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Synthesis of a Small Molecule Nitrosocysteine Inhibitor of Caspase-1 Activity

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ABSTRACT Caspase-1 is an enzyme that is overactive in autoimmune and autoinflammatory diseases, cleaving pro-interleukin-1 β to the cytokine interleukin-1 β (IL-1 β), which leads to inflammatory symptoms. The inhibition of caspase-1 will cause a decrease in the concentration of interleukin-1 β (IL-1 β), resulting in the reduction of inflammatory symptoms. Recent research has revealed that the appending of a nitric oxide (NO) or nitroxyl (HNO) donating group to non-steroidal anti-inflammatory drugs (NSAIDs) reduced, or avoided, the side effects caused by currently available treatments. A small molecule based on a known caspase-1 inhibitor with a nitrosocysteine appended on it was synthesized to look at the effect of NO donation on caspase-1.

INTRODUCTION

Caspase-1 is an enzyme that is overactive in autoimmune and autoinflammatory diseases such as Alzheimer's disease, Crohn's disease, Rheumatoid Arthritis, and more.¹ The enzyme is responsible for the cleavage of pro-interleukin-1 β into the cytokine interleukin-1 β (IL-1 β). The abundance of IL-1 β leads to the inflammatory symptoms of the aforementioned diseases.¹ It does so by inducing gene expression of inducible nitric oxide synthase (iNOS) in glial cells, thus producing nitric oxide (NO)².

NO has a regulatory effect on immune response, in that lower concentrations of NO limits the inflammatory response and, in higher concentrations, it results in observed inflammatory symptoms.^{2,3} An effective inhibitor for caspase-1 will cause a decrease in the concentration of IL-1 β as the activity of the enzyme decreases, leading to a reduction in inflammatory symptoms.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the current drug options for treating some auto-inflammatory diseases. Most NSAIDs have gastrointestinal, renal, and hepatic side effects.² Recent research has revealed that an inhibitor

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containing a nitric oxide (NO) or nitroxyl (HNO) donating group could avoid the side effects caused by currently available NSAIDs.² Caspase-1 is known to have an active site thiol group, which makes it susceptible to oxidation by NO. A small molecule based on a known caspase-1 inhibitor with a nitrosocysteine appended on was designed and synthesized to look at the effect of NO donation on caspase-1, similar to the NSAID-NO inhibitors mentioned above.

Two parts are necessary for the analysis of the activity of caspase-1. First, a substrate is needed to simulate the environment necessary for the overactivity of caspase-1 and to produce a measurable quantity of activity. Second, an inhibitor must be used to decrease the activity of the enzyme through competitive, non-competitive, or mixed inhibition. Since the active site would be directly targeted, the inhibitor will likely be competitive and reversible. In this case, the NO group is presumably donated from the inhibitor to a thiol group located on the active site of caspase-1 (a process called trans-nitrosation), modifying the active site and blocking nucleophilic attack on the substrate by the active site thiol and subsequent cleavage of the substrate.

METHODS

General Methods: All reagents were purchased from commercial sources and used as received. Caspase-1 and fluorogenic peptide substrate for caspase-1 (WEHD-AMC) were purchased from Enzo Life Sciences. NMR spectra were taken on a Bruker 300 MHz spectrometer. Enzyme assays were performed in 96-well plate format on a BioTek Synergy H1 multimodal plate reader. All purifications were done using silica gel and gradients of dichloromethane/methanol or hexanes/ethyl acetate as detailed below.

Route A:

Preparation of A2: 2-phenoxybenzoic acid (100-200 mg) was added to a vial with a stir bar. Approximately 1 mL of thionyl chloride was added to the vial and covered with parafilm. The reaction vial was then placed in a hot oil bath at

60°C and left for the mixture to reflux for 24 hours. After 24 hours, the vial was removed from the hot oil bath and left to stir for a minimum of five days. Once the reaction was completed, as determined through TLC (95% DCM:5% MeOH), the product was extracted four times using sodium bicarbonate and dried with sodium sulfate. The product was washed with dichloromethane and solvent was removed with a rotary evaporator; washing was repeated twice more. Observations: Yellow, grainy solid product. Yield of **A2**: 77.9%. **A2** ¹H NMR (300 MHz, CD₃OD) δ: 7.91 (d, 2H), 7.19-7.47 (m, 16H), 6.92-7.02 (t, 3H).

Preparation of A3: Crude **A2** product (0.1 M in dry DCM) was reacted under nitrogen with 1.2 equivalents of S-trityl-cysteine, and excess DMAP. The reaction was left to stir for a minimum of two days and the reaction was determined complete through use of TLC (95% DCM:5% MeOH). The product was extracted three times using 1 M hydrochloric acid. Solvent was removed with a rotary evaporator. The desired product was isolated through column chromatography using DCM and methanol (0%-7%) and checked for purity using ¹H NMR in CD₃OD. Observations: Crude product: tar-like, off-white substance. Isolated product: pale, yellow solid. Yield of **A3**: 7.62%. ¹H NMR (300 MHz, CD₃OD) δ: 8.03 (d, 1H), 7.51 (t, 1H), 7.26-7.36 (m, 12H), 7.16-7.26 (m, 12H), 7.09 (d, 2H), 6.95 (d, 1H), 4.57 (t, 1H), 2.66 (m, 2 H).

Route B:

Preparation of B1: 2-phenoxybenzoic acid (100-200 mg) was combined with 1.5 molar equivalents of (L)-S-tritylcysteine amide, 1.5 molar equivalents of EDC, 1 molar equivalent of HOBT, 4-8 molar equivalents of DMAP and dissolved in DCM (0.1 M equivalent). The reaction was left to stir at room temperature for 24 hours. The reaction was monitored via TLC (1:1 Hexanes:EtOAc). The product was extracted four times with 1 M HCl, washed once with saturated NaCl, and dried over Na₂SO₄. Solvent was removed with a rotary evaporator. Column chromatography was used to isolate the product using a 2:1 Hexanes:EtOAc first to remove all highly mobile byproducts, and then followed by 1:1 Hexanes:EtOAc to remove the

desired product from starting material. The fractions containing the product were combined and solvent was removed with a rotary evaporator. An ^1H NMR in CDCl_3 was taken to check for purity. Observations: Off-white solid. Yield of **B2**: 55% ^1H NMR (300 MHz, CDCl_3) δ : 8.26 (d, 1H), 8.17 (dd, 1H), 7.39-7.48 (dt, 1H), 7.36 (d, 3H), 7.33 (d, 4H), 7.25 (d, 1H), 7.28 (d, 1H) 7.18 (s, 3H), 7.16 (s, 5H), 7.12 (d, 2H), 6.99 (d, 2H), 6.89 (d, 1 H), 6.19 (s, 1H), 5.45 (s, 1H), 4.44 (q, 1H), 2.82 (q, 1H), 2.53 (q, 1H).

Preparation of B2: A mass of **B1** was diluted with 1 mL of DCM and chilled on ice. A solution of 4:2:1 by volume of trifluoroacetic acid (TFA):DCM:triisopropylsilane (TIPS) was made for the reaction. **B1** had a final concentration of 0.1 M in the solution. The TFA:DCM:TIPS solution was added dropwise to **B1** and left to stir for an hour. Deprotection is indicated by the solution turning a bright yellow, followed by turning clear, indicating completion of the reaction. The solution was then washed with DCM and rotovapped to remove the TFA and repeated twice more. The product was dried under vacuum to remove all TIPS. Removal of excess TIPS was checked with ^1H NMR in CDCl_3 . After confirmation of all TIPS being removed, the product was isolated using column chromatography starting with 100% DCM and finishing with 95% DCM: 5% MeOH. The fractions containing the product were combined and solvent was removed with a rotary evaporator. An NMR in CDCl_3 was taken to check for purity. The product was dried under vacuum to remove all solvents before finally being dissolved in DMSO to make a 20 mM solution. The solution was stored in the freezer at 0°C . Observations: White solid. Yield of **B2**: 10% with 95% purity. ^1H NMR (300 MHz, CDCl_3) δ : 8.69 (d, 1H), 8.23 (dd, 1H), 7.37-7.51 (m, 3H), 7.16-7.30 (m, 2H), 7.10 (d, 2H), 6.94 (d, 1H), 6.47 (s, 1H), 5.46 (s, 1H), 4.94 (m, 1H), 3.26 (m, 1H), 2.70 (m, 1H).

Preparation of B3: At least 50 μL of a 0.2 M NaNO_2 in 0.5 M HCl solution was made using a small mass of NaNO_2 . Equivalent volumes (50 μL) of 20 mM **B2** and 0.2 M NaNO_2 in 0.5 M HCl were reacted together in a centrifuge tube

and covered with foil to reduce the reaction's exposure to light. The reaction was left for 30 minutes. The nitrosylated **B3** was diluted to make 500, 250, and 50 μM dilutions in DMSO.

Caspase-1 inhibition assay of B3: The inhibition assay was done at 100 μL total volume and in triplicate in a 96-well plate format. The assay was monitored at room temperature for 60 minutes in HEPES buffer (50 mM HEPES, 10 mM NaCl, 0.5% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM DTT pH 7.4). Caspase-1 was activated with 100 μM DTT on ice for 30 minutes and held constant for all assays with 5 nM caspase-1. The substrate WEHD-AMC was held constant for all assays at 10 μM . After enzyme activation, the inhibitor and caspase-1 were left to incubate for 30 minutes before the addition of WEHD-AMC substrate. The reactions were monitored immediately after the addition of the WEHD-AMC to the wells. The percent activities of caspase-1 were calculated from the control screening of caspase-1 containing no inhibitor. The concentration of product, 7-amino-4-methylcoumarin (AMC) was calculated from the corresponding standard curve. Percent activity versus inhibitor concentration was plotted in Origin and fit using a non-linear curve fit to approximate IC_{50} values of **B3** from the dose-response curve.

RESULTS

An initial synthesis (Route A) was proposed to make a small molecule nitrosocysteine inhibitor, starting with 2-phenoxybenzoic acid. Generally, the synthesis was ineffective and the final product could not be obtained for testing with caspase-1. Because of this, a second, more efficient synthesis (Route B) was developed that yielded a nitrosocysteine-containing caspase-1 inhibitor. Route A is shown in Figure 1.

The first step began with 2-phenoxybenzoic acid, **A1**. The reaction was refluxed with thionyl chloride to generate **A2**, an acid chloride. In the first attempt, a minor extraction was performed with HCl, and the reaction yielded a dark brown, grainy product. In the second reaction, **A2** was fully extracted, resulting in the pale yellow solid seen in this and the remaining two attempts. The

crude **A2** product was reacted with (L)-S-tritylcysteine, along with TEA and DMAP, and dissolved in DCM to form **A3**. The product **A3** was successfully made in all the attempted reactions; however it was isolated only once. The yield was so small that the next step could

not be taken. Only the first two steps of the Route A synthesis were completed: the substitution of the carboxylic acid to form **A2**, and the addition of S-trityl-cysteine to the acid chloride to form **A3**.

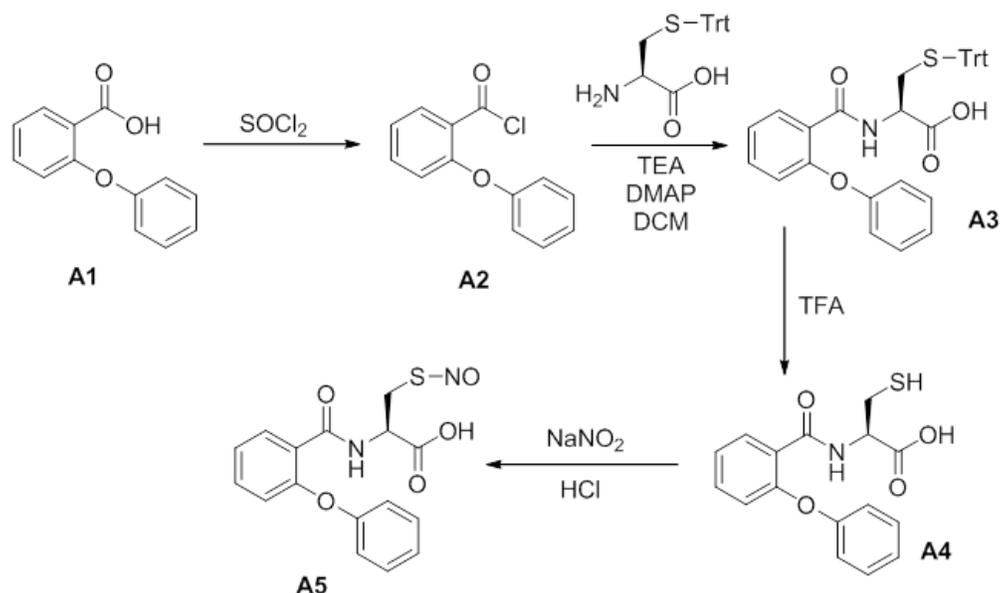


Figure 1. Route A synthesis of nitrosocysteine inhibitor using (L)-S-tritylcysteine.

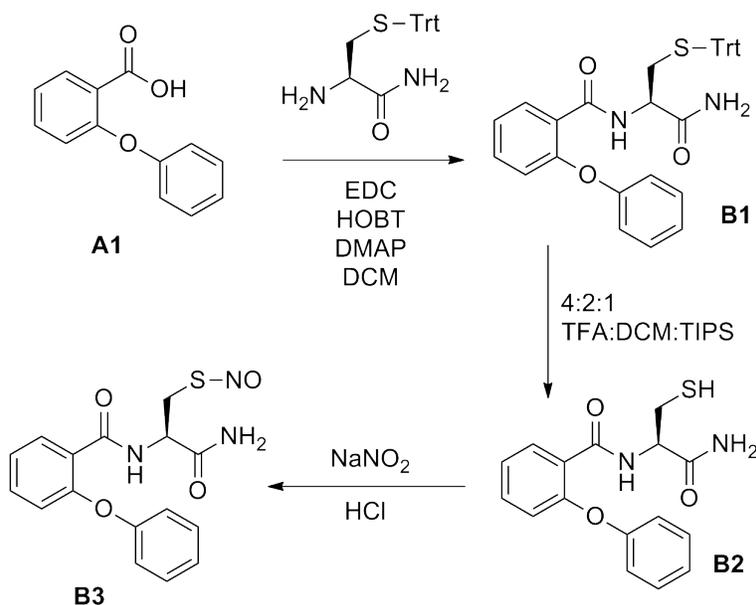


Figure 2. Route B synthesis of nitrosocysteine inhibitor using (L)-S-tritylcysteine amide.

A second synthesis was proposed using (L)-S-tritylcysteine amide instead of (L)-S-tritylcysteine. Route B is shown in Figure 2.

The reaction begins again with 2-phenoxybenzoic acid, **A1**. However the carboxylic acid is directly amidated using (L)-S-

tritylcysteine amide with EDC, HOBT, and DMAP dissolved in DCM. The product **B1** was made successfully in multiple reactions. After a small scale reaction was completed with high success, a scale-up reaction was done to generate a larger amount of material to use for the deprotection step. The S-trityl group on **B1** was initially deprotected using only TFA (about 1 mL for 10 mg of **B1**) and no DCM or TIPS. However, the product could not be isolated from the removed trityl group. A second attempt was made using a 4:2:1 solution of TFA:DCM:TIPS. The first attempt with the 4:2:1 solution of TFA:DCM:TIPS was unsuccessful and the product could not be isolated from the excessive

amount of TIPS. Another attempt was made using less of the 4:2:1 solution of TFA:DCM:TIPS. The reaction was completed within an hour and the final **B2** product was isolated using column chromatography. Enough material was made to save and use in the final step. The nitrosylation of **B3** occurred with NaNO_2 dissolved in 0.5 M HCl and was tested against caspase-1. Preliminary biochemical results showed **B3** displayed an IC_{50} $5.5 \pm 2 \mu\text{M}$ (Figure 3). Additional studies need to be completed to determine the mechanism of inhibition prior to precise determination of IC_{50} values.

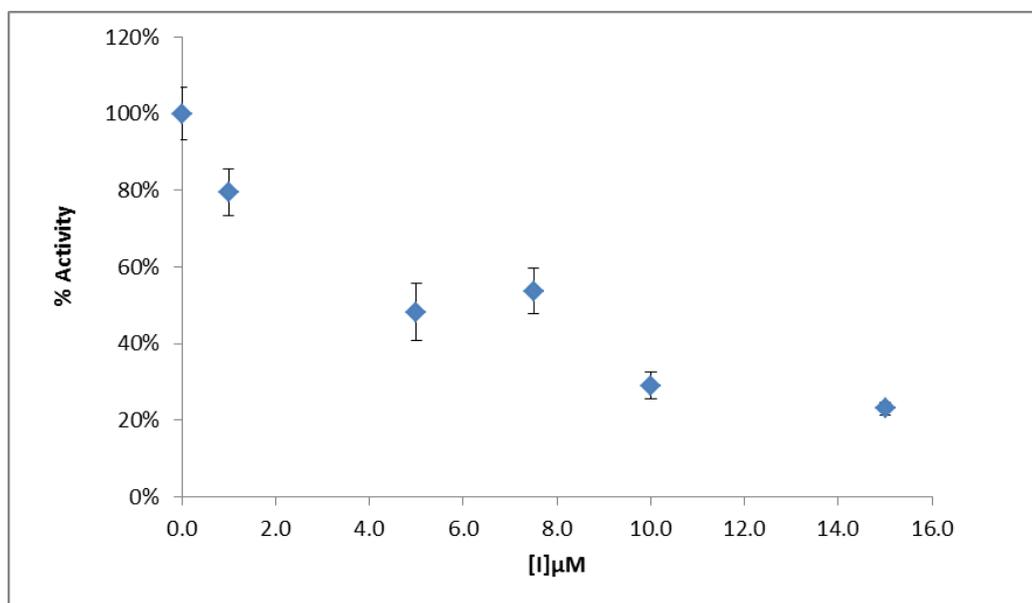


Figure 3. IC_{50} curve of **B3** showing reduction of % activity of caspase-1 versus concentration of inhibitor (μM).

DISCUSSION

As stated earlier, the Route A synthesis was unsuccessful, mainly due to the unsuccessful formation of **A3**. The biggest issue is that **A3** and the (L)-S-tritylcysteine had so similar an R_f value that **A3** could not be removed from the column without contamination from the cysteine starting material. The reason the yield had been so small (<10%) in the second attempt of isolating **A3** was because most of the fractions contained the (L)-S-tritylcysteine. There were also multiple side products from the reaction

indicating that the reaction was not entirely efficient, despite having gone to completion.

The failure to produce a workable amount of **A3** after multiple reactions led to a redesign of the synthesis. Instead of having **A1** react with thionyl chloride to make an acid chloride before adding the cysteine, the synthesis was shortened by adding the (L)-S-tritylcysteine amide directly to **A1**. Recognizing that the free carboxylic acid caused a major issue in the isolation of **A2**, the next reaction used an amidated version of the cysteine, (L)-S-tritylcysteine amide as starting

material. The amido group is less acidic than the carboxylic acid, which in turn created a simpler separation via silica gel column chromatography. **B1** was made easily within 24 hours and the workup removed excess EDC and HOBT from the product. However, it was not entirely effective in removing all the cysteine starting material. Column chromatography was used to isolate the product without contamination from either the side products or cysteine starting material, as checked by ^1H NMR. The NMR spectra showed an alkyl peak at 4.44 ppm, indicating that the reaction had occurred to form **B1**. After the initial success of the reaction on a small scale, the procedure was repeated to generate more material to work with. The scale-up was successful, pointing to the reproducible nature of this pathway.

Deprotection of **B1** was first attempted with pure TFA without any DCM solvent or TIPS. The reaction successfully removed the trityl group, as observed by the solution turning a bright yellow. However, **B2** could not be isolated from the removed trityl group through any extraction method. With additional research, the 4:2:1 TFA:DCM:TIPS solution was found and used in attempt to deprotect **B1** and remove the trityl group from the solution.⁵ TIPS is needed in order to remove the deprotected trityl group. TFA readily removes the trityl group, turning the solution a bright yellow.⁵ The bright yellow color is the trityl cation that was removed in the reaction.⁵ As the TIPS binds to the removed trityl cation, the solution becomes clear again, indicating the completion of the reaction.⁵ An excess of the solution was used the first time and the product was unsalvageable from excess TIPS. TIPS has a high boiling point and cannot be easily removed through use of a rotary evaporator. The reaction was repeated and **B2** was successfully made with about 95% purity of **B2**. Several of the ^1H NMR peaks had shifted,

providing evidence that the trityl group had been replaced with hydrogen. For example, the alkyl peak that was seen in the NMR for **B1** was at 4.44 ppm, but, in **B2**, it was observed at 4.94 ppm. Along with the peak shifts, there was a dramatic drop in the number of aromatic protons, providing another strong indicator that the trityl group had been successfully removed. The small yield is primarily due to spillage of the dissolved product before it was dried under vac; however, there was still enough **B2** product to use for the next reaction. The 20 mM stock of **B2** was stored at 0°C in the freezer and was used to test the nitrosylation reaction and the effect of NO donation to caspase-1.

The nitrosylation reaction occurred approximately 30 minutes before it was tested against caspase-1. The initial results showed that the **B3** inhibitor has an IC_{50} of about $5.5 \pm 2 \mu\text{M}$. This means that the inhibitor is capable of reducing caspase-1 activity to 50 % at a concentration of 5.5 μM . However, these results have not been replicated. The product of the nitrosylation reaction in sodium nitrite also needs to be quantified, which can be done using spectrophotometric methods to determine the exact amount of small molecule S-NO inhibitor we are starting with. After presenting more evidence that the **B3** inhibitor is working and reducing caspase-1 activity, Michaelis-Menten kinetics of the inhibitor will be studied along with reversibility.

A small molecule nitrosocysteine inhibitor was successfully made and tested against caspase-1. Initial results have shown successful inhibition of caspase-1 using a NO donor small molecule inhibitor. Similar small molecules can be synthesized and tested against caspase-1 to study the effect of inhibition with differing substituents on the aromatic ring.

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AUTHOR CONTRIBUTIONS

C.E.K designed the project. C.A.S carried out the project and wrote the paper.

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