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Structure–activity relationship studies of salubrinal lead to its active biotinylated derivative

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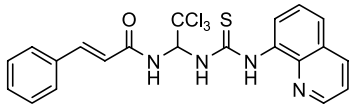
Abstract—The synthesis and structure–activity relationships (SAR) of salubrinal, a small molecule that protects cells from apoptosis induced by endoplasmic reticulum (ER) stress, are described. It is revealed that the trichloromethyl group greatly contributes to the activity. Based on the SAR results, salubrinal was converted into a biotinylated derivative which retains activity and can be used as a biological tool for target identification.

The endoplasmic reticulum (ER) stress response, a signaling pathway that connects ER with the nucleus, is essential for cellular homeostasis of all eukaryotic cells.[1] However, excessive and uncorrected ER stress can induce cell apoptosis, which has been implicated in many important pathologies, including diabetes, Alzheimer's disease, and viral infection.[2] Therefore, a better understanding of apoptotic pathway activated by ER stress will provide insight into the development of therapeutics for important human diseases. In a screen for compounds that protect mammalian cells from apoptosis induced by ER stress, we identified a small molecule, termed salubrinal (**1**).[3] Salubrinal protects cells against ER stress-induced apoptosis by selectively inhibiting the translation initiation factor eIF2α dephosphorylation. It blocks eIF2α dephosphorylation via inhibiting the protein complex GADD34/PP1 consisting of the general cellular serine/threonine phosphatase PP1 and the non-enzymatic cofactor GADD34, which targets eIF2α for dephosphorylation by PP1. As a specific inhibitor of eIF2α dephosphorylation, salubrinal is also useful in a disease model. It inhibits eIF2α dephosphorylation mediated by a herpes simplex virus encoded protein and blocks viral replication in vitro and in vivo.[3] These results suggest that the inhibition of eIF2α dephosphorylation may be a potent therapeutic strategy in diseases involving ER stress and viral infection, and that such small molecule as salubrinal might find therapeutic use in these diseases.



Salubrinal (1)

Whether salubrinal inhibits the GADD34/PP1 complex via direct binding or an indirect signaling event is yet unclear. A more detailed understanding of the molecular mechanism of salubrinal action is currently under investigation. We describe herein our efforts to investigate the structure–activity relationships (SAR) of salubrinal and to convert it into a biotinylated derivative as a biological tool for target identification.

The simplest modifications for salubrinal involves varying the substituents at its terminuses. The synthesis of these analogs is outlined in Scheme 1. Reaction of amides with anhydrous chloral gave chloralamides **2**, which were subsequently transformed into the corresponding amines **3** via two successive substitution reactions.[4] Addition of these amines to separately prepared isothiocyanates resulted in *N*,*N*′-disubstituted asymmetric thioureas **4**.[5] Compound **4l** was directly obtained by reduction of **4k** with $FeCl_2$ in 79% yield. The structures of **4a**–**4q** are shown in Table 1.

Keywords: Apoptosis; Inhibitor of eIF2α dephosphorylation; Synthesis.
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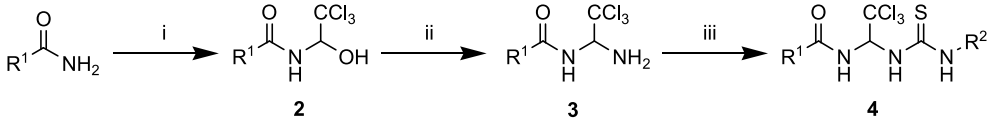


Scheme 1. Reagents and conditions: (i) CCl_3CHO, toluene, 90 °C, 80–97%; (ii) (1) PCl_5, Et_2O, (2) NH_3, Et_2O, 0 °C, 60–80%; (iii) R^2—N=C=S, THF, 60 °C, 60–95%.

Table 1. Structures of compounds **4a**–**4q** and EC_{50} values of salubrinal (**1**) and compounds **4**–**12**

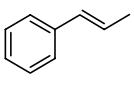
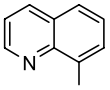
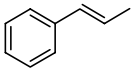
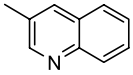
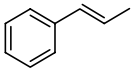
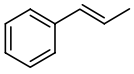
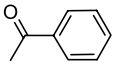
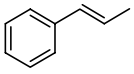
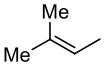
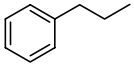
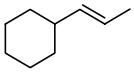
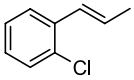
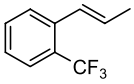
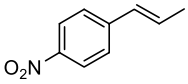
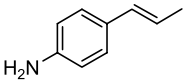
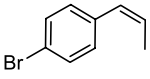
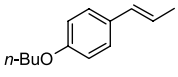
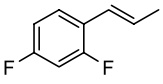
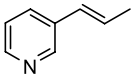
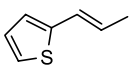
Compound	R^1	R^2	EC_{50} (μM)
1			15
4a			NA
4b			40
4c			>100
4d		–*n*-Bu	24
4e			NA
4f			NA
4g			33
4h			29
4i			40
4j			38
4k			28
4l			56
4m			50
4n			NA
4o			72
4p			30

Table 1 (*continued*)

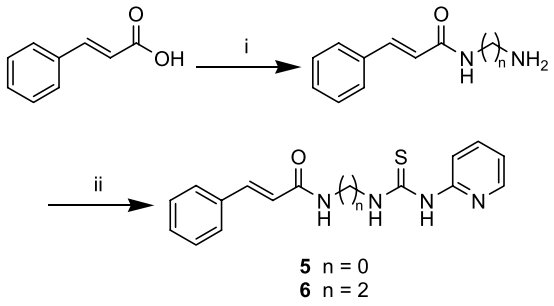
Compound	R^1	R^2	EC_{50} (μM)
4q			41
5			TX
6			TX
7			TX
8			51
9			75
10			NA
11			NA
12			NA

NA: not active up to 100 μM. TX: toxic.

In order to explore whether the trichloromethyl unit is necessary for the activity, analogs **5**–**10** were synthesized. As outlined in Scheme 2, compounds **5** and **6** were readily prepared via condensation of cinnamic acid with hydrazine and ethylenediamine respectively and subsequent addition to 2-isothiocyanatopyridine. Following the same procedure as for the synthesis of **4**, compounds **7**–**10** were obtained starting from the reaction of cinnamide with a range of aldehydes, as indicated in Scheme 3.[6] Compound **10** was further transformed into **11** and **12** via reduction and subsequent cyclization as described in Scheme 4.[7]

Compounds were assayed for their ability to protect the rat pheochromocytoma cell line PC12 from apoptosis induced by ER stress. In this assay, PC12 cells were treated with tunicamycin (Tm) and different doses of compounds for 37 h and then cell viability was assessed by cellular ATP content. Tm is an inhibitor of protein glycosylation in the ER and thus results in the accumulation of misfolded proteins, which will induce ER stress and subsequent apoptosis.[10] A treatment with 100 μM of zVAD.fmk, a broad-spectrum caspase inhibitor, served as a positive control. Salubrinal inhibited ER stress-mediated apoptosis in a dose-dependent manner with a median effective concen-tration (EC_{50}, as defined by the concentration at which the compound produces half-maximal rescue from cell death versus the Tm-only control) at about 15 μM and rescued about 35% of PC12 cells at 100 μM.[3] The cytoprotection activity of other analogues was assessed and the EC_{50} values are listed in Table 1.

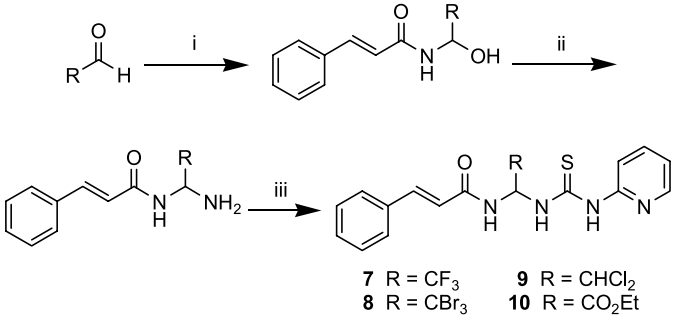
As shown in Table 1, Compounds **4a**–**4d** and salubrinal bear the same R^1 group but different R^2 groups. Structurally compound **4a** is almost the same as salubrinal



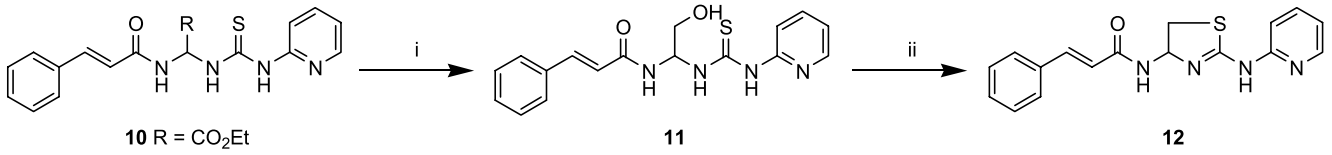
Scheme 2. Reagents and conditions: (i) for **5**: (1) oxalyl chloride, DMF, CH_2Cl_2, (2) hydrazine, Et_2O, 0 °C, 35%; for **6**: (1) HOSu, DCC, THF, (2) ethylenediamine, 60%; (ii) 2-isothiocyanatopyridine, EtOH, 60 °C, 44–78%.

except for the substitution position at the quinoline ring. However, **4a** displayed no activity. Replacement of the quinoline ring by pyridyl, benzoyl, or alkyl groups resulted in derivatives **4b**–**4d**. The potency of compound **4c** was much lower than salubrinal, whereas compound **4b** and **4d** had a close EC_{50} to that of salubrinal. Compound **4b** even demonstrated improved efficacy (the maximum protection from cell death at the optimal concentration) over salubrinal, therefore we retained the pyridine ring instead of quinoline ring in the following structural modifications.

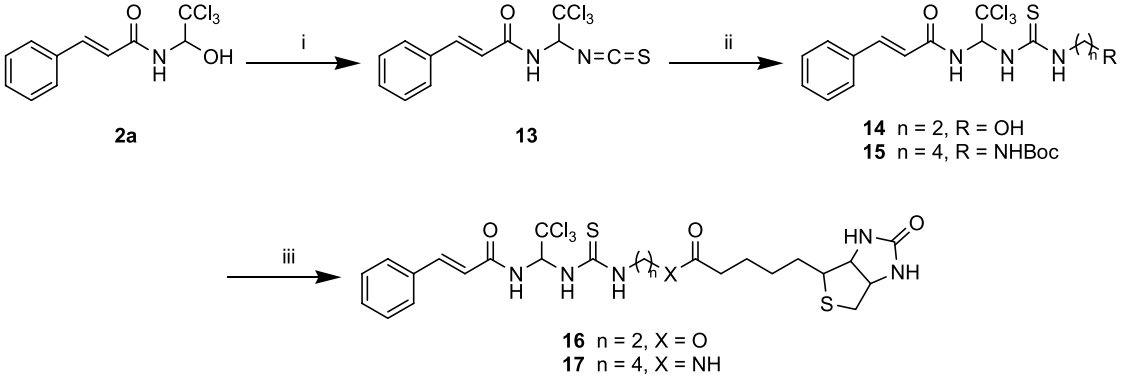
Next, the cinnamide system was modified and analogues **4e**–**4q** were synthesized. Most of them displayed cytoprotection activity except compounds **4e**, **4f**, and **4n**. Activity results of compounds **4b**, **4e**, and **4h** indicated that a cyclic moiety as R^1 might be favored for the activity. Comparison of compounds **4b**,**4f**, and**4g** suggested that a proper distance between the cyclic moiety and the trichloromethyl group might play some role for the activity. Activity results of compounds **4b**, **4g**, and **4m** demonstrated that the (*E*)-double bond in the leading compound might be unimportant because it could be changed into a (*Z*)-double bond or a single bond without much loss of activity.



Scheme 3. Reagents and conditions: (i) for **7**–**9**: cinnamide, toluene, 90 °C, 80–90%; for **10**: cinnamide, THF, rt, 65%; (ii) (1) PCl_5, Et_2O, (2) NH_3, Et_2O, 0 °C, 40–80%; (iii) 2-isothiocyanatopyridine, THF, 60 °C, 70–90%.



Scheme 4. Reagents and conditions: (i) $NaBH_4$, LiCl, THF/EtOH, 85%; (ii) PPh_3, DEAD, THF, 45%.



Scheme 5. Reagents and conditions: (i) (1) $SOCl_2$, Et_2O, (2) KSCN, acetone, 73%; (ii) for **14**: $HOCH_2CH_2NH_2$, $CHCl_3$, 70%; for **15**: $BocNH(CH_2)_4NH_2$, $CHCl_3$, 80%; (iii) for **16**: biotin, EDC, DMAP, DMF, 60%; for **17**: (1) TFA, CH_2Cl_2, (2) biotin, EDC, HOBt, DIPEA, DMF, 60%.

Substituents on the phenyl ring or replacement of it by other heterocycles seemed to have little influence on the activity except that a long chain attachment on the phenyl ring was unfavored (compound **4n**).

Next, the role of the trichloromethyl group was investigated. Removing the trichloromethyl unit resulted in analogues **5** and **6**, both of which were toxic to cells. Replacement of the trichloromethyl group by other multihalomethyl groups resulted in compounds **7**–**9**. Interestingly, a trifluoromethyl substituent completely eliminated the cytoprotection activity (compound **7**), whereas tribromomethyl (compound **8**) and dichloromethyl (compound **9**) substituents were tolerated. Other modifications on the trichloromethyl group afforded compounds **10**–**12**, which were all inactive. By considering all these results together, it is clear that the trichloromethyl group in the leading compound is crucial for the activity.

As mentioned above, salubrinal protects cells against ER stress-induced apoptosis via inhibiting eIF2α dephosphorylation (namely inducing eIF2α phosphorylation). We tested the analogues that showed rescue activity and found they also induced eIF2α phosphorylation,[3] which suggested that these analogs protected cells by the same mechanism.

After getting a clear SAR pattern, we turned to introduce a biotin moiety as an affinity tag into salubrinal, in the hope of using such a tagged reagent to purify the molecular target of salubrinal. According to the different activities of compounds **4d** and **4n**, we chose to link biotin from the R^2 side and used 2-hydroxyethylamine or 1,4-diaminobutane as a linker. Thus, compound **2a** was converted into the corresponding isothiocyanate **13** via two successive substitution reactions.[8] Addition of 2-hydroxy-ethylamine and monoprotected 1,4-diaminobutane to **13** afforded thiourea **14** and **15**, respectively. Condensation of **14** and deprotected **15** with biotin gave the corresponding biotinylated derivatives **16** and **17** (Scheme 5).[9]

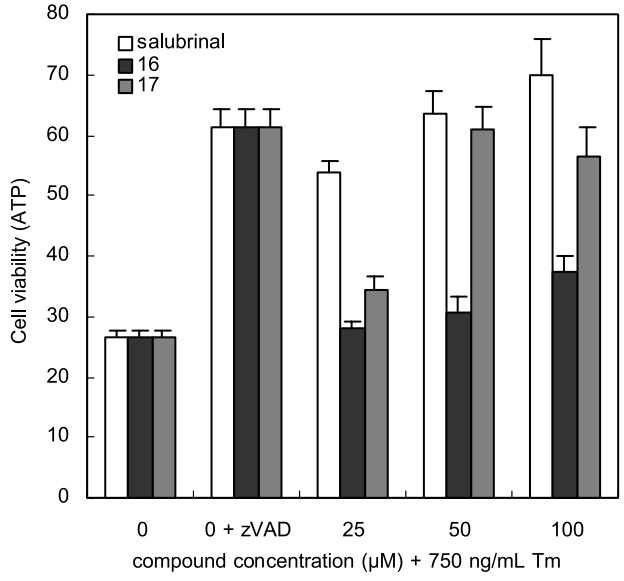


Figure 1. Dose-dependent protection of PC12 cells treated with Tm by salubrinal, compounds **16** and **17**, assessed by cellular ATP content. An amount of 100 μM of zVAD.fmk serves as a positive control. Error bars represent standard deviation.

We tested compounds **16** and **17** for the ability to protect PC12 cells from Tm-induced apoptosis. Compound **16** was almost inactive up to 100 μM. However, compound **17** exhibited considerable cytoprotection activity at the concentration range from 50 to 100 μM, and the efficacy was quite close to that of salubrinal, as shown in Figure 1. These results encourage us to use compound **17** as an affinity reagent to identify the binding target of salubrinal. The results will be reported elsewhere in due course.

Acknowledgments

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8. Drach, B. S.; Sinitsa, A. D.; Kirsanov, A. V. *Zh. Obshch. Khim.* **1970**, *40*, 934.
9. All the synthesized compounds were well charac-terized by ^{1}H NMR, MS, IR and elemental analysis. Analytical data for compound **17** are as follows: ^{1}H NMR (CD_3OD, 300 MHz) δ : 1.28 (q, J = 7.2 Hz, 2H), 1.37–1.58 (m, 8H), 2.03 (t, J = 7.2 Hz, 2H), 2.54 (d, J = 13.2 Hz, 1H), 2.78 (dd, J = 12.6, 5.1 Hz, 1H), 3.02–3.08 (m, 3H), 3.42 (m, 2H), 4.15 (dd, J = 7.8, 3.9 Hz, 1H), 4.35 (dd, J = 7.8, 4.5 Hz, 1H), 6.56 (d, J = 15.3 Hz, 1H), 7.24–7.52 (m, 6H), 7.88 (m, 1H); HRMS (ESI): Calcd for $C_{26}H_{35}Cl_3N_6O_3S_2Na$ ($M+Na^+$): 671.1170. Found 671.1180.
10. For a review, see: Kaufman, R. J. *Genes Dev* **1999**, *13*, 1211.