Translesion Synthesis Inhibitors as Anti-Cancer Adjuvant Agents

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Translesion Synthesis Inhibitors as Anti-Cancer Adjuvant Agents

Abstract

The translesion synthesis (TLS) pathway is a major mechanism through which cancer cells replicate past DNA lesions and promote chemoresistance. TLS allows cancers to survive genotoxic chemotherapy and increases the rate of mutation in tumors leading to drug-resistant cells. Cancer cells use a set of specialized low-fidelity TLS DNA polymerases to copy over lesions with Rev1 serving as a key scaffolding protein. Suppressing Rev1 activity sensitizes cancers to genotoxic chemotherapy and reduces the onset of chemoresistance by decreasing tumor mutation rate. Disruption of the Rev1/polζ-dependent TLS selective inhibitors has demonstrated the ability to sensitize cancer cells to platinating agents and reduce mutagenesis in tumors. This study identifies small molecule Rev1/polζ-dependent TLS inhibitors and validates the anti-cancer effects of combination cisplatin and TLS inhibitors. Our results demonstrate that TLS inhibitors do not have cell death-inducing activity on their own and may have additional inhibitory effects on cell proliferation and survival. TLS inhibitors indicate a strong promise for an effective and safe adjuvant agents for platinating agents in chemotherapy.

Introduction

The TLS pathway is the primary mechanism through which proliferating cells can tolerate DNA damage during replication. TLS allows cancers to survive genotoxic chemotherapy and increases the rate of mutation in tumors leading to drug-resistant cells.\textsuperscript{1-5} Cancer cells use TLS DNA polymerases to replicate past DNA lesions and promote chemoresistance.\textsuperscript{6} Inhibition of TLS has demonstrated the ability to sensitize cancer cells to platinating agents and reduce mutagenesis in tumors. Cisplatin is the first line therapy for treating many types of cancers such as malignant melanoma and Non-Hodgkin's lymphoma. Although cisplatin is a potent anti-
cancer agent, cancer cells tend to gain resistance with more exposure to cisplatin and related platinum chemotherapy. Combination of therapy with TLS inhibitors may reduce both the dose of platinating agents and associated toxic side effects, as well as help avert chemoresistance. Therefore, small molecule inhibitors of TLS are emerging as a promising new class of adjuvant agents for first-line cancer chemotherapy.

Within the TLS pathway, cancer cells use a set of specialized low-fidelity TLS DNA polymerases to copy over DNA lesions. These multi-protein complexes that act in this process are comprised of the Y-family DNA polymerases Rev1, polη, polι and/or polκ and the B-family polymerase polζ. Rev1 serves as a key scaffolding protein that assembles active TLS polymerases on proliferating cell nuclear antigen. The Rev1 C-terminal domain (Rev1-CT) binds Rev1-interacting regions (RIR) from polκ, polι and polη while interacting with the accessory Rev7 subunit of polζ. Cells deficient in Rev1 exhibit increased sensitivity to DNA damage and a significantly reduced mutation rate. Deletion of the Rev1-CT domain confers a similar phenotype, which suggests that this domain is critical for the cellular function of the TLS pathway. Finally, suppressing Rev1 activity in vitro and in vivo sensitizes cancers to genotoxic chemotherapy and reduces the onset of chemoresistance by decreasing tumor mutation rate.

Rev1/polζ-dependent TLS selective inhibitors may offer a promising therapeutic strategy by sensitizing cancers to genotoxic chemotherapy, reducing the onset of chemoresistance, and decreasing tumor mutation rate. The Hadden Lab has identified small molecules that disrupt Rev1-CT/Rev3/7 protein-protein interactions using FRET (fluorescence resonance energy transfer). This project will validate screening using MTS cell proliferation assay and clonogenic assay to determine inhibitory effects of combination cisplatin and TLS inhibitors on cell proliferation and survival.
Methods

Two assays were designed to evaluate whether TLS inhibitors increase cell sensitivity and avert chemoresistance to cisplatin, respectively: MTS cell proliferation assay and clonogenic assay. The cells that were used in these assays were U-2 OS (ATCC® HTB-96™) human osteosarcoma cells. The U-2 OS cells were untreated as a negative control, treated with DMSO as a vehicle control, and treated with cisplatin as a positive control. U-2 OS cells were suspended in U-2 OS growth media, which contains DMEM mixed with penicillin/streptomycin solution, and 10% FBS.

FRET High-throughput Screening

FRET high-throughput screening was used to identify which small molecule inhibitors interact with Rev1-CT and Rev 7/3. Rev1-CT was conjugated to eCFP and Rev 7/3 was conjugated to eYFP. The buffer used in this assay was 10 mM HEPES, 50 mM NaCl at pH 7.4 and 1% DMSO was to titrate solutions to final concentrations. A stock concentration of 178 µM Rev1CT-eCFP was diluted to a final concentration of 2 µM. Stock concentrations of 156 µM or 132.7 µM Rev7/3-eYFP were diluted to a final concentration of 6.4 µM. Compounds were tested at a final concentration of 10 µM using the buffer. Each well in the 384-well plate had a final volume of 20 µL. The four controls used were 5 µL each of Rev1-CT-eCFP and Rev7/3-eYFP with 10 µL 1% DMSO in FRET buffer, 5 µL each of Rev1-CT-eCFP and Rev7/3-eYFP with 10 µL of 20 µM unlabeled Rev7/3, 5 µL of Rev1CT-eCFP with 15 µL FRET buffer, and 5 µL of Rev7/3-eYFP with 15 µL of FRET buffer.

5 µL of Rev1-CT-eCFP was added to respective wells with either 10 µL of each compound or controls. The plate was covered with aluminum foil and left to incubate for 30 minutes at 25 °C. Then 5 µL of Rev7/3-eYFP was added to each well. Then plate was covered.
again and incubated for an additional 10 minutes at 25 °C before reading. The plate was excited at 433 nm and read at 2 emissions: 490 nm and 530 nm.

To quantify the data, a FRET ratio was determined using the following formula:

$$\frac{F_m - F_a - F_d (D_m / D_d)}{D_m}$$

where

- F = Fret channel
- D = Donor channel
- d = donor sample alone
- a = acceptor alone
- m = mix of donor and acceptor

The FRET channel represents the value at the 530 nm emission and the donor channel is the value at the 490 nm emission. $F_a$, $F_d$, and $D_d$ remained the same throughout the calculation because they were the control wells. Consequently, the FRET ratios of the compounds were compared to the FRET ratio of the unlabeled Rev7/3. Compounds that had a similar or lower FRET ratio were considered hits. The IC$_{50}$ of each compound calculated to measure the concentration at which there was 50% inhibition of the Rev7/3 binding to Rev1CT.

**MTS Cell Proliferation Assay**

The MTS cell proliferation assay was used to determine the compounds’ ability to increase cell sensitivity to cisplatin. This assay is similar to the MTS cell proliferation assay used in project with ITZ analogues. At first, MTS cell proliferation assays were performed to determine whether TLS inhibitors had any cell death-inducing activity. TLS inhibitors are not intended to demonstrate cell death-inducing activity, because they are used as adjuvant agents to cisplatin. The vehicle control was treated with 1% DMSO to account for DMSO used to prepare the TLS inhibitors. 3,000 U2-OS cells were seeded in a 96-well plate at 37°C for 24 hours, suspended in fresh media, then treated with either DMSO or TLS inhibitors at 50 and 25 µM.
After each of the wells were drugged with respective compounds, the plates were incubated at 37°C for another 72 hours. 20 µL of a 20:1 MTS and PMS mixture were added to each well and incubated at 37°C for 3 hours. The absorbance of each sample was consequently measured using a spectrophotometer. Thereafter, the absorbance values were used to calculate percent viability of each treatment. After determining TLS inhibitors have no cell death-inducing activity, these TLS inhibitors were tested with cisplatin in subsequent experiments to determine whether co-treatment increases cell sensitivity with cisplatin.

For the following experiments, U2-OS cells were seeded in a 96-well plate at 37°C for 24 hours, suspended in fresh media, then the vehicle controls were treated with 1% DMSO and 2% DMSO to account for DMSO used to prepare cisplatin and co-treatment with cisplatin and TLS inhibitors, respectively. The positive controls were treated with cisplatin at 100, 75, 50, 25, 10, 5, 1, and 0.5 µM. The experimental wells were treated with cisplatin at these concentrations with TLS inhibitors at 50 and 25 µM as well as TLS inhibitors at these concentrations alone. After each of the wells were drugged with respective compounds, the plates were incubated at 37°C for another 72 hours. 20 µL of a 20:1 MTS and PMS mixture were added to each well and incubated at 37°C for 3 hours. The absorbance of each sample was consequently measured using a spectrophotometer at 490 nm and these values were used to calculate percent viability of each treatment to determine half maximal inhibitory concentration (IC₅₀) of each treatment.

**Clonogenic Assay**

The clonogenic assay is an in vitro cell survival assay that measures the ability of a single cell to grow into a colony. This assay was used to determine the compounds’ ability to enhance the anti-cancer activity of cisplatin. 1,000 U2-OS cells were seeded in 6-well plates at 37°C for 24 hours, suspended in fresh media, then the vehicle controls were treated with 1% DMSO and
2% DMSO to account for DMSO used to prepare cisplatin and co-treatment with cisplatin and TLS inhibitors, respectively. The positive controls were treated with cisplatin at 10 µM and the experimental wells were treated with cisplatin at these concentrations with TLS inhibitors at 25 and 50 µM. After each of the wells were drugged with respective compounds, the plates were incubated at 37°C for another 72 hours, suspended in fresh media, and incubated at 37°C for another 7 days. After incubation, the media was removed from the plates and a fixative 50% methanol and 10% glacial acetic acid was added to the cells for 20 minutes. Then the fixative was removed from the plates, and Coomassie Brilliant Blue R-250 in MeOH:AcOH:H2O was added (46.5:7:46.5; v/v/v) to the cells. The dye was removed after 20 minutes and colonies ≥ 50 cells in diameter were scored to determine IC₅₀ of each treatment.

Results

FRET High-throughput Screening

TLS inhibitors 2 (IC₅₀ = 9.2 ± 4.7 µM) and 4 (IC₅₀ = 3.1 ± 2.3) were found to interact with Rev1-CT and Rev 7/3 and rescreened to rule out false positives using FRET high-throughput screening. The other compounds demonstrated in Table 1 were structurally similar to compounds 2 and 4 and were selected for additional testing. The IC₅₀ of these compounds were calculated as well. All of these compounds moved on to additional in vitro studies in either the MTS cell proliferation assay or clonogenic assay.

MTS Cell Proliferation Assay

First, TLS inhibitor candidates alone were tested in U2-OS cells at 100, 75, 50, 25 µM using the MTS cell proliferation assay to determine whether compounds had cell death-inducing activity and the results are demonstrated in Figure 1. Cisplatin, compound 1, demonstrated potent cell death-inducing activity at concentrations from 100 µM to 0.5 µM. All of the TLS
compounds tested 2, 3, 4, 5, 6, 9, and 10 at all concentrations from 100 µM to 0.5 µM demonstrated similar effects in U2-OS cells to DMSO and untreated controls (IC$_{50}$ > 100), which suggests that these TLS inhibitor candidates do not have cell death-inducing activity. These candidates progressed to additional studies testing the co-treatment of cisplatin and TLS inhibitors in the MTS cell proliferation assay.

Consequently, cisplatin at concentrations from 100 µM to 0.5 µM and co-treatment of cisplatin and TLS inhibitors at 25 µM and 50 µM were tested in U2-OS cells using the MTS cell proliferation assay and the results are demonstrated in Figure 2.

Comparing between the compounds tested, compounds 2, 3, 4, 5, and 6, co-treatment of cisplatin and compound 2 at 25 µM (IC$_{50}$ = 10.54 ± 9.13) was the most potent in inhibiting cell viability and at 50 µM (IC$_{50}$ = 15.5 ± 1.87) demonstrated similar activity. Co-treatment of cisplatin and compound 6 at 50 µM (IC$_{50}$ = 10.58 ± 1.54) was also relatively more potent than other treatments, but was the least potent co-treatment of cisplatin and TLS compounds at 25 µM (IC$_{50}$ = 34.63 ± 6.93). Co-treatment of compounds 3, 5, and 6 at 50 µM (IC$_{50}$ = 19.41 ± 3.03, 13.86 ± 0.82, 10.58 ± 1.54) were more markedly potent than at 25 µM (IC$_{50}$ = 27.71 ± 3.70, 19.08 ± 11.3, 34.63 ± 6.93), which suggests that these compounds are dose dependent. Co-treatment of cisplatin and compound 4 had similar activity at both concentrations (IC$_{50}$ at 25 µM = 30.77 ± 0.27; IC$_{50}$ at 50 µM = 30.49 ± 1.96) and was the least potent. Co-treatment with compounds 3 and 6 at 50 µM were more potent than cisplatin alone. Co-treatment with compounds 2 and 5 at both concentrations were more potent than cisplatin alone. Co-treatment with compound 4 at both concentrations was not more potent than cisplatin alone.

Comparing between all compounds, co-treatment of cisplatin and compound 10 at 50 µM (IC$_{50}$ = 3.1 ± 1.9) was the most potent and co-treatment at 25 µM (IC$_{50}$ = 11.0 ± 2.5) was
relatively more potent than all other co-treatments at 25 µM. Co-treatment of cisplatin and compound 9 at 50 µM (IC₅₀ = 8.6 ± 2.8) was relatively more potent in inhibiting cell viability and co-treatment at 25 µM (IC₅₀ = 8.8 ± 1.0) was the most potent of all other co-treatments at 25 µM. Co-treatment of cisplatin and compound 8 at 25 µM (IC₅₀ = 12.6 ± 6.9) was also relatively more potent than all other co-treatments at 25 µM. Co-treatment of cisplatin and compound 7 at both concentrations (IC₅₀ at 25 µM = 24.4 ± 6.9; IC₅₀ at 50 µM = 15.3 ± 4.3) and co-treatment of cisplatin and compound 8 at 50 µM (IC₅₀ = 19.6 ± 7.5) at had moderate potency compared to all other treatments. Co-treatment of compounds 7 and 10 at 50 µM were more markedly potent than at 25 µM, which suggests that these compounds are dose dependent. Co-treatment of cisplatin and compound 8 and 9 had similar activity at both concentrations.

These results suggest that most of the TLS inhibitors, except for compound 4, may be considered as adjuvant agents to platinating therapy. Treatments with compounds 7, 8, 9, and 10 advanced to additional studies in the clonogenic assay to assess the compounds’ ability to enhance the anti-cancer activity of cisplatin.

**Clonogenic Assay**

Cisplatin at 10 µM, compounds 7, 8, 9, and 10 alone at 25 µM and 50 µM, and co-treatment of cisplatin and these compounds were tested in U2-OS cells using the clonogenic assay and the results are demonstrated in Figure 3 and 4. All of the tested TLS inhibitors alone had similar activity to DMSO, which suggests that these TLS inhibitor candidates do not have cell death-inducing activity. These results are comparable to the findings of compounds 9 and 10 alone (IC₅₀ > 100) in the MTS cell proliferation assay listed in Table 1. Most co-treatments with compounds at 25 µM, except compound 9, were more potent than cisplatin alone. Co-treatment with compound 8 at 25 µM was the most potent and co-treatment with compound 9 at 25 µM
was the least potent compared to other treatments that were tested. All co-treatments with the compounds at 50 µM were more potent than cisplatin alone. Co-treatment with compound 7 at 50 µM was the most potent and co-treatment with compound 9 was the least potent of all other treatments. All co-treatments with TLS inhibitors at 50 µM were more potent than co-treatment with TLS inhibitors at 25 µM, which suggests that these compounds are dose dependent. Most co-treatments with TLS inhibitors at either concentration, except compound 9 at 25 µM, were more potent than cisplatin alone. These results suggest that all of the TLS inhibitors that were tested may be considered as an adjuvant therapy to platinating agents.
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<th>Cisplatin IC&lt;sub&gt;50&lt;/sub&gt; + 25 µM Compound</th>
<th>Cisplatin IC&lt;sub&gt;50&lt;/sub&gt; + 50 µM Compound</th>
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Figure 1. MTS cell proliferation assay with cisplatin and TLS inhibitors at concentrations of 25 µM and 50 µM, respectively. Data represent the Ave ± SEM of at least 2 separate experiments.
Figure 2. MTS cell proliferation assay with cisplatin, 1, alone at concentrations from 100 µM to 0.5 µM and TLS inhibitors, 2, 3, 4, 5, 6, alone at concentrations from 100 µM and 0.5 µM.
Figure 3. Clonogenic assay with TLS inhibitors alone and co-treatment of cisplatin with 25 µM TLS inhibitors.

Figure 4. Clonogenic assay with TLS inhibitors alone and co-treatment of cisplatin with 50 µM TLS inhibitors.
Discussion

The TLS pathway is a major mechanism cancer cells use to bypass DNA lesions, survive genotoxic chemotherapy, and promote chemoresistance. Inhibition of TLS may offer a new strategy to sensitize cancer cells to genotoxic chemotherapy, help avert chemoresistance, and decrease tumor mutation rate. In the clinical setting, combination therapy with a TLS inhibitor may reduce associated toxic side effects and reduce the onset of chemoresistance in cancer patients managed on platinating agents.

By interacting with Rev1-CT and Rev 7/3, TLS candidates were identified using FRET high-throughput screening. These compounds were then tested using in vitro cellular assays to validate their abilities in inhibiting the TLS pathway. This study explores TLS inhibitors as potential adjunct agents to platinating therapy towards increasing cell sensitivity using the MTS cell proliferation assay and clonogenic assay, respectively. Our preliminary data demonstrates that the TLS inhibitors tested have no cell death-inducing activity alone and several candidates provide additional inhibitory effects with platinating agents towards cell viability and survival.

In the MTS cell proliferation assay, most of the TLS inhibitors studied, except for compound 4, provided additional inhibitory effects to cell proliferation at either one or both concentrations, 25 µM and 50 µM. Comparing between all the compounds, most co-treatments with compounds at both concentrations, except compound 9 at 25 µM, provided additional inhibitory effects to cell proliferation compared to cisplatin alone. These results indicate that most of the TLS inhibitors may be promising candidates for adjunctive therapy with platinating agents towards increasing cancer cell sensitivity. Platinating agents may be used at lower concentrations combined with TLS inhibitors and reduce associated side effects, such as nausea, vomiting, adverse hematologic effects, neurotoxicity, and nephrotoxicity.
In the clonogenic assay, all of the TLS inhibitors that were tested may be considered as adjuvant agents to platinating therapy. All of the tested TLS inhibitors alone, compound 9 and 10, had similar activity to DMSO, which indicates that these candidates do not have cell death-inducing activity similar to findings from the MTS cell proliferation assay. Most co-treatments with compounds at both concentrations, except compound 9 at 25 µM, were more potent than cisplatin alone and may be promising candidates as adjunct therapy to platinating agents towards averting chemoresistance. The addition of TLS inhibitors may reduce cancer cell resistance to platinating agents and allow patients to stay on therapy for a longer period of time.

Altogether, this study demonstrates that TLS inhibitors do not cause cell death-inducing activity on their own and are favorable as adjuvant agents. Most of the TLS inhibitors studied may be considered as promising adjuvant agents to platinating therapy towards increasing cancer cell sensitivity and/or averting chemoresistance. Overall, TLS inhibitors may be considered as safe and effective adjunct agents towards complex regimens involving platinating agents and other chemotherapy.
References


