HHS Public Access

Author manuscript

J Med Chem. Author manuscript; available in PMC 2022 May 18.

Published in final edited form as:

J Med Chem. 2021 November 11; 64(21): 15593–15607. doi:10.1021/acs.jmedchem.1c00419.

Development of a Redox-Sensitive Spermine Prodrug for the Potential Treatment of Snyder Robinson Syndrome

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Abstract

Snyder Robinson Syndrome (SRS) is a rare disease associated with a defective spermine synthase gene and low intracellular spermine levels. In this study, a spermine replacement therapy was developed using a spermine prodrug that enters cells *via* the polyamine transport system. The prodrug was comprised of three components: a redox-sensitive quinone “trigger”, a “trimethyl lock (TML)” aryl “release mechanism”, and spermine. The presence of spermine in the design facilitated uptake by the polyamine transport system. The quinone–TML motifs provided a redox-

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Author Contributions

O.P. created the prodrug design and co-wrote the manuscript with M.P.T. M.P.T. also synthesized the prodrug, performed the stability studies, and assisted V.S. in the *in vitro* evaluations and HPLC analyses of polyamine pools. X.T. and R.G.Z. performed the *Drosophila* evaluations and R.G.Z. provided experimental write-ups for the *Drosophila* experiments.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00419.

CV details and profile for prodrug **1**, polyamine levels in CMS-23916 cells treated with prodrug **1** (at 50 μM or 5 μM), CDAP (100 μM), or Spm (5 μM), graph showing an increased intensity of Mitoview 633 staining (APC-A) in CMS-23916 cells pretreated for 6 h with NAC (N) (2 mM) followed by 24 h incubation period, polyamine levels in CMS-6233 (mutant) fibroblasts cultured for 24 h in the presence of aminoguanidine (1 mM) with or without treatment of prodrug **1** or Spm (5 μM) for 24 h, ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectra of compounds **1** and **5**–**8**, elemental analyses for compound **1**, HPLC purity checks for compounds **6** and **8** (PDF)

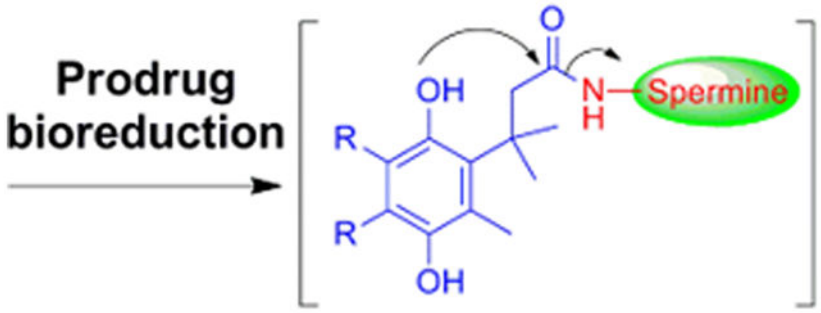
Bioactivities observed (CSV)

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jmedchem.1c00419

The authors declare no competing financial interest.

sensitive agent, which upon intracellular reduction generated a hydroquinone, which underwent intramolecular cyclization to release free spermine and a lactone byproduct. Rewardingly, most SRS fibroblasts treated with the prodrug revealed a significant increase in intracellular spermine. Administering the spermine prodrug through feeding in a *Drosophila* model of SRS showed significant beneficial effects. In summary, a spermine prodrug is developed and provides a lead compound for future spermine replacement therapy experiments.

Graphical Abstract



INTRODUCTION

Snyder Robinson Syndrome (SRS) is a rare disease first described by Snyder and Robinson in 1969[1] with <100 reported cases worldwide. In 2003, Cason et al. demonstrated that this syndrome resulted from a mutation in the spermine synthase gene (SMS).[2] SMS protein converts spermidine (SPD) to spermine (SPM) *via* the transfer of an aminopropyl group. As shown in Figure 1, the aminopropyl unit comes from the amino acid methionine after its conversion first to *S*-adenosylmethionine (SAM) and then to decarboxylated *S*-adenosylmethionine (dc-SAM) by *S*-adenosylmethionine decarboxylase (SAMDC).[3] Biochemically, SMS patients have low levels of intracellular spermine and elevated spermidine/spermine ratios (due to low to no SMS activity). With a low level of SPM, the patients with SRS often present with osteoporosis, scoliosis, facial asymmetry, speech abnormalities, seizures, and low bone density.[4,5] This phenotype, in part, may stem from the role of spermine in calcium import[6–10] and other processes.[11] Another phenotype from this X-linked disorder is impaired intellectual function, presumably due to reduced spermine levels in the brain. Indeed, polyamines are important for neurodevelopment because mutations in other enzymes in the polyamine pathway (ODC, DHPS, and eIF5A) have also been linked to neurodevelopmental disorders.[12–15]

Recently, Casero et al. demonstrated that lymphoblastoid/fibroblast cells derived from SRS patients are capable of importing exogenous spermine and its analogues (Me_2SPM) into the cell, and their import results in a significant decrease in the intracellular spermidine pools.[16,17] While our understanding of this disease has led to new diagnostic genetic tools, there are, unfortunately, no cures and physicians are relegated to treating patient symptoms. Attempts to treat SRS patients with spermine have been unsuccessful.[18] The limited literature in this area suggests that the administration of spermine (SPM) by intraperitoneal injection or through dietary supplementation led to toxicity or low patient compliance.[19,20]

Quinones have been studied over several decades due to their interesting electronic and biological properties. Natural and synthetic quinones are an important class of biologically active agents due to the reversible quinone/hydroquinone equilibrium in biological systems.[21] Example bioactive quinones are shown in Figure 2 and include coenzyme Q, vitamin K, Mitomycin C (chemotherapeutic agent), and doxorubicin (anticancer agent).[22] Depending upon their substituents, most quinones are reduced by various bio-reductive enzymes to hydroquinones.[23] Due to the unique redox potential associated with quinones, the reduction can be through a one-electron pathway to produce a semiquinone or through a two-electron pathway to hydroquinones.[24] Generally, quinones are typical substrates for oxidoreductases and can be reduced by accepting two electrons from NADPH in a reaction catalyzed by diaphorase to form hydroquinones.[25]

Beyond the unique redox abilities of quinone systems, quinones containing a trimethyl lock motif are found in several prodrug designs due to their intracellular reduction to the reactive hydroquinone form. As shown in Scheme 1, the hydroquinone undergoes a rapid intramolecular lactonization to release an appended bioactive component (Scheme 1).[26] The “trimethyl lock” (TML) is a highly versatile molecular release system, which dramatically increases the rate of lactonization of trialkyl-substituted quinone propionic acid derivatives. The trimethyl lock concept was first described by Carpino et al.[24] and applications of this drug-release technology have been expanded by other research groups.[27,28] Due to their special conformational restriction and bio-reductive release, TMLs have been used in modern drug design and cell imaging.

This paper describes our efforts to harness the TML technology to form a spermine prodrug, where the displaced drug in Scheme 1 is spermine. Here, we describe the synthesis and bioevaluation of the spermine prodrug for its ability to rebalance intracellular polyamine pools in fibroblasts derived from patients with Snyder Robinson Syndrome (SRS). We demonstrate that the prodrug, indeed, releases free spermine and can rebalance intracellular polyamine pools in select SRS fibroblasts. Consistent with a functioning polyamine transport system, exogenous spermine was able to enter and rebalance polyamine pools in all SRS fibroblasts tested. In contrast, the prodrug selectively delivered spermine to SRS fibroblasts with specific mutations. Further experiments revealed that the prodrug unresponsive cells imported the prodrug but failed to effectively convert it to spermine suggesting a lower cellular reduction potential in specific SRS cell types. This is consistent with the known mitochondrial defects noted in SRS cells with specific mutations.[29,30] Further experiments showed that one could convert the recalcitrant fibroblast line to a prodrug-responsive line *via* preincubation with *N*-acetylcysteine (NAC).

RESULTS AND DISCUSSION

Ubiquinone (UQ), also known as coenzyme Q (CoQ), is a lipophilic redox-active molecule present in all eukaryotes (Figure 2). In UQ, the redox-active benzoquinone group is conjugated with the lipophilic side chain (polyisoprenoid), which makes it lipid soluble. UQ plays an important role in mitochondrial energy generation. Due to the redox-active benzoquinone head group, UQ can easily accept electrons to form ubiquinol inside the cells.[31] In an effort to develop a novel treatment for Snyder Robinson syndrome, we

designed a spermine prodrug by incorporating important features of ubiquinone and the trimethyl lock system in single molecule to deliver spermine efficiently inside cells (see compound **1** in Figure 3). Unlike spermine, the prodrug requires a two-step process involving cellular uptake and intracellular reduction to deliver spermine. This requirement provided the advantage of a targeted intracellular delivery system, which was enhanced in cells with active polyamine import. However, this advantage also presented the potential scenario where certain SRS cells with defective mitochondria may be able to import the prodrug but may have insufficient cellular reduction potential to reduce the quinone and release spermine.

Synthesis.

The prodrug **1** was synthesized, as outlined in Scheme 2. Initially, the commercially available quinone **4** was reduced using aqueous sodium dithionite to form hydroquinone **5** in a 90% yield. Next, treatment of hydroquinone **5** with methyl 3,3-dimethylacrylate under acidic conditions led to the lactone **6** in good yield (72%) using a reported procedure.[24]

Opening of the TML lactone **6** was challenging and was explored under various reaction conditions (Table 1). An initial experiment was conducted with lactone **6** in the presence of *N*-bromo succinimide (NBS) in 10% aq. acetonitrile and the expected product **7** was isolated in 25% yield (Table 1, entry 1). Next, the lactone-opening reaction was attempted using NBS at 0 °C but failed to improve the product yield (Table 1, entry 2). In another attempt, we used NBS as the oxidant in a combination of acetonitrile:acetone:- water (4.5:4.5:1), which slightly improved the product yield (Table 1, entry 3). Furthermore, the use of sodium hydroxide to open lactone and air oxidation failed to deliver the desired product **7** (Table 1, entry 4).

Next, we used pyridinium dichromate (PDC, 1 equiv) as the oxidant, which delivered the acid **7** in a slightly improved yield. Interestingly, the use of more equivalents of PDC (2 and 4 equiv) in *N*,*N*-dimethylformamide (DMF) afforded the desired acid **7** in 40 and 65% yields, respectively (Table 1, entries 6 and 7). The polyamine component was introduced *via* the trisubstituted *tert*-butoxycarbonyl (Boc)-protected spermine **3** reported earlier by our group.[32] Our early efforts to couple the synthesized acid **7** with **3** provided the amide **8** in poor yield. The reason for this poor yield was likely due to the unstable nature of acid **7**. To overcome this problem, the crude acid **7** was maintained in solution and condensed with the Boc-protected spermine motif **3** using 1-[bis-(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxide hexafluoro-phosphate (HATU) as the coupling reagent and provided the amide **8** in 70% yield after two steps (lactone-opening and -coupling reaction). Finally, the Boc groups in **8** were cleaved using 4 M HCl in dioxane to give the pure spermine prodrug **1** as its trihydrochloride salt in 85% yield.

The prodrug was then characterized by ^{1}H NMR, ^{13}C NMR, high-resolution mass spectrometry (HRMS), and elemental analysis; all of which were consistent with its structure. In addition, cyclic voltammetry (CV) experiments were conducted at pH 7.4 and showed the expected redox profile for the quinone–hydroquinone interchange (Supporting Information, Figure S1).

Biological Evaluation.

We first evaluated the toxicity of spermine, prodrug **1**, and lactone **6** (which is the byproduct of prodrug spermine release) in wild-type (CMS-24949) and SMS mutant (CMS-26559, CMS-6233, and CMS-23916) fibroblast cells received from the Greenwood Genetic Center (Greenwood, South Carolina). As shown in Table 2, spermine, prodrug **1**, and lactone **6** were relatively nontoxic in all tested fibroblasts (IC_{50} = >100 μM) after 72 h incubation at 37 °C. Polyamines are sensitive to degradation by amine oxidases present in bovine serum and are often tested in the presence of the amine oxidase inhibitor aminoguanidine (AG). AG was found to be nontoxic in the tested cells (wild-type and mutant cells) up to a concentration of 1.0 mM and was co-dosed with each compound to slow compound degradation.

Casero et al. reported that SRS fibroblast cells can rebalance their intracellular polyamine pools upon treatment of exogenous Spm (5 μM).[16] Based on our toxicity studies and this previous report, we dosed fibroblasts (wild-type and SMS mutant) and measured spermine levels inside the cells in the presence (5 μM) and absence of either spermine or prodrug **1** in the presence of aminoguanidine (1 mM) as a bovine serum amine oxidase inhibitor. Successful spermine delivery by either spermine or prodrug would appear as increased spermine levels by high-performance liquid chromatography (HPLC) analysis of the lysed (prewashed) cell pellets. Briefly, cells are incubated for 72 h with each agent and then the cells are washed, lysed, and the intracellular polyamines were extracted into a perchloric acid buffer. The polyamines are then *N*-dansylated and quantified by HPLC using authentic standards of each *N*-dansyl polyamine metabolite (Figure 4).

The polyamine distributions in SRS patient-derived fibroblast cell lines are shown in Figure 4 and Table 3. As shown in Figure 4a, the untreated wild-type (WT; CMS-24949) fibroblast cells had significant levels of intracellular Spm (8.65 nmol/mg protein) and demonstrated a low Spd:Spm ratio (0.2). When the wild-type fibroblast cells were treated with either exogenous Spm (5 μM) or prodrug **1** (5 μM), the polyamine levels were not significantly altered presumably due to homeostatic controls, which allow for the interconversion of polyamine levels. We noted high intracellular spermine levels compared to spermidine in the untreated wild-type fibroblasts (i.e., low Spd/Spm ratios (0.2) in the WT cell line), which were consistent with the lower Spd/Spm ratio findings of Murray-Stewart et al. in wild-type fibroblasts.[16] In contrast, the CMS-26559 (SMS mutant) fibroblast cells had a highly skewed Spd:Spm ratio (49) (Figure 4b). Interestingly, treatment of CMS-26559 mutant cells with exogenous Spm (5 μM) or prodrug **1** (5 μM) rewardingly increased the intracellular Spm concentration but also significantly reduced the intracellular level of Spd. This significant shift in polyamine pools resulted in decreased Spd:Spm ratios for the spermine (ratio 0.25) and prodrug treatments (1.39), respectively. In addition, we also observed that the total polyamine level (Put, Spd, and Spm) in CMS-26559 cells was more than triple that of WT cells (37.0 vs 10.8 nmol/mg protein), and treatment with either Spm or prodrug **1** slightly reduced the total polyamine levels to ~33.2 nmol/mg protein. The intracellular polyamine analysis (Table 3) and the literature report by Larcher et al.[33] suggest that CMS-26559 mutant cells have the most severe mutation with regard to limited to nonexistent SMS activity. The good response by this cell line validated our strategy and provided hope

for new therapies for these “most severe” SMS mutations in terms of rebalancing their polyamine pools.

Two other SMS mutant fibroblast cell lines (CMS-6233 and CMS-23916) were tested for their intracellular polyamine pools. In CMS-6233 fibroblast cells, the basal Spm concentration (~7.0 nmol/mg protein) was near the observed Spm level in untreated WT cells (~8.65 nmol/mg protein) with a Spd:Spm ratio of ~1.75, as shown in Figure 4c. The treatment of CMS-6233 cells with Spm or prodrug **1** (5 μM) for 72 h increased the intracellular Spm level up to 15.6 and 8.7 nmol/mg protein, respectively. The noted increases in Spm were associated with decreased Spd pools. The concentration of total polyamines was not much affected with Spm or prodrug **1** treatment; however, the Spd:Spm ratio was decreased from ~1.75 to ~0.21 (Spm) and ~1.02 (prodrug), respectively. This provided another example of an SMS mutant cell line that responded to spermine and prodrug therapy by rebalancing intracellular polyamine pools while maintaining total polyamine levels presumably *via* homeostatic processes. In CMS-23916 (mutant) cells, the Spd:Spm ratio was ~3.3 and treatment with Spm or prodrug **1** reduced the Spd:Spm ratio to ~0.56 and ~2.62, respectively. This cell line was unique in that the response to spermine was significantly different than the prodrug. The HPLC analysis showed that very little change occurred in polyamine pools in the presence of the prodrug compared to spermine treatment. In this regard, the SRS cell line (CMS-23916) was essentially recalcitrant and unresponsive to the prodrug at 5 μM.

The most encouraging finding of this work is that the prodrug **1** showed significant outcomes in cells with a virtually complete loss-of-function SMS protein (CMS-26559). Collectively, these SRS cell line investigations suggested that all mutant SMS cells are able to import spermine and rebalance their polyamine pools and reduce their Spd/Spm ratios. However, not all SRS cells responded equally to prodrug therapy. Surprisingly, the mutational status of each cell resulted in different prodrug responses even though exogenous spermine was able to enter and replenish spermine pools (Table 3).

How could one SMS mutation give rise to a prodrug-responsive cell and a different SMS mutation cause the cells to be unresponsive to the prodrug? Using the responsive (CMS-26559) and unresponsive (CMS-23916) cell lines, we further investigated their prodrug response properties. We considered several hypotheses to explain our results.

First, we investigated whether one could improve prodrug performance at a higher dose (>5 μM) in the recalcitrant cell line CMS-23916. To test this hypothesis, we treated CMS-23916 cells with a 10-fold higher concentration of prodrug **1** (50 μM) and analyzed the intracellular polyamine levels using HPLC. The intracellular polyamine pools suggested that the use of a 10-fold higher concentration of prodrug gave only modest increases in the intracellular Spm concentration with the Spd:Spm ratio ~1.5 (Supporting Information, Figure S2). The result obtained from this experiment suggested that the use of higher concentrations of prodrug **1** (50 μM) treatment provided only modest gains in the recalcitrant cell line CMS-23916.

Second, we noted that the initial Spm concentration in CMS-23916 cells was higher than in the prodrug-responsive CMS-26559 cells. We hypothesized that the higher starting Spm

levels in CMS-23916 may be responsible for the moderate response of **1** in these cells. To probe this hypothesis, we decreased the intracellular Spm level in CMS-23916 cells using *N*-cyclohexyl-1,3-diaminopropane (**CDAP**), a known inhibitor of SMS. CDAP at a nontoxic dose of 100 μM reduced intracellular Spm levels by 66% (Supporting Information, Figure S3). However, the combination of CDAP (100 μM) with **1** (5 μM) failed to rebalance the Spm pool efficiently. The outcome of this experiment suggested that the higher starting Spm levels in CMS-23916 cells were not the reason for the minimal response to prodrug **1**.

With this knowledge in hand, we envisioned two possible explanations for the differential response to the prodrug: (a) defects in import and (b) defects in cell reduction potential. The fact that all of the tested SMS mutant cells responded well to Spm treatment at 5 μM suggested that polyamine import was active and functional. Indeed, a prior report by Murray-Stewart et al. demonstrated that this was the case in SRS lymphoblasts and fibroblasts.[16,17] Since one end of spermine is capped as an amide group in the prodrug **1**, one of the charges on spermine is masked and the prodrug is essentially a substituted spermidine derivative. To study the rate of prodrug and Spm uptake in SMS mutant cells, we determined the kinetic parameters associated with competitive uptake of ^{3}H-spermidine in the two mutant cell lines (CMS-26659 and CMS-23916) and the data are shown in Table 4.

Remarkably, the transport parameters are similar in both cell lines, suggesting that they have approximately the same affinity for spermine, the same affinity for prodrug, and similar affinities for Spd uptake. The V_{max} values were slightly higher in the responsive cell line (CMS-26559) but did not seem large enough to explain the observed differences. The fact that spermine and prodrug had approximately the same affinity (K_i value) for binding to the putative polyamine transport receptor and that all cells responded to spermine but not prodrug suggested that differential import of spermine over prodrug was not the case. These experiments effectively ruled out defective import as a reason for the difference in spermine rescue in the responsive and unresponsive cell lines.

High intracellular polyamine levels are known to inhibit the import of exogenous polyamines.[35,36] This property would be expected to limit prodrug and spermine import *via* the polyamine transport system into SRS cells. Fortunately, this was not the case. For example, as shown in Figure 4 and Table 4, SRS cells with relatively high polyamine levels like CMS-26559 (total polyamines: ~37 nmoles/mg protein) had similar V_{max} values (V_{max} = 0.50 pmol/μg protein/min) when compared to CMS-23916, which had significantly lower intracellular polyamines (~22 nmoles/mg protein total polyamines), and V_{max} of 0.33 pmol/μg protein/min. This suggested that high intracellular polyamine load in these cells does not significantly inhibit import under these conditions. In short, there seems to be sufficient uptake to provide therapeutic benefit as demonstrated by the delivery of spermine into cells by either spermine or prodrug *in vitro*.

Previous studies have demonstrated that the SMS deficiency impairs mitochondrial function.[29,30] The dysfunction of mitochondria is linked to the oxidative state of the cell because mitochondria can be damaged by reactive oxygen species (ROS). Because the ROS hydrogen peroxide is a byproduct of spermidine catabolism, we speculated that the oxidative stress generated from the metabolism of the imbalanced polyamine pools in SRS cells may

affect mitochondrial function and lower cellular reduction potential.[29,30] In short, ROS load could explain why certain SRS cells were recalcitrant to the prodrug. Since polyamine import activity was still operative in the recalcitrant cell line, we speculated that the prodrug entered these cells but failed to release spermine due to a lower cellular reduction potential caused by ROS.

To investigate this possibility, we compared the mitochondrial membrane potential (MMP) in the recalcitrant (CMS-23916) and high-responding (CMS-26559) cell lines by JC-1 and Mitoview 633 staining. JC-1 is a cationic dye that aggregates when it enters the mitochondria of cells with a normal MMP and upon excitation fluoresces in the red region of the electromagnetic spectrum. In contrast, in cells with impaired mitochondria, the JC-1 dye enters the mitochondria to a lesser degree and predominantly emits green fluorescence as its monomeric form in the cytosol. Comparison of the JC-1 staining pattern in both cell lines by fluorescence-activated cell sorting (FACS) revealed that in high-responding CMS-26559 cells, 78.2% of cells showed green and red fluorescence and only 2.7% cells exhibited green fluorescence. However, in the recalcitrant CMS-23916 cells, 31.1% cells showed green fluorescence (~11-fold higher than CMS-26559 cells), indicating impaired membrane potential in these cells (Figure 5A).

To confirm these findings, we also stained both the cell lines with Mitoview 633, which is another far-red fluorescent mitochondrial dye (absorbance/emission at 622/648 nm). The dye is membrane permeable and becomes brightly fluorescent (red) upon accumulation in the mitochondria. Cells with high MMP would exhibit a higher intensity of red fluorescence. As shown in Figure 5b, CMS-26559 cells treated with MitoView 633 (100 nM) for 15 min exhibited an ~10-fold higher intensity of red fluorescence by flow cytometry compared to the CMS-23916 cells. Taken together, these observations clearly indicate that the recalcitrant cell line CMS-23916 has defective mitochondria resulting in a reduced redox potential inside the cells.

Fortunately, cellular ROS load can be lowered by the use of small-molecule antioxidants such as *N*-acetylcysteine (NAC), *N*-acetylcysteine amide (AD4), or *N*-2-mercaptopropionil glycine (*N*-2-MPG).[29,37]

To test this hypothesis, we treated these cells with NAC and observed a significant recovery of mitochondrial “health” using the MitoView probe after the 24 h incubation period where NAC and prodrug were co-incubated (see Figure S4 in the Supporting Information) after an initial 6 h preincubation with NAC only. We then examined intracellular polyamine levels in the recalcitrant CMS-263916 cells pretreated for 6 h with NAC (2 mM) to reduce ROS followed by prodrug addition (5 μM) and continued incubation together for 24 h. The data obtained in the presence of NAC was then compared with the prodrug only (5 μM, 24 h). As shown in Figure 6, the combination of prodrug (5 μM) and NAC (2 mM) significantly increased the intracellular spermine concentration compared to untreated (UT). This experiment demonstrated that recalcitrant SRS cells can be made prodrug responsive by pretreatment with the antioxidant NAC.

Prodrug Stability Studies.

The fetal bovine serum (FBS) used contains amine oxidases, which can degrade amine-containing molecules such as the prodrug **1** or spermine. An advantage of the prodrug design is that one end of the spermine molecule is sequestered in an amide bond effectively shielding it from amine oxidase degradation. In addition, the prodrug still presents a single polyamine end for recognition by the polyamine transport system or degradative amine oxidases. To assess its stability, prodrug **1** was incubated in complete media Dulbecco's Modified Eagle Medium (DMEM) containing 15% FBS for 24, 48, and 72 h at 37 °C. We then measured the amount of prodrug **1** remaining over time by HPLC in the presence and absence of aminoguanidine (AG, 1 mM, a known inhibitor of amine oxidases, see Figure 7).

Spermine has two aminopropyl termini, whereas the prodrug **1** contains a single aminopropyl terminus. Both compounds are expected to be substrates for amine oxidases, which can give rise to hydrogen peroxide.[38] This is a common issue in polyamine research and researchers often add the amine oxidase inhibitor aminoguanidine (**AG**) to inhibit the degradative action of these oxidases during the course of the experiment. Interestingly, in the absence of **AG** in CMS-26599 and CMS-23916 cells, spermine is more toxic than the prodrug consistent with its two aminopropyl motifs (see footnote, Table 2). One would, therefore, expect to see a slower rate of degradation of the amine-based compound in the presence of **AG**. As shown in Figure 7, the rate of decomposition of the prodrug was, indeed, slower in the presence of **AG**, which directly supports amine oxidases as being a major degradation pathway for this prodrug compound. After 24 h, there was 93% prodrug **1** remaining in the presence of **AG** (1 mM) but only 65% remaining in the absence of **AG**. The estimated half-life of the prodrug in the absence of **AG** was 72 h (where 50% of the prodrug still remained intact). In the presence of 1 mM **AG**, 64% of the prodrug remained intact after 72 h, suggesting that the protective effect of **AG** diminishes in longer-term experiments >24 h under these conditions. Importantly, inspection of the media after 72 h incubation at 37 °C with the prodrug showed that no spermine was released (data not shown). This ruled out premature spermine release as a mechanism of prodrug spermine delivery.

Due to its time-dependent stability, a new experiment was conducted over 24 h with CMS-6233 cells, prodrug, and **AG** (1 mM). Both exogenous spermine and prodrug delivered spermine (Spm) to cells, suggesting that the intact prodrug gives a similar response over 24 h, as seen at 72 h (see Figure S5 in the Supporting Information). We noted that the relative increase in Spm pools was the same after 24 h as it was after 72 h, suggesting that the prodrug enters cells and delivers Spm within the first 24 h (comparing **CMS-6233** data in Figures 4 and S5). Taken together, these data suggested that future experiments could be conducted over 24 h in the presence of **AG** (1 mM) as increased incubation times did not lead to further increases in Spm pools with either prodrug **1** or exogenous Spm.

Additional stability studies with NAC and prodrug **1** in the presence of AG (1 mM) showed that 85% of the prodrug remained after 24 h incubation at 37 °C, which is similar to the 93% prodrug remaining after 24 h in the absence of NAC. This indicates that the majority of the prodrug remains intact in the presence of NAC in the absence of cells. In addition, no lactone was detected by HPLC when the prodrug was treated with NAC (2 mM) in

PBS at 37 °C for 72 h suggesting no premature spermine release in the presence of NAC. Therefore, the prodrug's architecture protects 50% of spermine's structural liability (*via* an amide bond), still utilizes the PTS for targeted delivery, and does not prematurely release spermine.

Having shown reasonable stability of the prodrug, we investigated the ability of spermine or prodrug **1** to extend survival in a *Drosophila* model of SRS.[29] The *Drosophila* SMS (dSms) gene is on chromosome III (autosome). We established a loss of the SMS allele, where homozygous ($dSms^{e/e}$) flies have less than the 0.05% dSms transcript level of wild-type flies.[29] Homozygous $dSms^{e/e}$ flies (both male and female) have elevated levels of spermidine, ROS, and aldehyde in the brain, as well as damaged mitochondria and lysosomes and shortened lifespan, recapitulating key pathological features of SRS patients.[29]

Before the survival study, we first evaluated the toxicity of the prodrug to the SMS mutant ($dSms^{e/e}$) and wild-type (*yw*) flies. As shown in Figure 8, the prodrug was well tolerated from 30 to 1000 μM in the SMS mutant adult female flies, with 300 μM providing the best response of the concentrations tested (panel a). In contrast, the prodrug was well tolerated from 30 to 300 μM in SMS mutant adult male flies with 100 μM providing the best response of the concentrations tested (panel b). Consistent with these studies, the prodrug was not toxic to adult female wild-type (*yw*) flies up to 1000 μM, whereas 1000 μM was toxic to adult male yw flies (Figure 8). We speculate that the difference in toxicity may be explained by the larger body size of female flies. As some toxicities or under-performance was noted at the higher doses in Figure 8, we re-tested both sexes of the SRS mutant flies at 100 μM.

Rewardingly, feeding $dSms^{e/e}$ flies with prodrug **1** significantly extended their median survival. As shown in Figure 9, 47% (female) and 43% (male) increases in median survival were noted in $dSms^{e/e}$ flies fed the prodrug (100 μM). In contrast, feeding spermine (Spm) at the same concentration showed no beneficial effect. These results illustrate the beneficial effect of prodrug 1 *in vivo* in an SRS model.

Finally, we measured spermine levels by HPLC in male SMS mutant flies treated for 20 days with or without prodrug **1** (100 μM in food). Rewardingly, as shown in Figure 10, treatment with prodrug **1** resulted in increased spermine levels consistent with spermine delivery *in vivo* and a rebalancing of Spd/Spm ratios was observed in treated SMS mutant male flies. We noted that spermidine and total polyamine levels also increased, which may be explained by differences between fly and human polyamine pathways, as observed by Nowotarski et al.[39]

It is important to note that there are limitations to our polyamine quantification in the fly model. Due to the low innate levels of spermine in the wild-type yw flies (~1 nmol/mg protein) and even lower levels in the dSms e/e fly samples and the biological variation encountered, the formal changes in spermine levels did not meet statistical significance but trended higher (Figure 10). The observed changes in the levels of Spd, total polyamines, and the Spd/Spm ratio all were statistically significant in the prodrug-treated arm compared to the untreated mutant flies (see the legend, Figure 10). Since the Spd/Spm ratios cancel

out systematic errors, they likely offer a better way to assess polyamine rebalancing in this model. We note that the clinical diagnosis for SRS in part relies on the Spd/Spm ratio, rather than the absolute value of Spd and Spm (because of the biological variability of polyamine amounts in patients). The fact that the prodrug rebalances the Spd/Spm ratio is, therefore, a significant finding.

CONCLUSIONS

A prodrug for spermine was developed, which is relatively nontoxic and releases free spermine inside human SRS fibroblast cells and SMS mutant male flies. This spermine delivery system was shown to rebalance spermidine/spermine ratios closer to those observed in wild-type human fibroblasts and wild-type flies, respectively. The most encouraging finding of this work is that prodrug **1** showed a significant beneficial effect in human SRS cells (CMS-26559) containing a complete loss-of-function SMS protein (a likely worst-case scenario in SRS). Our work provides a rare example of using the quinone–TML approach to deliver bioactive amines to human cells; an approach that could be applied to other amine-containing drugs.[27] This approach requires the reactive hydroquinone to displace an amine group from an amide linkage; an organic reaction that is typically disfavored at room temperature by phenols. In summary, these results suggest that a therapeutic intervention for SRS may be possible by harnessing the polyamine transport system to deliver a spermine prodrug and using the cell's own reduction potential to release spermine to rebalance polyamine pools. We recognize that there are limitations associated with the *Drosophila* model in terms of the physiological differences between flies and humans and that humans may respond differently to the prodrug. Nevertheless, *Drosophila* retain highly conserved molecular pathways and have approximately 75% of the genes linked to human disease. As a result, they offer a rapid and cheap model to evaluate potential drug candidates.[40] Future work will evaluate this approach in the available SRS mouse model (*C57BL/6J-Sms*^{em2Lutzy}*/JSms*^{G56S}, The Jackson Laboratory) to bring the technology one step closer to humans.

EXPERIMENTAL SECTION

Materials.

Silica gel (32–63 μm) and chemical reagents were purchased from commercial sources and used without further purification. All solvents were distilled prior to use or purchased as an analytical grade. All reactions were carried out under atmospheric pressure unless a N_2 atmosphere was specified. ^{1}H and ^{13}C NMR spectra were recorded at either 400 (or 500 MHz) or 100 (or 125 MHz), respectively. The newly synthesized prodrug **1** provided satisfactory elemental analysis and was tested at ≥95% purity. HRMS was performed on an Agilent 6230 time-of-flight (TOF) mass spectrometer.

Biological Studies.

Snyder Robinson Syndrome fibroblast cell lines were received from Greenwood Genetic Center (GGC) in Greenwood, SC, and were negative for mycoplasma by PCR. The wild-type (CMS-24949) and mutant (CMS-26559, CMS-6233, and CMS-23916) cells were

grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamine in a humidified 5% CO_2 atmosphere at 37 °C. For uptake experiments, the wild-type (0.25×10^6 cells per 10 cm dish) and mutant (0.65×10^6 cells per 10 cm dish) cells were grown in the presence of 1 mM aminoguanidine (**AG**) to inhibit extracellular oxidation of spermine by bovine serum amine oxidase present in the culture medium. The wild-type fibroblast cells and SMS mutant cells were then incubated in the presence or absence of exogenous spermine (Spm) or prodrug **1**. For the NAC-containing experiments (NAC alone or combination of NAC with prodrug **1**), cells were pretreated with NAC for 6 h prior to prodrug addition (NAC was not removed) and the time of prodrug exposure was identical for all experiments containing the prodrug. Intracellular polyamine levels were determined by HPLC after *N*-dansylation.

Determination of Mitochondrial Membrane Potential in Cells.

SMS mutant (CMS-26559 and CMS-23916) cells were seeded in a 48-well plate at a density of 60,000 cells per well and incubated for 24 h. On the following day, the culture media in each well was replaced with either media (250 μL) containing MitoView 633 dye (100 nM) and incubated at 37 °C for 15 min or media containing 1X JC-1 reagent and incubated at 37 °C for 30 min. At the end of the incubation, cells were washed with PBS twice, trypsinized, and collected for FACS analysis by a flow cytometer using an appropriate excitation/emission setting or detection channel. Excitation/emission wavelengths for JC-1 were 510/527 nm (monomer) and 585/590 nm (aggregate) and absorbance/emission wavelengths for MitoView 633 were 622/648 nm, respectively.

Radiolabeled Spermidine Uptake Cells.

Cells (100,000 cells/well) were seeded in a 24-well plate and incubated with 5% CO_2 for 24 h at 37 °C. The medium was then changed with preheated Hanks Balanced Salt Solution (HBSS, containing Ca^{2+} and Mg^{2+}) at 37 °C. Cells were treated with prodrug **1** and Spm at different concentrations (0, 1, 3, and 5 μM) followed by addition of 1 μM of ^{3}H-Spd (Perkin-Elmer Inc., Boston, MA). Cells were incubated at 37 °C for 15 min. *Note*: ^{3}H-Spd was used in these competition experiments because the prodrug represents a substituted spermidine derivative as one end of its spermine chain is capped as an amide. The cells were then washed with cold HBSS and lysed with 0.1% sodium dodecyl sulfate (SDS) in water (300 μL). Cell lysates were then transferred to an Eppendorf tube and centrifuged at 15,000 rpm for 15 min. A sample of each supernatant (200 μL) was transferred into a scintillation vial containing 2 mL of Scintiverse BD, and the resulting scintillation counts were measured using a Beckman Coulter LS6500 scintillation counter. The amount of protein was determined using the Pierce BCA protein assay kit from the remaining lysate volume (approximately 100 μL) to normalize the radioactive counts obtained (pmol ^{3}H-Spd/μg protein). K_i and K_m values were determined using double reciprocal Lineweaver–Burk plots. The K_i value was determined from the equation $K_i = IC_{50}/(1 + (L + K_m))$, where IC_{50} is the concentration of either prodrug **1** or Spm required to block 50% of the relative uptake of ^{3}H-Spd and L is the concentration of ^{3}H-Spd used in the assay (1 μM). The $K_m = -1/x$ intercept.

HPLC Method.

The method of Minocha was used with modifications.[41] The biological samples (~2 million cells harvested by trypsinization) were homogenized using a hypersonic needle for 5 s (typically three times) in the presence of 150 μL of 0.2 M perchloric acid buffer ($HClO_4$/1 M NaCl) and 50 μL of 0.9% aqueous NaCl solution. *Note:* for Figure 10, the flies were raised on fly food containing prodrug **1** at 100 μM for 20 days. In the whole fly experiments, batches containing 10 flies in a 1 mL Eppendorf tube were separately homogenized in the presence of the above buffer using a hypersonic needle for 5 s (five times at a setting of 5). The obtained milky solution was then centrifuged at 5000 rpm for 10 min to give a clear supernatant, which was separated from the remaining pellet. The total volume of the supernatant layer was measured by a pipette and recorded for each sample. The remaining cell pellet was saved and used for protein level determination using the Bicinchoninic Acid (BCA) method. Each supernatant (100 μL) was transferred to a 4 mL glass vial, the internal standard 1,7-heptanediamine (10 μL, 400 μM in 2% perchloric acid solution) was added, and the resulting mixture was vortexed for 30 s. Next, an aqueous carbonate buffer (pH 9.5, 240 μL) was added to each vial followed by freshly prepared dansyl chloride solution (400 μL) in dry acetone (20 mg/mL) was added. All vials were then capped, vortexed for good mixing, and incubated at 60 °C in a rotary shaker for 60 min. After this incubation, 25 μL of L-alanine (100 mg/mL in water) was added to quench the remaining dansyl chloride in each vial and the incubation was continued for an additional 15 min. The solvent was evaporated to dryness using a rotary evaporator to give a colorless solid residue. The residue was then dissolved in water (300 μL) and chloroform (1.0 mL) was added to this water solution, and the mixture was vortexed for 30 s. The reaction mixture was then centrifuged at 1000 rpm for 4 min to obtain a clean layer separation. The bottom chloroform layer (containing the dansyl polyamines) was transferred separately into respective vials. The chloroform layer was then evaporated to dryness under reduced pressure to give a residue. Methanol (1.0 mL) was added to the residue and the sample was vortexed to dissolve the dansylated polyamines. The methanol solution (containing the dansylated polyamines) was passed through a C18 plug and additional methanol (0.5 mL) was passed through the plug to generate approximately 1.5 mL of a filtered methanol solution. The solution (40 μL) was then injected into the HPLC for polyamine analysis. The retention times and fluorescence detector response factors for each polyamine were determined using authentic standards.

Statistical Analysis.

All experiments were performed in triplicate at least two independent times unless otherwise stated. Excel 2019 and GraphPad Prism 8.4.2 were used to perform two-way ANOVA for HPLC assay statistical analyses.

HPLC Conditions.[42]

A 0–40% gradient of mobile phase A [100% acetonitrile (ACN)] and B [25 mM sodium acetate buffer (pH 5.94) containing 3% 1-propanol and 10% ACN] was run for 10 min at 1 mL min^{-1} to elute the dansylated polyamines within the same run; the gradient was then raised from 40 to 100% ACN in 17 min at a flow rate of 2.5 mL min^{-1}.

Stability Study of Prodrug 1.

A solution of prodrug **1** (0.4 mL, 5 mM in PBS) was added to a vial containing cell culture media (3.6 mL, DMEM media supplemented with 15% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine). The mixture was thoroughly mixed using vortex for 20 s and incubated in a humidified 5% CO_2 atmosphere at 37 °C. At 0, 24, 48, and 72 h, samples (750 μL) were taken and mixed with methanol (750 μL). The mixture was filtered through a 0.45 μm nylon syringe filter and the same volume (40 μL) was injected into the HPLC for analysis. The % remaining was calculated by dividing the area under the curve (AUC) for the prodrug peak (6.4 min) at each time point by the AUC for the prodrug at time zero × 100%.

Drosophila Culture and Prodrug Feeding.

Flies were maintained on a cornmeal–molasses–yeast medium at 22 °C, 65% humidity, and 12 h light/12 h dark. Prodrug **1**, spermine, or solvent was added to freshly made liquid *Drosophila* food cooled to 50 °C. The food was thoroughly mixed with drug solutions, transferred into new vials, and allowed to solidify for 30 min. Newly enclosed adults were transferred to fresh food supplemented with prodrug, spermine, or solvent and raised at 22 °C. Viable flies were counted every day. Flies were transferred to fresh food vials containing their respective treatment every three days until all flies completed their lifespan or the duration period of the experiment was completed, e.g., Figure 10: 20 days.

Synthesis of Prodrug (1).—To an ice-cold solution of compound **8** (0.2 mmol) in dichloromethane (DCM, 1 mL) was added 4 M HCl in dioxane (1 mL). The resulting reaction mixture was allowed to stir at room temperature for 45 min. The solvent was then evaporated to dryness to provide a light brown solid. Diethyl ether (5 mL) was added to the solid and the mixture was sonicated for 2 min. The solvent was then decanted and the remaining solid was dried under reduced pressure to give the hydrochloride salt of **1** as a light brown, highly hygroscopic solid. **1**: Light brown solid, yield 85%, ^{1}H NMR (400 MHz, D_2O) δ 4.07 (s, 3H), 4.07 (s, 3H), 3.19−3.15 (m, 6H), 3.14−3.10 (s, 8H), 2.13−2.08 (m, 6H), 1.82−1.78 (m, 6H), 1.21 (s, 3H), 1.05 (s, 3H); ^{13}C NMR (125 MHz, D_2O) δ 196.7, 196.2, 192.5, 177.0, 149.5, 149.2, 61.4, 61.2, 48.0, 47.0, 44.5, 44.0, 42.1, 36.5, 24.8, 23.7, 22.7, 22.4, 16.3; HRMS *m*/*z* calcd for $C_{24}H_{43}N_4O_5$ $[M + H]^+$ = 467.323; found: 467.316; anal. $C_{24}H_{45}Cl_3N_4O_5.0.5H_2O$, CHN.

Synthesis of Hydroquinone (5).—To a solution of quinone **4** (5.4 mmol) in diethyl ether (25 mL) was added a solution of $Na_2S_2O_4$ (16.2 mmol) in water (25 mL).[43] The resulting reaction mixture was stirred at room temperature for 1 h. After the starting material was consumed as evidenced by thin-layer chromatography (TLC), water was added (15 mL) and extracted using diethyl ether (2 × 50 mL). The combined diethyl ether layer was dried over anhydrous sodium sulfate, filtered, and concentrated to dryness to give pure hydroquinone **5**. **5**: Colorless solid, yield 90%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.49 (d, J = 0.7 Hz, 1H), 5.39 (s, 1H), 5.27 (s, 1H), 3.91 (s, 3H), 3.88 (s, 3H), 2.17 (d, J = 0.7 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 141.6, 140.4, 139.1, 137.2, 119.4, 111.3, 60.8, 60.7, 15.4; HRMS *m*/*z* calcd for $C_9H_{16}NO_4$ $[M + NH_4]^+$ = 202.1074; found: 202.1083.

Synthesis of Lactone (6).[24]—To a mixture of hydroquinone **5** (5.4 mmol) and methyl 3,3-dimethylacrylate (7.0 mmol) was added methanesulfonic acid (10 mL).[24] The resulting reaction mixture was stirred at 70 °C for 90 min. Upon completion of the reaction, the contents were cooled to room temperature. Cold water (50 mL) was added and the mixture was extracted using ethyl acetate (2 × 30 mL). The combined organic layers were washed with saturated sodium bicarbonate solution (30 mL), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give the crude product. The crude product was then recrystallized from 10% ethyl acetate:hexanes to produce the pure lactone **6** in 72% yield, which matched the literature ^{1}H spectrum.[24] **6**: Colorless solid, yield 72%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.76 (s, 1H), 3.97 (s, 3H), 3.90 (s, 3H), 2.57 (s, 2H), 2.33 (s, 3H), 1.45 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.7, 143.9, 138.6, 138.4, 138.3, 126.4, 116.4, 61.5, 61.2, 45.9, 35.8, 27.7, 13.7; HRMS *m*/*z* calcd for $C_{14}H_{19}O_5$ $[M + H]^+$ = 267.1227; found: 267.1234.

Synthesis of Acid (7).—To a solution of lactone **6** (4.13 mmol) in DMF (5 mL) was added pyridinium dichromate (PDC, 16.52 mmol) slowly at room temperature.[24] The reaction mixture was stirred at rt for 4 h. After consuming the starting material, as indicated by TLC, water (25 mL) was added and the mixture was extracted using diethyl ether (2 × 30 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness to give acid **7** in 65% yield as a yellow oil. We observed that the obtained acid was unstable when exposed to prolonged light and air. For this reason, it was made and immediately consumed in the next step.

7: Yellow oil, yield 65%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.97 (s, 3H), 3.90 (s, 3H), 3.06 (s, 2H), 2.15 (s, 3H), 1.46 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 186.4, 184.5, 177.7, 149.8, 145.3, 142.4, 137.7, 60.9, 60.4, 47.1, 38.2, 28.9, 14.0; HRMS *m*/*z* calcd for $C_{14}H_{19}O_6$ $[M + H]^+$ = 283.1176; found: 283.1178.

Synthesis of Amide (8).—To a solution of acid **7** (0.12 mmol) in DMF (1 mL) were added HATU (0.36 mmol) and diisopropylethyl-amine (DIPEA, 0.36 mmol) at room temperature. The resulting reaction mixture was stirred at 50 °C for 15 min. Next, a solution of tri-Boc-spermine **3**[44] (0.13 mmol in 0.2 mL of DMF) was added and the solution was stirred overnight. After the starting materials were consumed (as indicated by TLC), the mixture was cooled to room temperature. Water (10 mL) was added and the product was extracted using ethyl acetate (2 × 10 mL). The combined organic layer was then washed with saturated aqueous $NaHCO_3$ (10 mL), followed by 1 N HCl (10 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated to give the crude product, which was then purified by column chromatography using ethyl acetate:hexanes as the eluent to provide the pure amide **8** in 70% yield. **8**: Light orange oil, yield 70%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.96 (s, 3H), 3.93 (s, 3H), 3.24 (s, 4H), 3.11 (s, 8H), 2.86 (s, 2H), 2.10 (s, 3H), 1.45 (d, J = 7.3 Hz, 35H), 1.42 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.4, 184.6, 171.8, 156.6, 156.5, 156.1, 156.0, 151.8, 141.9, 135.3, 79.9, 79.8, 77.2, 60.7, 60.2, 49.3, 46.7, 43.1, 38.2, 35.2, 28.6, 28.5, 13.8; HRMS *m*/*z* calcd for $C_{39}H_{66}N_4NaO_{11}$ $[M + Na]^+$ = 789.4620; found: 789.4611.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Charles Schwartz at the Greenwood Genetic Center for supplying the SRS cell lines and control wt fibroblasts and the Snyder Robinson Foundation for financial support of this work (UCF25068A04 to O.P.). The authors would also like to acknowledge Dr. Thomas Andl at UCF for help with the flow cytometry experiments. The HRMS equipment at the University of Florida core lab was funded *via* a grant from the NIH (NIH S10 OD021758-01A1). This research was also supported in part by the National Institutes of Health (NIH) R01NS109640 (to R.G.Z.).

ABBREVIATIONS

ACN	acetonitrile
AD4	*N*-acetylcysteine amide
AG	aminoguanidine
APAO	acetylpolyamine oxidase
BCA	bicincho-ninic acid
CDAP	*N*-cyclohexyl-1,3-diaminopropane
DFMO	difluoromethylornithine
DIPEA	*N,N*-diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
HATU	(1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluoro-phosphate
MAT	methionine adenosyl transferase
MCHA	4-methylcyclohexylamine
Me_2SPM	dimethylspermine
NAC	*N*-acetylcysteine
***N*-2-MPG**	*N*-2-mercaptopropionil glycine
ODC	ornithine decarboxylase
SAM	*S*-adenosyl methionine
dc-SAM	decarboxylated *S*-adenosylmethionine
SAMDC	*S*-adenosylmethionine decarboxylase

SMOX	spermine oxidase
SMS	spermine synthase
SPD	spermidine
SPM	spermine
SRS	Snyder Robinson Syndrome
SSAT	spermidine/spermine N^1-acetyltransferase
TML	trimethyl lock
UQ	Ubiquinone

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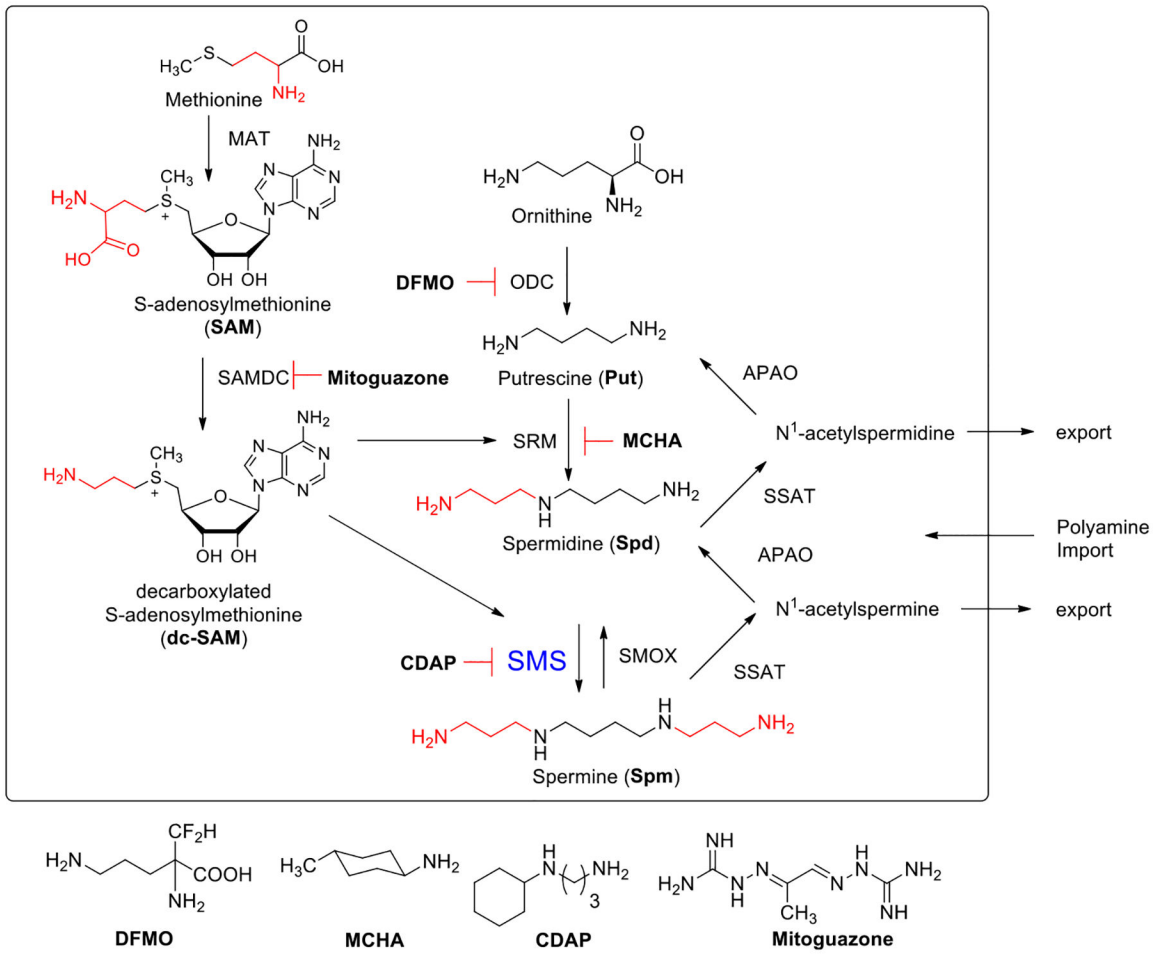


Figure 1.

Polyamine metabolism, SMS, and specific inhibitors of polyamine biosynthesis. Ornithine is converted to putrescine *via* ornithine decarboxylase (**ODC**). Methionine is converted to *S*-adenosylmethionine (**SAM**) *via* the enzyme methionine adenosyl transferase (**MAT**). SAM is then converted to decarboxylated *S*-adenosylmethionine (**dc-SAM**) *via* the action of *S*-adenosylmethionine decarboxylase (**SAMDC**). Putrescine is converted to spermidine *via* spermidine synthase (SRM) and an aminopropyl fragment derived from dc-SAM. Similarly, spermidine is converted to spermine *via* spermine synthase (SMS) and dc-SAM. Back conversion can occur *via N*-acetylation using spermidine/spermine N^1-acetyltransferase (**SSAT**) to form N^1-acetyl derivatives, which can be oxidized by acetylpolyamine oxidase (**APAO**) to generate the respective polyamine. N^1-acetylpolyamines can also be excreted by cells to maintain intracellular polyamine levels and exogenous polyamines can be imported to increase intracellular polyamine pools *via* the polyamine transport system. Spermine oxidase (**SMOX**) allows direct conversion of spermine to spermidine. Difluoromethylornithine (**DFMO**) is an ODC inhibitor, 4-methylcyclohexylamine (**MCHA**) is an inhibitor of SRM and *N*-cyclohexyl-1,3-diaminopropane (**CDAP**) is an inhibitor of SMS, and mitoguazone is a known SAMDC inhibitor.

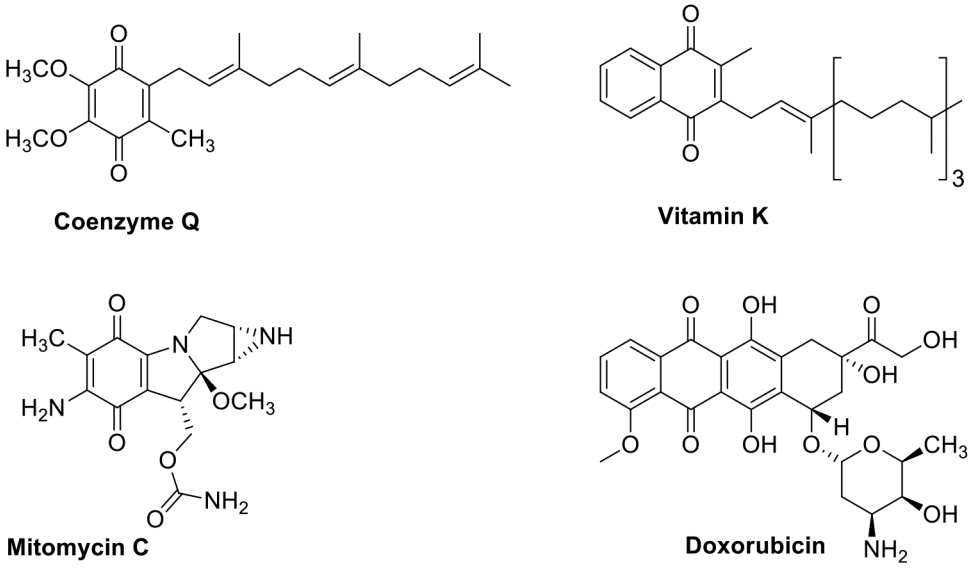


Figure 2.
Natural and synthetic biological important quinones.

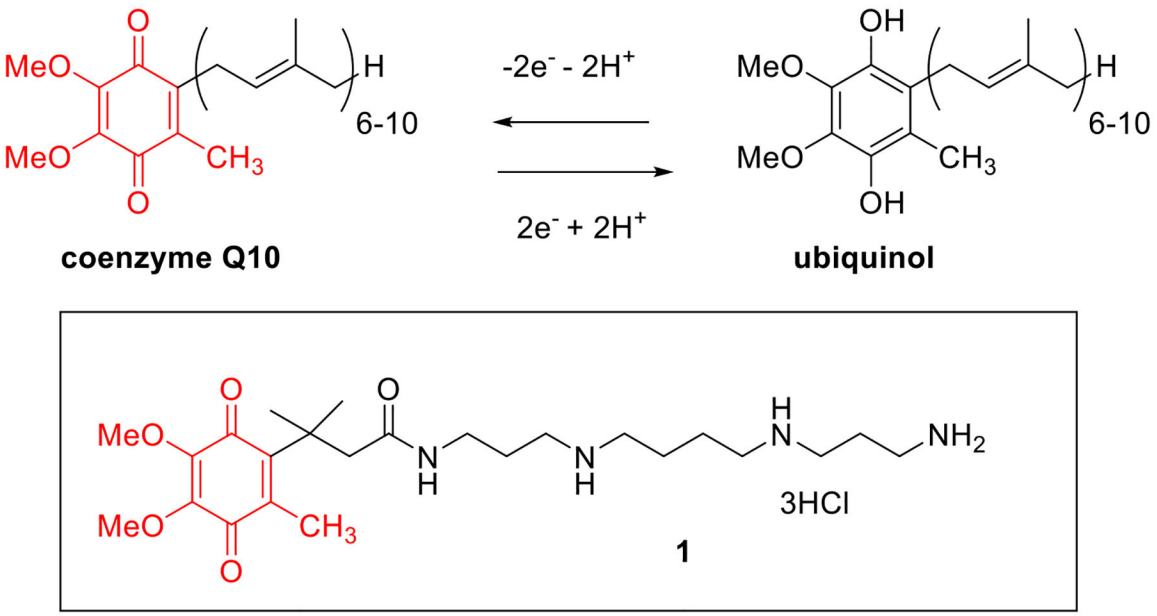


Figure 3.
Naturally occurring redox of coenzyme Q10 to ubiquinol and the structure of prodrug **1** (in the box).

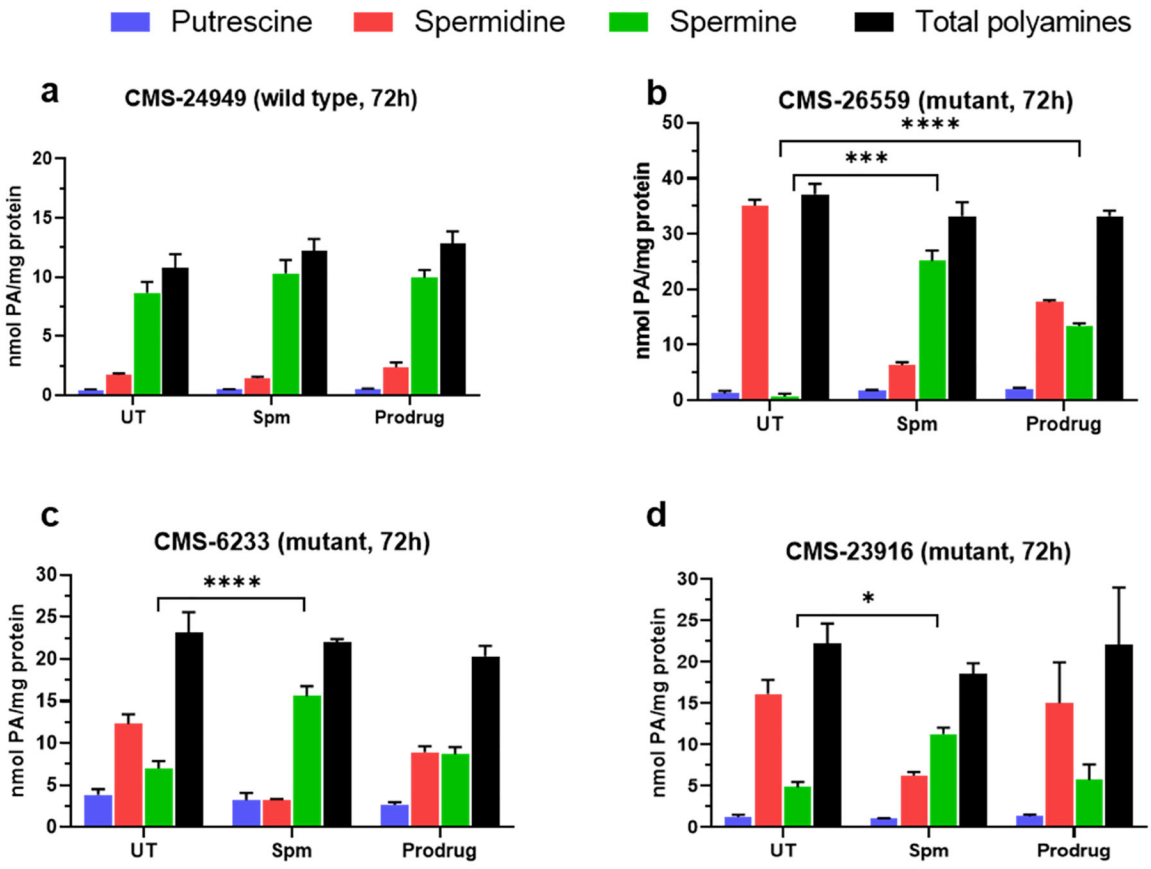


Figure 4.

Polyamine levels in wild-type (a: CMS-24949) and SMS mutant (b: CMS-26559, c: CMS-6233, and d: CMS-23916) fibroblast cell lines with or without treatment of Spm or prodrug **1** (5 μM) in the presence of AG (1 mM) after 72 h incubation at 37 °C. Values represent data from the experiment performed in triplicates ± SD, *$p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

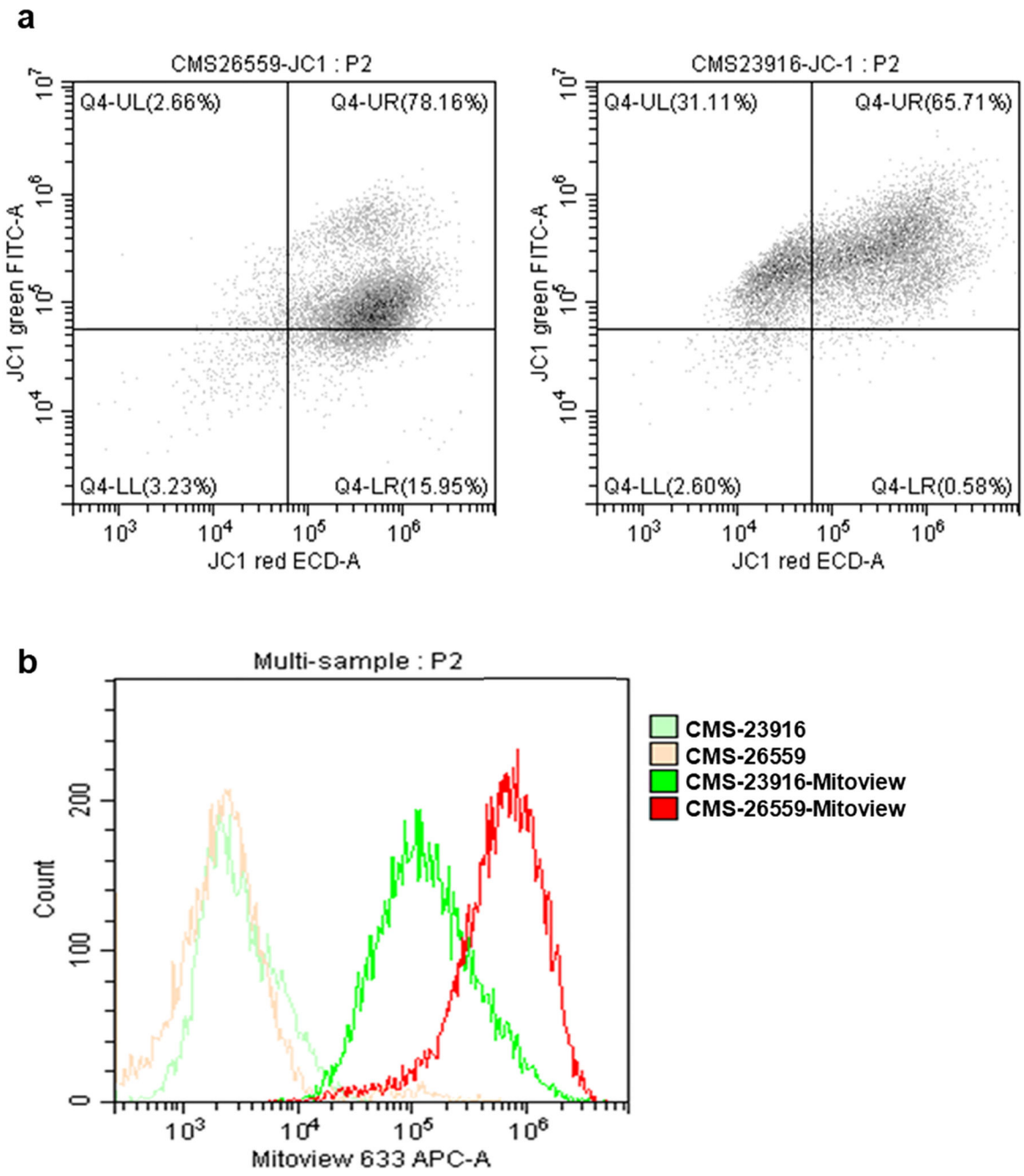


Figure 5.

FACS analysis to determine the mitochondrial membrane potential (MMP) of CMS-23916 and CMS-26559 cells by JC-1 (panel a) and Mitoview 633 staining (panel b). (Panel a) Two scatter parameters FITC-A and ECD-A were used for the JC-1 flow cytometric gating strategy (dot plot). (Panel b) Graph showing a reduced intensity of Mitoview 633 staining (APC-A) in CMS-23916 cells compared to CMS-26559 cells.

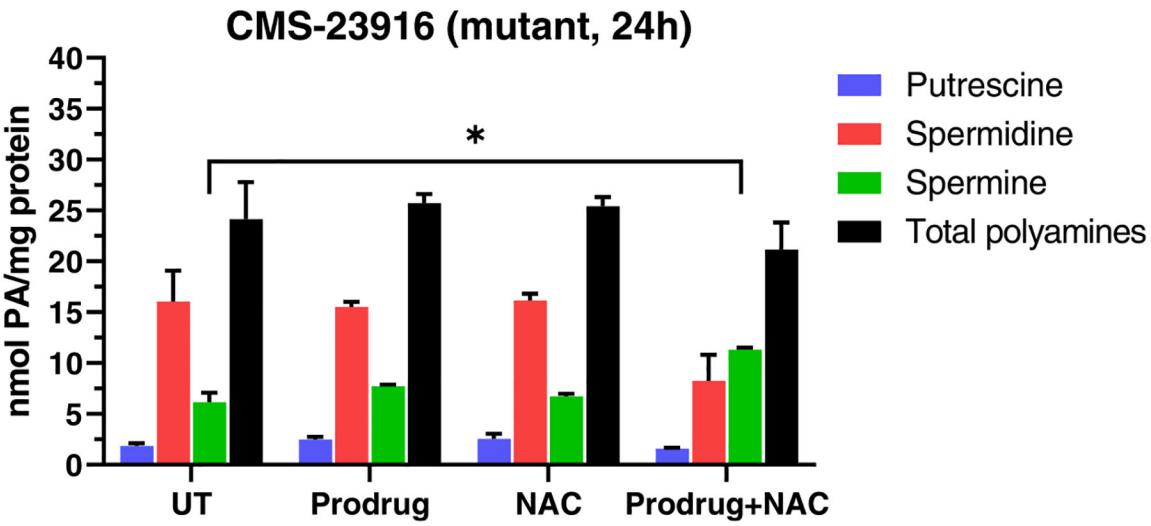


Figure 6.

Polyamine levels in CMS-23916 (mutant) fibroblast cell lines untreated (UT) or treated with prodrug **1** (5 μM) only, NAC (2 mM) only, or a combination of prodrug **1** (5 μM) and NAC (2 mM). Values represent data from the experiment performed in triplicates ± S.D. *$p <$ 0.05.

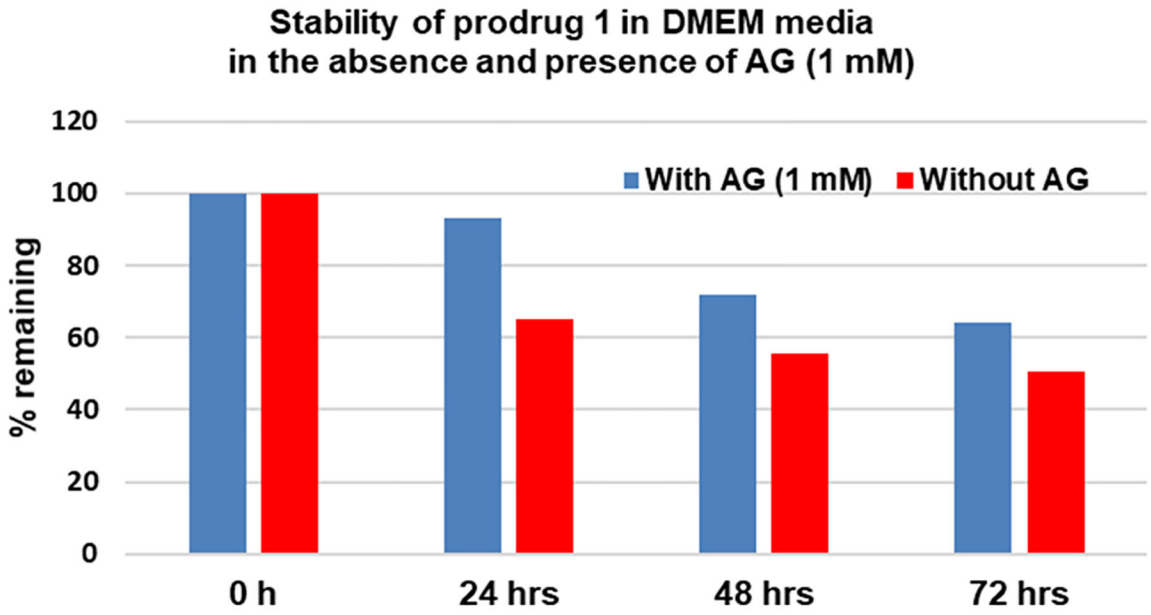


Figure 7.

HPLC study on the stability of prodrug **1** in DMEM media in the absence and presence of the amine oxidase inhibitor **AG** (1 mM). The data is shown as % remaining, where the starting (t = 0 h) area under the curve (AUC, 0 h) for the prodrug peak (eluting at the 6.4 min retention time) was set to 100% and data calculated at 24 h, for example, as 100% × AUC 24 h/AUC 0 h. These were conducted as single experiments comparing prodrug versus media only with and without **AG**.

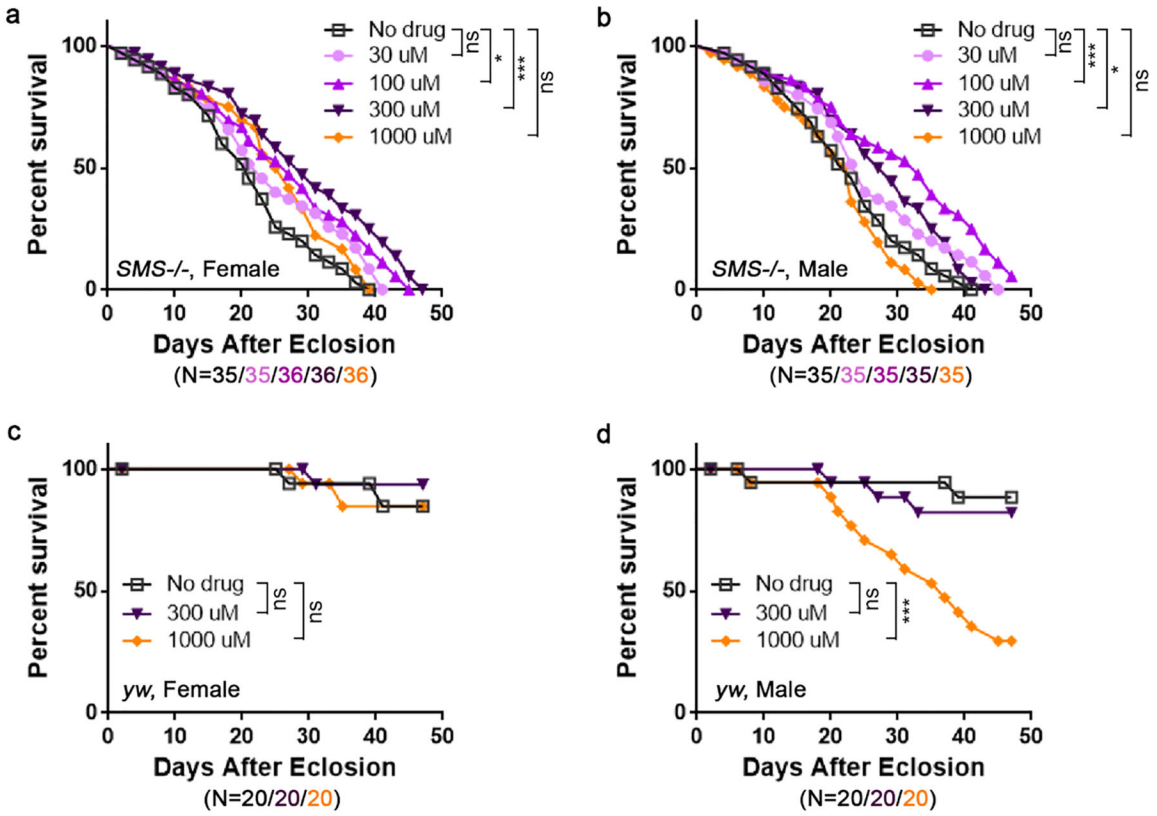


Figure 8.

Cytotoxicity experiments in *Drosophila* with the prodrug. The dSms e/e flies (panel a, female; panel b, male) or *yw* flies (panel c, female; panel d, male) are maintained on a cornmeal–molasses–yeast medium with the prodrug at the indicated concentration from the first day after the eclosion. Log-rank (Mantel–Cox) test; ns: nonsignificant, * $p < 0.05$, *** $p < 0.001$.

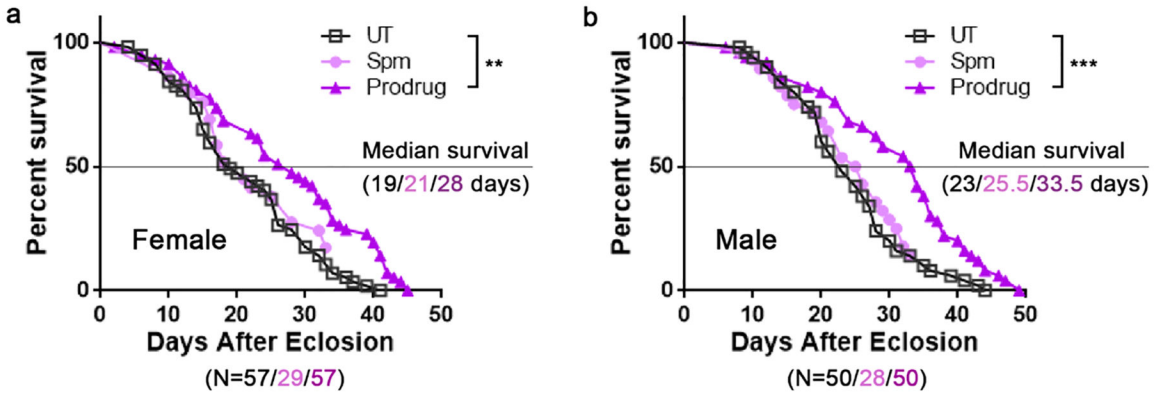


Figure 9.

Survival studies of SRS flies provided spermine or prodrug **1** feed. The dSms e/e flies (panel a, female; panel b, male) are maintained on a cornmeal–molasses–yeast medium with spermine or the prodrug (100 μM) from the first day after eclosion. Log-rank (Mantel–Cox) test; ** $p < 0.01$, *** $p < 0.001$. A significant extension of median survival was observed in both sexes, where UT = untreated, Spm = spermine, and Prodrug = prodrug **1**. N is the # of flies per experimental arm.

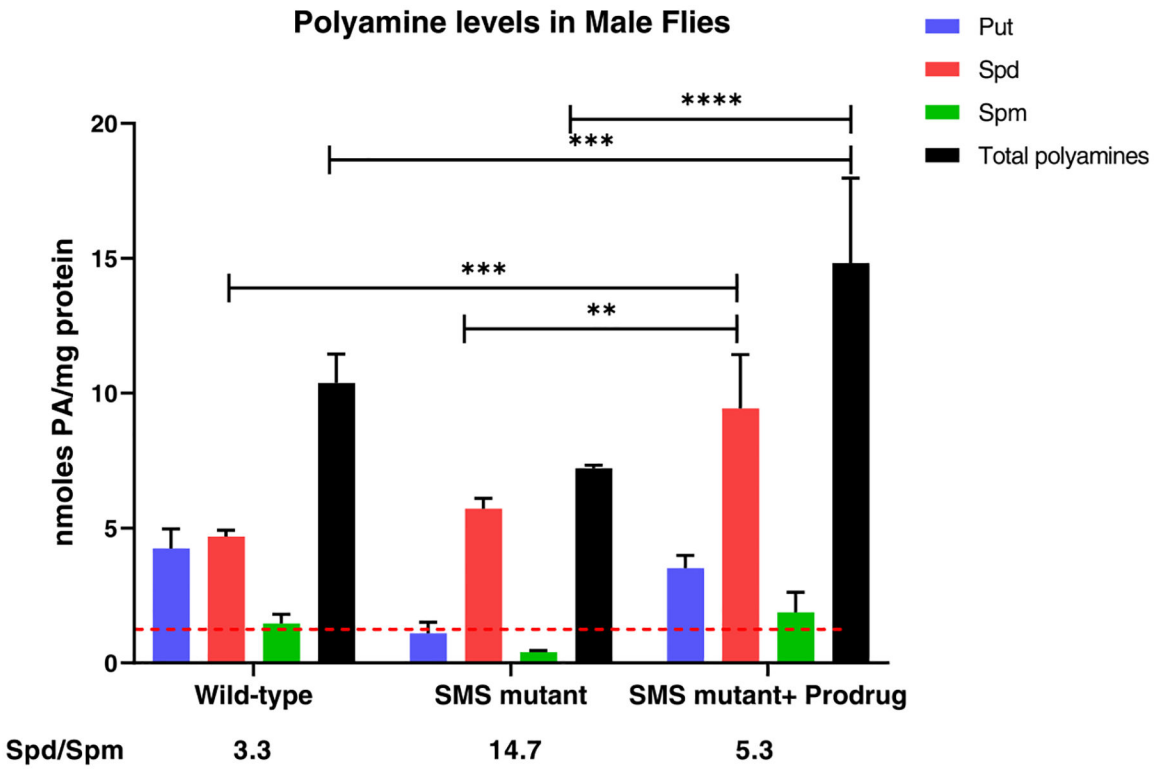
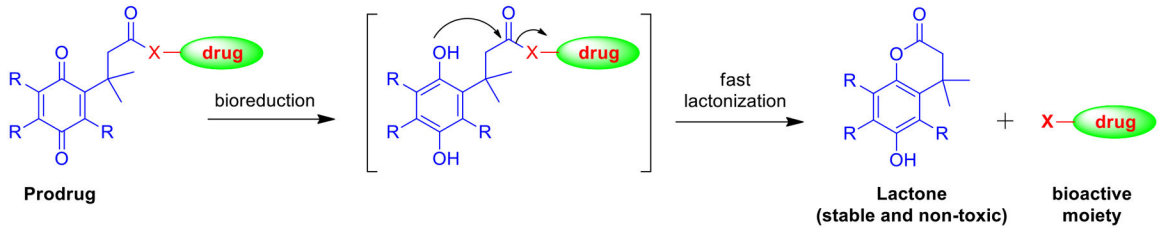
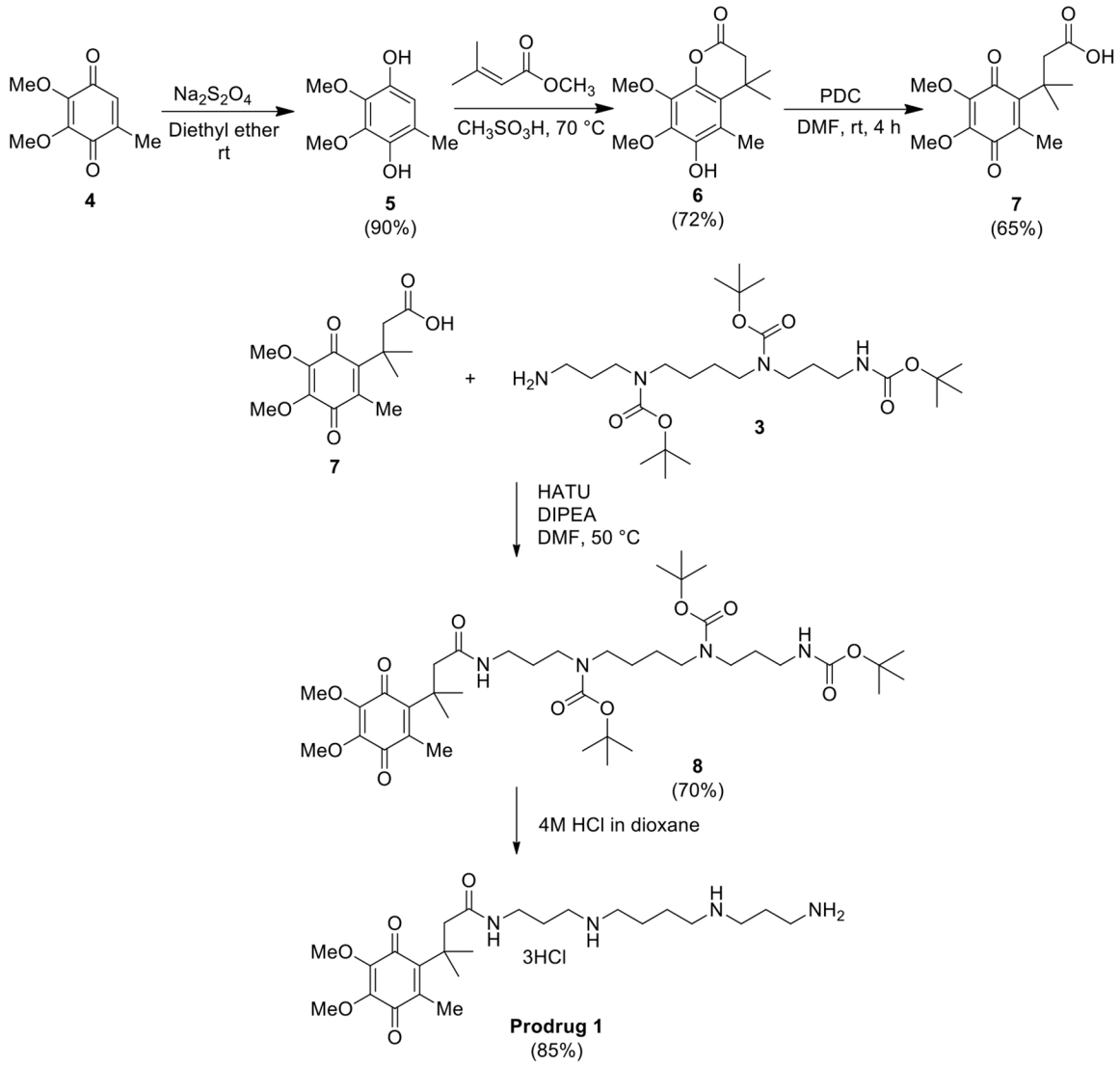


Figure 10.

Polyamine levels (nmoles/mg protein) in SMS mutant male flies in the presence and absence of prodrug **1**. The untreated wild-type control is provided on the left and a red dotted line indicating spermine levels is included for comparison. The Spd/Spm ratios are listed under each experimental arm. Values represent data from the experiment performed in triplicates. Two-way analysis of variance (ANOVA) ****$p < 0.0001$, ***$p < 0.0004$ (total polyamines), ***$p < 0.0002$, **$p < 0.0028$ (Spd). Statistical comparisons of Spd/Spm ratios: wild-type vs SMS mutant (****, p value <0.0001), wild-type vs SMS mutant plus the prodrug (not significant, $p = 0.15$), and SMS mutant vs SMS mutant plus the prodrug (****, $p < 0.0001$).



Scheme 1.
Principle of the Molecular Release of TML Quinone Systems

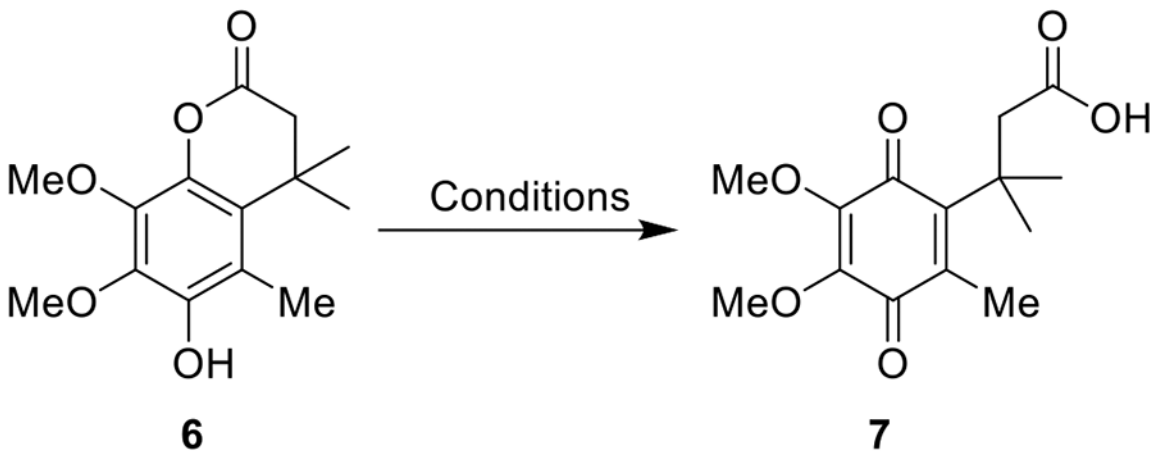


Scheme 2.
Synthesis of Spermine Prodrug 1

J Med Chem. Author manuscript; available in PMC 2022 May 18.

Table 1.

Optimization of the Reaction Conditions for Opening of the TML Lactone



6 **7**

entry	reagent	solvent	temp (°C)	time (h)	yield (%)
1	NBS (1.5 equiv)	aq. CH_3CN (10%)	rt	1	25
2	NBS (1.5 equiv)	aq. CH_3CN (10%)	0	2	25
3	NBS (1.5 equiv)	CH_3CN:acetone:H_2O	0	2	32
4	aq. NaOH (1 M)	CH_3CN	rt	48	trace
5	PDC PDC (1 equiv)	DMF	rt	4	30
6	(2 equiv)	DMF	rt	4	40
7	PDC (4 equiv)	DMF	rt	4	65

Table 2.

[a]72 h IC_{50} Values (in μM) of Spermine, Prodrug 1, and Lactone 6 in Wild-Type (CMS-24949) and SMS Mutant (CMS-26559, CMS-6233, and CMS-23916) Fibroblast Cells

entry	compound	CMS-24949	CMS-26559	CMS-6233	CMS-23916
1	spermine	>500	356.7 ± 10.9	422.1 ± 20.7	>500
2	prodrug **1**	326.7 ± 3.7	198.5 ± 4.7	244.1 ± 4.0	>500
3	lactone **6**	>100	>100	>100	>100

[a]IC_{50} values are listed in micromolar units (μM ± SD) and were determined in the presence of AG (1 mM).

Note: the 72 h IC_{50} values of spermine were 14.6 ± 2.2 and 14.9 ± 3.8 μM and for the prodrug 22.1 ± 5.7 and 26.3 ± 11.5 μM in the absence of AG in CMS-26559 and CMS-23916 cells, respectively, suggesting an increase in toxicity in the absence of AG. The fact that spermine is more toxic than the prodrug in the absence of AG is consistent with spermine's two exposed aminopropyl ends, which are targets for degradation by amine oxidases.

Table 3.

Effects of Spm or Prodrug 1 on Polyamine Distribution in SRS Patient-Derived Cell Lines[33,34] in the Presence of AG (1 mM)[a,b]

cells	treatment	Put	Spd	Spm	Spd/Spm	mutations
CMS-24949	UT	0.41 ± 0.10	1.72 ± 0.13	8.65 ± 0.94	0.20	control[c]
	Spm	0.47 ± 0.05	1.44 ± 0.12	10.32 ± 1.12	0.14	
	prodrug	0.53 ± 0.04	2.36 ± 0.42	9.95 ± 0.64	0.24	
CMS-26559	UT	1.27 ± 0.40	35.10 ± 1.04	0.71 ± 0.47	49.43	c.908_911 DEL[d]
	Spm	1.72 ± 0.13	6.34 ± 0.52	25.11 ± 1.86	0.25	
	prodrug	2.06 ± 0.20	17.68 ± 0.36	13.42 ± 0.46	1.32	
CMS-6233	UT	3.86 ± 0.68	12.28 ± 1.14	7.00 ± 0.87	1.75	c.329+5 G>A[e]
	Spm	3.20 ± 0.89	3.20 ± 0.15	15.61 ± 1.17	0.21	
	prodrug	2.69 ± 0.30	8.93 ± 0.73	8.72 ± 0.80	1.02	
CMS-23916	UT	1.26 ± 0.24	16.14 ± 1.69	4.84 ± 0.64	3.33	c.335c>t[f]
	Spm	1.05 ± 0.06	6.27 ± 0.41	11.28 ± 0.78	0.56	
	prodrug	1.32 ± 0.21	15.04 ± 4.92	5.74 ± 1.88	2.62	

[a] Data are expressed in nmol/mg protein.

[b] UT= untreated, Spm = spermine treatment (5 μM), Prodrug = Prodrug **1** treatment (5 μM).

[c] Control = wild-type.

[d] Frameshift mutation that results in premature stop codon leading to complete loss of SMS function.

[e] Intronic variant resulting in the presence of a fraction of normal SMS protein and a fraction of SMS protein missing 22 amino acids.

[f] Results in P112L mutation, which decreases the stability of the monomer and significantly affects the stability of the SMS dimer.

Table 4.

Kinetic Parameters of ^{3}H-spermidine Uptake by CMS-26559 and CMS-23916 Cells Treated with Spm or Prodrug 1[a]

cells	K_m (μM)	V_{max} (pmol/μg protein/min)	K_i Spm (μM)	K_i prodrug (μM)
CMS-26559	9.44 ± 2.01	0.50 ± 0.10	3.30 ± 0.10	2.50 ± 0.43
CMS-23916	6.55 ± 1.40	0.33 ± 0.07	3.41 ± 0.19	3.28 ± 0.13

[a]Experiments were performed once in triplicate.

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