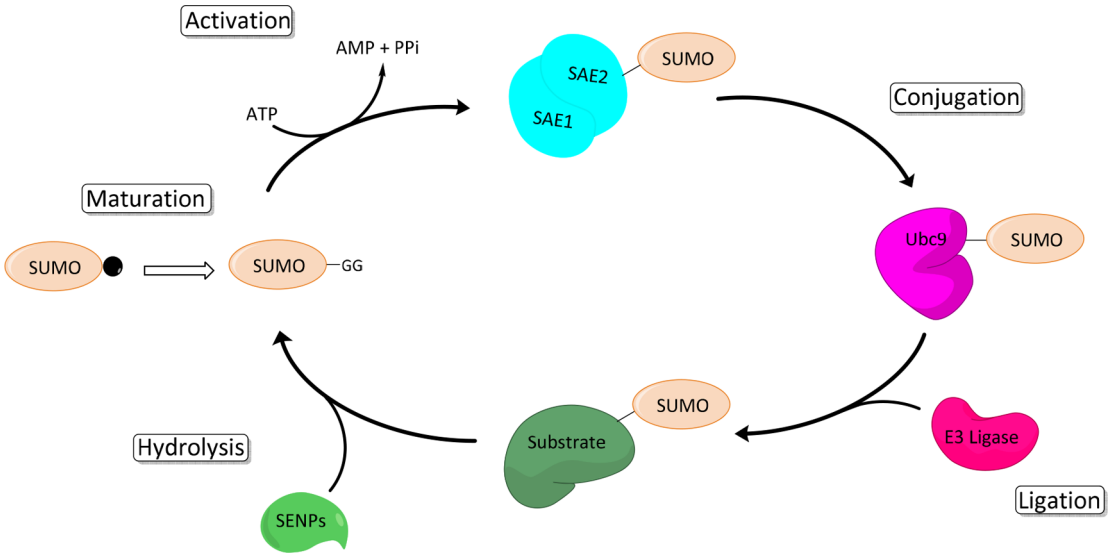

Figure 1. SUMOylation catalytic cycle: (1) maturation, (2) activation, (3) conjugation, (4) ligation, and (5) hydrolysis.

a SUMOylation inhibitor, no assessment of its structure–activity relationships nor its ability to inhibit SUMOylation has occurred. The original study demonstrated ginkgolic acid as an inhibitor of the E1 enzyme and showed that methylation of the acid was not tolerated, however, acetylation of the hydroxyl moiety did not affect inhibitory activity. Furthermore, salicylic acid, which is identical to ginkgolic acid but lacks the hydrocarbon tail, was completely inactive.[10] In addition, ginkgolic acid inhibits other biological processes as well, which complicates its use as an inhibitor of SUMOylation.[18] Therefore, efforts to develop more potent and selective inhibitors are highly desired. Herein, we report preliminary structure–activity relationships between ginkgolic acid/anacardic acid and the inhibition of SUMOylation.

Initial studies aimed to determine the significance and optimal position of the alkyl chain and its effect on SUMOylation. Four 2-hydroxybenzoic acid scaffolds were chosen to generate all possible regioisomers as presented in Figure 2, including the 2,6-substitution pattern present in the natural product.

Synthesis of these ginkgolic acid derivatives commenced with commercially available dihydroxybenzoic acids, which were condensed with acetone to provide the corresponding acetonides, **3a–d**, in modest yields. The free phenols were subsequently converted to the corresponding trifluormethylsulfonates, **4a–d**, via reaction with trifluoromethanesulfonic anhydride. Initially, the triflates were coupled with different primary alkenes via a Heck reaction. The 11-, 13-, and 15-carbon chains were chosen to mimic the natural products, which have alkyl chains ranging from 13–17 carbons. After the Heck coupling, the alkenes were reduced or left unsaturated prior to base-mediated hydrolysis of the acetonide (Scheme 1).

The initial library of compounds was screened for its ability to inhibit RanGAP1 SUMOylation in vitro at 50 μM. All compounds were soluble in the reaction media at this concentration. RanGAP1 is a GTPase activating protein that is involved in the transportation of proteins to and from the nucleus. RanGAP1 associates with the nuclear core complex and initiates transport only after SUMOylation has occurred.[19] Furthermore, it does not require an E3-ligase for SUMOylation, which makes the use of this recombinant system useful

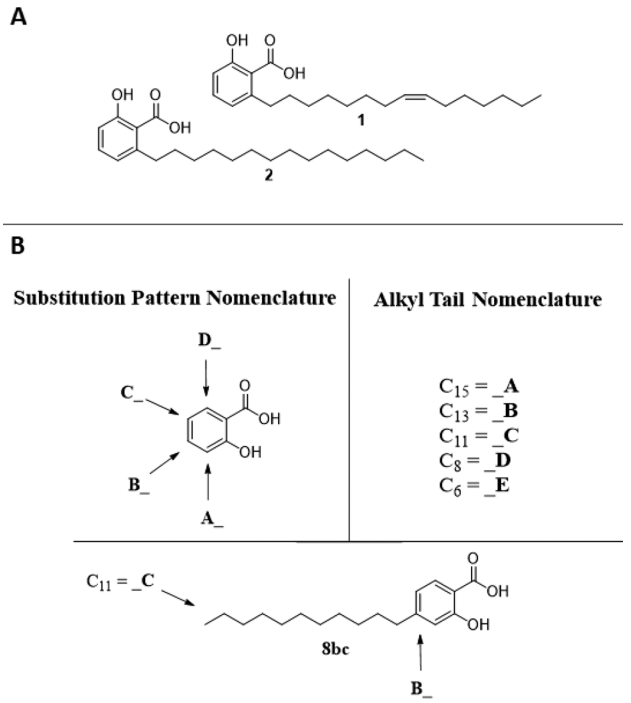

Figure 2. (A) Ginkgolic acid (**1**) and anacardic acid (**2**). (B) Compound numbering scheme. The first letter represents position of the alkyl tail, and the second letter represents alkyl tail length. Example compound **8bc** shown.

for the evaluation of SUMOylation inhibitors. The presence of SUMOylated RanGAP1 was probed via Western blot analysis (Figure 3). The results obtained from this in vitro assay are summarized in Table 1. The data from the initial screen suggest that any alteration of the substitution pattern about the central aromatic ring completely ablates SUMOylation inhibition. All 2,6 disubstituted analogues (**6da–6dc** and **8da–8dc**) were able to successfully inhibit RanGAP1 SUMOylation at 50 μM, suggesting that unsaturation did not exhibit a negative effect on activity. To determine whether there was an optimal alkyl tail length, the compounds manifesting inhibitory activity at 50 μM were screened at 5

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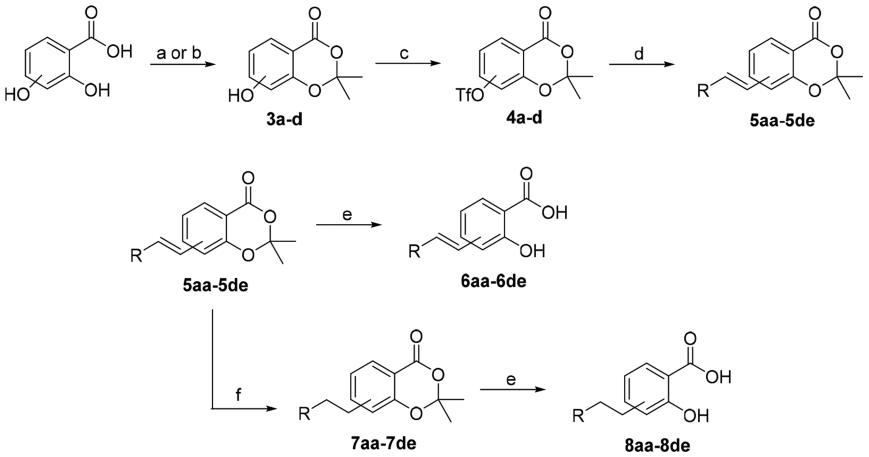
Scheme 1. Synthesis of Ginkgolic/Anacardic Acid Analogues[a]



[a](a) $SOCl_2$, DMAP, acetone, 1,2-DME, 24 h (77%); (b) trifluoroacetic anhydride, trifluoroacetic acid, acetone, 24 h (27−53%); (c) Tf_2O, pyridine, CH_2Cl_2, 3 h (35−89%); (d) alkene, $Pd(dppf)Cl_2$, K_2CO_3, DMF, 75 °C, 12 h (38−86%); (e) KOH, DMSO, 80 °C, 2 h (42-94%); (f) H_2, EtOAc, Pd/C, 16 h (64−98%).

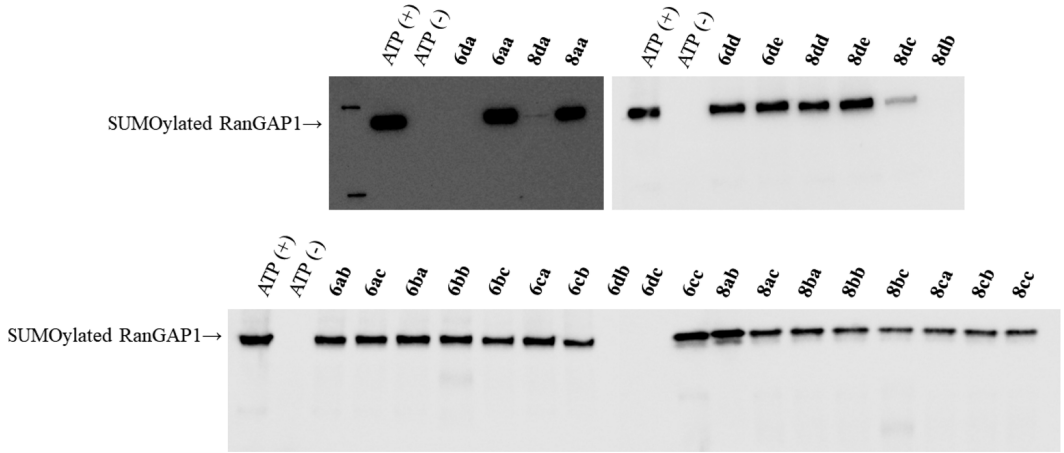


Figure 3. Western blot results from initial library screen. All compounds were tested at 50 μM.

μM, which revealed the unsaturated compounds, **6db** and **6dc**, to be most active (Supporting Information (SI), Figure S1).

The length of the alkyl chain for ginkgolic acids range from 13 to 17 carbons, but the initial study identified ginkgolic acid as a SUMOylation inhibitor that contained a C15 side chain. However, the data presented herein demonstrate that an alkyl chain of 11 carbons is also an effective inhibitor. To determine whether compounds with shorter alkyl chains could also inhibit SUMO-E1, 6- and 8-carbon analogues were synthesized as shown in Scheme 1. As other substitution patterns had proven deleterious toward SUMOylation inhibition, only derivatives based on the 2,6-substitution pattern were pursued. Compounds **6dd**, **6de**, **8dd**, and **8de**, shown in Figure 4, were screened at 50 μM and found to be completely inactive at the inhibition of SUMOylation. Furthermore, the results clearly show that a long alkyl chain is required for ginkgolic acid derivatives to inhibit SUMO-E1.

Compounds **6db**, **6dc**, **8db**, and **8dc** were chosen for further evaluation, whereas **8ab** was used as an inactive control, compound **8db** is fully saturated ginkgolic acid derivative, anacardic acid, **2**, from Figure 1. After establishing the ability of these compounds to inhibit RanGAP1 SUMOylation in vitro, the cellular activity of the lead compounds was investigated. The highly metastatic prostate cancer cell line, PC3, as well as the triple negative breast cancer cell line, MDA-MB-231, were treated with compounds and overall SUMOylation levels were probed via Western blot analysis. Consistent with the in vitro results, global SUMOylation levels were significantly diminished at 10 and 20 μM in PC3 cells after 24 h treatment. Consistent with the preliminary studies, treatment with compound **8ab** did not affect SUMOylation at either concentration tested (Figure 5A). Similar results were obtained in MDA-MB-231 (SI, Figure S2). Additionally, the ability of the compounds to inhibit SUMOylation was independent of the SUMO isoform (SI, Figure S3).

The original study demonstrated ginkgolic acid to manifest activity against SUMOylation and identified SUMO E1 as the target.[10] While the mechanism of action was not investigated, it is believed that due to structural similarities between the lead compounds and ginkgolic acid, **6db**, **6dc**, **8db**, and **8dc** are likely manifesting a similar mechanism of inhibition.

Prior studies demonstrated that the inhibition of SUMOylation via silencing or small molecule inhibition induces cell death via an autophagy-mediated apoptosis mechanism.[17] Increased cleavage of Poly(ADP-ribose) polymerase (PARP) is a classic hallmark of apoptosis,[20] and indeed, treatment with

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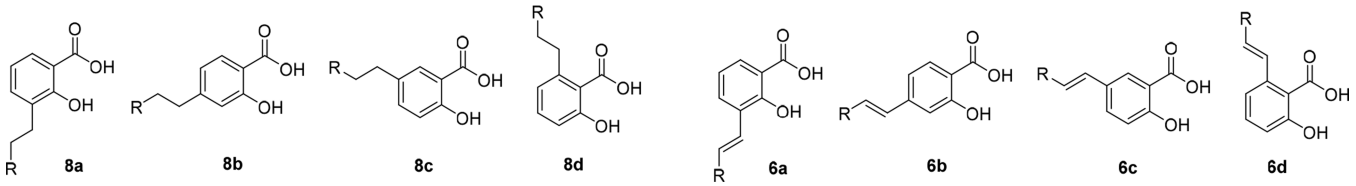
Table 1. Results of Initial Library Screen



	saturated			unsaturated		
substitution pattern	tail length	entry	active at 50 μM	tail length	entry	active at 50 μM
2,3	pentadecyl ($C_{15}H_{31}$)	**8aa**	no	pentadecenyl ($C_{15}H_{29}$)	**6aa**	no
2,4		**8ba**	no		**6ba**	no
2,5		**8ca**	no		**6ca**	no
2,6		**8da**	yes		**6da (2)**	yes
2,3	tridecyl ($C_{13}H_{27}$)	**8ab**	no	tridecenyl ($C_{13}H_{25}$)	**6ab**	no
2,4		**8bb**	no		**6bb**	no
2,5		**8cb**	no		**6cb**	no
2,6		**8db**	yes		**6db**	yes
2,3	undecyl ($C_{11}H_{23}$)	**8ac**	no	undecenyl ($C_{11}H_{21}$)	**6ac**	no
2,4		**8bc**	no		**6bc**	no
2,5		**8cc**	no		**6cc**	no
2,6		**8dc**	yes		**6dc**	yes

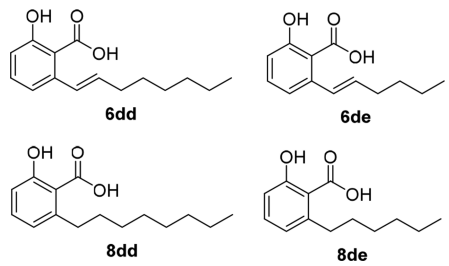


Figure 4. C8 and C6 ginkgolic acid derivatives.

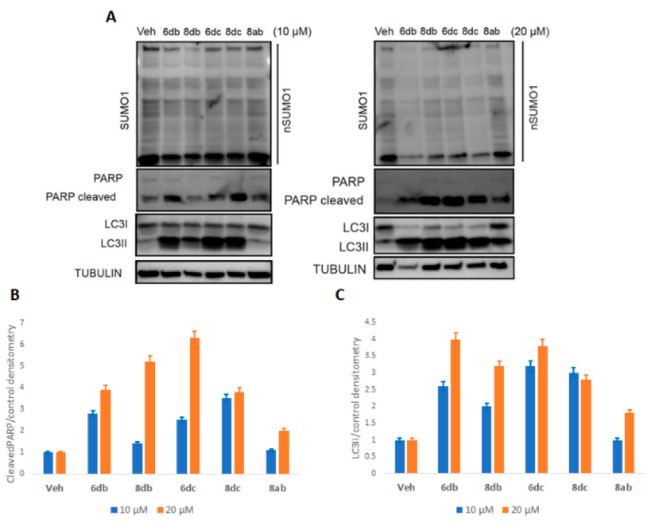


Figure 5. (A) Effects of lead compounds on global SUMOylation with SUMO1, apoptosis, and autophagy related factors on PC3 cells after 24 h treatment. (B) Densitometric quantification of the cleaved PARP data from A using ImageJ. (C) Densitometric quantification of the LC3II data from A using ImageJ. All experiments were performed at least three times, and the data are presented as the mean ± SEM.

either 10 or 20 μM of compounds **6db**, **6dc**, **8db**, or **8dc** led to increased levels of cleaved PARP (Figure 5B). Treatment of PC3 cells with 10 μM **8ab** did not induce levels of cleaved PARP or LC3II, however, treatment with 20 μM **8ab** did lead to increased levels of both species, albeit to a lesser extent than the active compounds, indicating that at the higher concentration, **8ab** may induce apoptosis, albeit an alternative mechanism of action not related with SUMO inhibition. Consistent with previous studies,[17] the active compounds also increased levels of LC3II, which is the phosphatidylethanolamine form of LC3 that is associated with the autophagosome (Figure 5C). Taken together, these results provide evidence that SUMOylation inhibition induces cell death via autophagy.

Cell viability was measured directly against both cell lines and at concentrations wherein SUMOylation was suppressed. Clearly, inhibition of cell growth was also affected by the administration of these compounds. Time course studies were also performed and demonstrated that compounds **6db**, **6dc**, **8db**, and **8dc** induced cell death after 24 h treatment, which is consistent with the immunoblot data linking inhibition of SUMOylation to cell viability. (Figure 6).

The IC_{50} values manifested by the five compounds were determined against both cancer cell lines. Previous studies have reported that *MYC* dependent cell lines such as MDA-MB-231 are more susceptible to SUMOylation inhibition.[21,22] Consistent with these reports, our compounds were generally more effective against MDA-MB-231 cells as compared to the PC3 cell line (SI, Figure S4). Compound **6db** was the most active against both cell lines and manifested IC_{50} values of 10.77 and 8.2 μM against PC3 and MDA-MB-231, respectively. Against PC3 cells, both unsaturated compounds were the most active, however, against MDA-MB-231 cells, the alkyl chain length appeared important for both C13 derivatives, **6db** and **8db**, which were most active. In addition, the IC_{50} values manifested by the active compounds correspond to the concentrations at which cleaved PARP and LC3II were induced. Compound **8ab** exhibited the weakest antiproliferative activity with IC_{50} values

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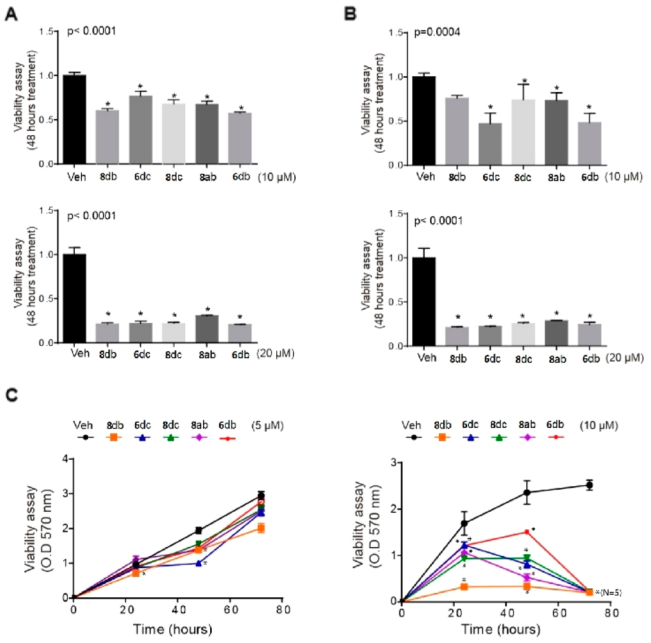

Figure 6. (A) The 48 h viability studies of four active and one inactive compound at 10 or 20 μM against PC3 cells. (B) The 48 h viability studies of four active and one inactive compound at 10 or 20 μM against MDA-MB-231 cells. (C) Time-dependent viability studies with two concentrations against PC3 cells (*$P \leq 0.05$ significant versus nontreated cells). All experiments were performed at least three times and the data are presented as the mean ± SEM.

of 14.8 and 12.8 against PC3 and MDA-MB-231 cells, respectively. The IC_{50} value manifested by **8ab** against PC3 cells is lower than the concentration at which **8ab** induced cleaved PARP and LC3II, suggesting that **8ab** affects cell viability by an alternative mechanism of inhibition.

SUMOylation is an important post-translational modification owing to the wide range of pathways that it modulates. In several cancers, the E1, E2, or SENPs have been altered to produce a dysregulated SUMOylation pathway and has now emerged as a novel target for the development of new cancer treatments. The *MYC* oncogenic pathway plays a key role in many cancers, and its activity relies upon the SUMOylation pathway to function properly, making the development of SUMOylation inhibitors even more important. In fact, numerous natural products have been discovered that inhibit SUMOylation, but most affect other enzymatic processes as well. For example, ginkgolic acid was the first natural product SUMOylation inhibitor discovered but also exhibits antidepressant, antifungal, and antimicrobial activities. Despite these concerns, ginkgolic acid continues to be used as a selective SUMOylation inhibitor. This study represents a first step toward the establishment of structure−activity relationships for ginkgolic acid as an inhibitor of the SUMOylation pathway. While ginkgolic acid and some of the inhibitors in this study contain unsaturation, the location of unsaturation did not affect inhibitory activity. This data highlights the nature of the alkyl chain, which is less important than the sterics, as compounds with shorter alkyl chain did not exhibit any activity in the initial in vitro assay. Future studies will further explore this trend and optimize the selectivity of these compounds toward SUMOylation inhibition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00353.

Synthetic experimental details, characterization of compounds, and biological data (PDF)

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Author Contributions

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

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SUMO, small ubiquitin-like modifier; SENP, sentrin-specific protease

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