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Research Article

Water-soluble SCR7 Can Abrogate DNA End Joining and Induce Cancer Cell Death

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ARTICLE INFO

Article history:
Received: 9 June, 2020
Accepted: 1 July, 2020
Published: 16 July, 2020

Keywords:
NHEJ inhibitor
DSB repair
nonhomologous end joining
ligase IV
apoptosis
cancer therapy

ABSTRACT

Small molecule inhibitors targeting DNA repair pathways in cancer cells is a novel and promising approach in cancer therapy, which can improve current therapeutic regimen. Although various attempts have been made for designing inhibitors against DNA damage response and repair proteins, reports on Nonhomologous End Joining (NHEJ) inhibitors are limited. Of the several chemical moieties identified, SCR7 and its oxidized form are novel and potent DNA Ligase IV inhibitors involved in the abrogation of DNA end joining thereby leading to cell death. In the present study, we have synthesized sodium salt of SCR7 to generate a water-soluble version of the molecule, referred to as water-soluble SCR7 (WS-SCR7). WS-SCR7 inhibits NHEJ in Ligase IV dependent manner, with a subtle effect on Ligase III at higher concentration. No effect on Ligase I mediated joining was observed. WS-SCR7 shows cytotoxicity in cancer cell lines, leading to induction of apoptosis in a dose-dependent manner.

Introduction

DNA double-strand breaks (DSBs) are the most deleterious DNA damage. When left unrepaired, DSBs can result in the accumulation of DNA breaks in the nucleus, chromosomal rearrangements and cell death [1, 2]. It is well established that induction of DSBs in cancer cells during radiotherapy or chemotherapy can lead to apoptosis and cell death. Recently, inhibition of DNA repair resulting in accumulation of DSBs is considered as an interesting and novel strategy to treat cancer [3, 4].

Repair of double-strand breaks occurs through two predominant pathways: Homologous Recombination (HR) and Nonhomologous DNA End joining (NHEJ) [5-9]. While HR requires homologous sequences from sister chromatid for the repair of DSBs, NHEJ is error-prone, sequence-independent and considered as a dynamic process involving the organization of multiple proteins [4, 10-13]. In the case of canonical NHEJ, one of the core proteins KU heterodimer binds to broken DNA, followed by processing of the ends by DNA-PKcs, Artemis, and finally ligation by Ligase IV/XRCC4/XLF [4, 10-13]. The majority of DSBs generated in human cells are repaired through NHEJ.

Over the years therapeutic success of inhibition of DNA repair pathways depends on the selection of an appropriate protein target that is either directly involved in oncogenesis or has synthetic lethal interactions with another repair protein [4]. Previous reports have revealed a novel small molecule inhibitor, SCR7, which can inhibit NHEJ in a Ligase IV dependent manner in human cells [14]. Studies in mouse tumor models have suggested its therapeutic potential by regression of tumor volume and increasing the lifespan. Recently, we have shown that parental SCR7 can get cyclized into a stable form with the same molecular formula and mass, which upon further oxidation can result in SCR7-pyrazine, which possesses a different molecular formula and molecular weight [15]. Although both forms of SCR7 inhibited NHEJ *in vitro* and *ex vivo*, SCR7-pyrazine was less specific inside the cells [15].

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http://dx.doi.org/10.31487/j.COR.2020.07.09

Multiple studies have also used SCR7 as a cancer therapeutic agent as well as a biochemical inhibitor [16-20]. Besides, several independent groups have reported a 2-19 fold increase in precise genome editing when SCR7 was used along with CRISPR-Cas9 constructs *ex vivo* and *in vivo* [3, 21-23].

Although SCR7 found multiple applications, its high hydrophobicity and DMSO solubility are few of the limitations for its clinical trial. In the present study, a water-soluble version of SCR7 has been synthesized, generating sodium salt of SCR7 and characterized with respect to inhibition of end joining and effect on cancer cell proliferation. WS-SCR7 works in Ligase IV dependent manner, leading to accumulation of DSBs and improved cell death.

Materials and Methods

I Chemicals and Reagents

Analytical grade reagents and chemicals were procured from Sigma Chemical Co. (St. Louis, MO, USA) and Sisco Research Laboratories (Andheri East, Mumbai, India). DNA modifying enzymes were from NEB (Ipswich, Massachusetts, USA). Foetal Bovine Serum (FBS), antibiotics Penicillin and Streptomycin (PenStrep) and tissue culture media were purchased from Lonza (Walkersville, MD, USA). Radiolabeled nucleotide, γ^{32}P-ATP was purchased from BRIT (Hyderabad, India).

II Cell Lines and Culture Conditions

Nalm6 (B cell precursor leukemia), CEM (T cell leukemia) and Molt4 cells were cultured in RPMI 1640 supplemented with 10% FBS and 100 µg/ml of Penicillin G and Streptomycin. Human cervical cancer cell line, HeLa was cultured in DMEM containing 10% FBS and PenStrep. Cells were grown at 37ºC in a humidified atmosphere containing 5% CO_2. HeLa, CEM, Molt4 were obtained from National Centre for Cell Science, Pune, India, while Nalm6 was from M. Lieber, USA.

III Chemical Synthesis and General Procedures

All reactions were monitored using TLC, performed with E. Merck silica gel 60 F254 aluminium plates (Kenilworth, NJ, USA) and were visualized under UV light. The following mobile phases were employed for TLC: chloroform, methanol, hexane and ethyl acetate in different ratios. ^{1}H NMR (400 MHz) spectra was recorded in DMSO-d6 solutions on a 400 MHz and 100 MHz spectrometer. Chemical shifts are reported in δ (ppm) relative to TMS as internal standard; coupling constants (J) are expressed in Hz.

IV Synthesis of SCR6 [3]

A suspension of 5,6-diamino-4-hydroxy-2-mercaptopyrimidine (1.0 g, 0.0063 mol) and benzaldehyde (0.67 g, 0.0063mol) in dimethyl formamide (30 mL) and acetic acid (10 mL) was stirred at room temperature for 16 h. The reaction mixture was then added slowly to ice-cold water, and the precipitated solid was filtered, washed with water and recrystallized from dimethyl formamide-ethanol as described. Yield: 0.93g (60%).

V Synthesis of SCR7 [4]

A suspension of SCR6 [3] (0.93 g, 0.0037mol) and benzaldehyde (0.4g,0.0037mol) in dimethyl formamide (30 mL) and acetic acid (3 mL) was refluxed at 200ºC for 8 to 10 h. The reaction mixture was transferred to ice-cold water, and the precipitated solid was filtered, washed with water and recrystallized from dimethyl formamide-ethanol as described before [14, 15] Yield: 0.44 g (35%).

VI Synthesis of Sodium Salt of 2-Mercapto-6,7-Diphenyl-7,8-Dihydropteridin-4-Ol [5]

A suspension of SCR7 [4] (0.44 g, 0.00131 mol) and NaOH (0.234 g, 0.00585 mol) in THF (30 mL) was refluxed at 50ºC for 12 h. Evaporation of solvents resulted in a yellow solid, which was washed in ethanol. The solid precipitate was allowed to dry. Yield: 0.231 g (60%); Yellow solid; ^{1}H NMR (400 MHz, DMSO-d6): 9.82 (s, 1H), 8.13 (s, 1H), 7.84 (d, J = 4 Hz, 2H), 7.36–7.21 (m, 8H). 5.92 (s,1H).

VII Ethics Statement

Wistar rats were maintained according to the guidelines of the Animal Ethical Committee, Indian Institute of Science. Indian National Law on animal care and usage was followed. The study design was approved by the Institutional Animal Ethics Committee (CAF/Ethics/526/2016), Indian Institute of Science, Bangalore, India.

VIII DNA End Joining Reactions Using Oligomeric DNA Substrate and Cell-Free Extracts

4-6 week old male Wistar rats, *Rattus norvegicus* were maintained in the Central Animal Facility, Indian Institute of Science, and organs were isolated following dissection. Rat testicular extract was prepared as described earlier [24-26].

Oligomeric DNA was purified using 8-12% denaturing PAGE as described earlier [27]. The 5' end-labeling of the oligomeric DNA was performed using T4 polynucleotide kinase in a buffer containing 20 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT and γ^{32}P-ATP at 37ºC for 1 h. Next, γ^{32}P-ATP end-labeled 75 nt oligomer was annealed with the unlabeled complementary oligomer in 10 mM NaCl and 1 mM EDTA in a boiling water bath for 10 min, followed by slow cooling [28].

DNA end joining reactions were performed in NHEJ buffer containing 30 mM HEPES-KOH (pH 7.9), 7.5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 50 µM dNTPs and 0.1 µg BSA at 25ºC. The reaction mix containing tissue extract (1 µg) was incubated for 30 min with the inhibitor, followed by the addition of radiolabeled substrate and further incubation for 1 h [14, 24, 26, 29]. Phenol/chloroform extraction was used to purify the reaction products, which were then resolved on 8% denaturing PAGE. The gel was dried, exposed, and the signal was detected using PhosphorImager FLA9000 (Fuji, Japan). Quantification of joined products was done using MultiGauge software and presented as PhotoStimulated Luminescence Unit (PSLU). GraphPad Prism (V5) software was used for calculating statistical significance.

IX End Joining Mediated by DNA Ligases

DNA ligases were overexpressed and purified from bacteria [14, 30]. His-tagged Ligase IV/XRCC4 bicistronic vector (pMJ4052) was a kind gift from Dr. Mauro Modesti. DNA Ligases were purified using Ni-NTA and Unosphere Q Anion exchange columns. End joining reactions were set up in NHEJ buffer with purified protein and inhibitor at 25ºC for 30 min, followed by incubation with radiolabeled nicked DNA substrate for 1 h. Reaction products were resolved on 12% denaturing PAGE, the signal was detected using PhosphorImager FLA9000 (Fuji, Japan) and analysed using GraphPad Prism (V5) software.

X Cytotoxicity

Cell viability was analysed by Trypan Blue dye exclusion assay as described previously [14, 31-33]. Briefly, 25,000 cells/ml were seeded in tissue culture grade 24-well plate and incubated for 48 h with varying concentrations of WS-SCR7 (10, 50, 100 and 250 μM). Cells were mixed with an equal volume of 0.4% Trypan blue (Sigma Chemical Co., St Louis, MO, USA) and counted using haemocytometer. Experiments were repeated three times, and error bars plotted using GraphPad Prism (V5) software.

XI Cell Cycle Analysis

Cell cycle analysis was performed as described before [34, 35]. Briefly, 25,000 cells/ml were seeded in 4 ml culture media and treated with WS-SCR7 (90 and 180 μM for Molt4) for 48 h. Harvested cells were washed with 1X PBS and fixed in 70% ethanol overnight at -20ºC. Fixed samples were centrifuged and subjected to RNaseA treatment overnight under shaking conditions at 37ºC. Finally, cells were stained with Propidium Iodide and analysed using CytoFLEX flow cytometer, Beckman Coulter (Brea, California, United States). A minimum of 10,000 cells were acquired, and data analysis was done using CytExpert software.

XII JC-1 assay

In order to identify changes in mitochondrial membrane potential, cells were treated with increasing concentration of WS-SCR7 (50 and 100 μM for Nalm6) for 48 h and subjected to JC-1 staining assay [31, 36, 37]. Cells were incubated in JC-1 dye (5,50,6,60-tetrachloro-1,1,3,30-tetraethylbenzimidazolcarbocyanamideiodide (Calbiochem, USA) (1 μM) at 37ºC for 20 min, with intermittent mixing. 2, 4-Dinitrophenol (4 mM) was used as the positive control. After 1X PBS wash, samples were acquired using CytoFLEX flow cytometer. Mitochondrial depolarization, indicated by a decrease in the red/green fluorescence intensity ratio, was represented in bar diagrams prepared using GraphPad Prism.

Results and Discussion

In our previous studies, we have reported SCR7 and its other forms as novel and potent inhibitor of NHEJ that can work in Ligase IV dependent manner [14, 15, 38]. SCR7 binds to the DNA binding domain of Ligase IV, thereby interfering with the binding of the protein to DSBs, leading to accumulation of unrepaired breaks inside cells and apoptosis. However, a major limitation of the molecule was its solubility in organic solvents such as DMSO. In order to increase its solubility and hence permeability in cells, a water-soluble version of SCR7 was synthesized by replacing one of the hydrogens with sodium in the moiety (Figure 1A). Molecular structure was verified using NMR studies (Figure 1B).

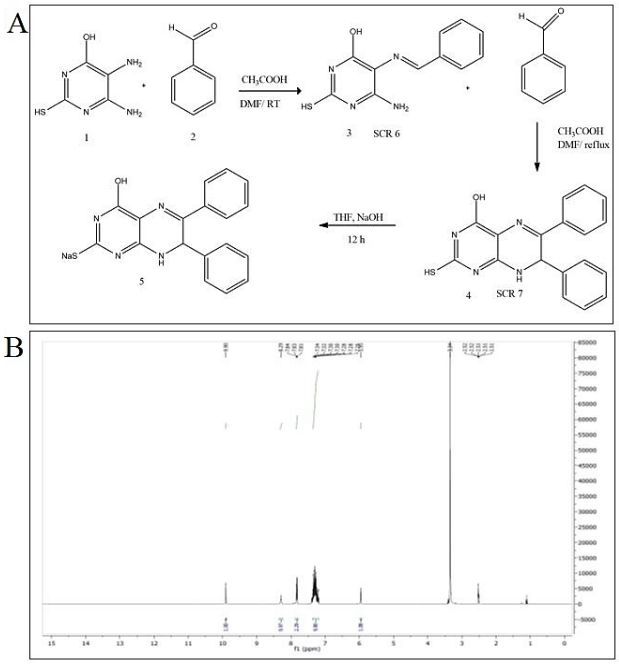

Figure 1: Synthesis and characterization of WS-SCR7. **A)** Synthetic route employed in obtaining WS-SCR7. **B)** Structural characterization of WS-SCR7 by ^{1}H-NMR spectroscopy.

A cell-free repair assay system was utilised to investigate the potential of WS-SCR7 to inhibit NHEJ. Previously, we have reported that testicular extracts are proficient in NHEJ compared to other tissue extracts [25, 26]. Hence, testicular extracts were incubated with increasing doses of WS-SCR7 (50, 100, 200, 400, 600, 800 and 1000 μM). Results revealed inhibition of end joining from 600 μM of WS-SCR7 onwards when the compound was incubated with γ^{32}-labeled double-stranded oligomeric DNA substrate in the presence of testicular proteins (Figure 2A). Ligase IV/XRCC4 is the crucial protein involved in ligation of breaks as the last step in the NHEJ pathway [39-42]. As reported earlier, SCR7 can inhibit purified Ligase IV/XRCC4 mediated joining at 200 μM [14, 15]. In the present study, the effect of WS-SCR7 on the inhibition of joining catalysed by purified Ligase IV/XRCC4 was investigated (Figure 2B). Results revealed a dose-dependent inhibition of joining catalysed by Ligase IV/XRCC4 from 200 μM, suggesting Ligase IV dependent effect of the water-soluble version at a concentration similar to that of SCR7. Effect of WS-SCR7 (50, 100, 200, 400, 600, 800 and 1000 μM) on Ligase I and Ligase III/XRCC1 mediated end joining was also investigated. We observed that WS-SCR7 did not inhibit Ligase I mediated end joining even at the highest concentration (1000 μM), similar to SCR7 (Figure 2C). However, Ligase III/XRCC1 catalysed end joining was inhibited from a concentration of 600 μM onwards (Figure 2D), which was consistent with our previous report [14].

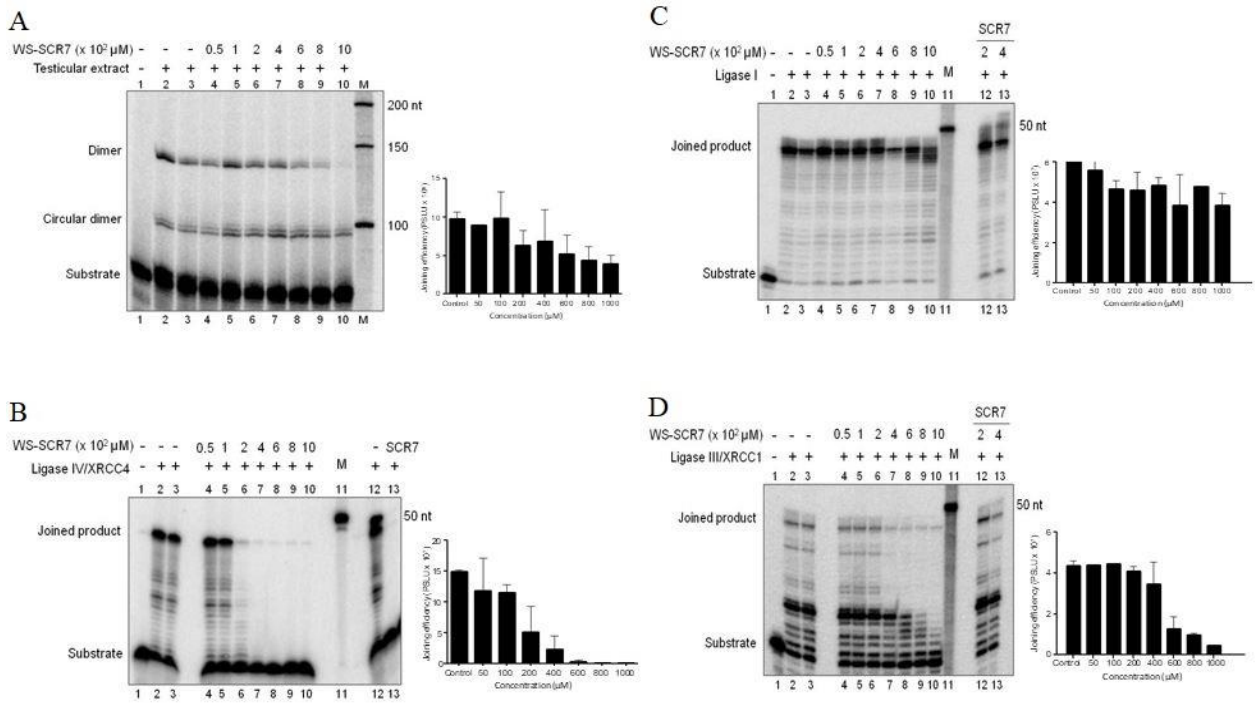

Figure 2: Effect of WS-SCR7 on NHEJ and evaluation of Ligase IV specificity. **A)** Inhibition of end joining of rat testicular extract in presence of increasing concentrations of WS-SCR7 (50, 100, 200, 400, 600, 800 and 1000 µM). Bar graph shows quantification of inhibition of end joining in presence of WS-SCR7 in a concentration-dependent manner. **B)** Inhibition of joining catalysed by purified Ligase IV/XRCC4 when increasing concentrations (50, 100, 200, 400, 600, 800 and 1000 µM) of WS-SCR7 was added. Bar graph depicts quantification of inhibition of Ligase IV/XRCC4 mediated joining. **C)** Evaluation of effect of WS-SCR7 on joining catalysed by Ligase I. **D)** Evaluation of joining by Ligase III/XRCC1, in presence of WS-SCR7 and bar graphs showing quantification of extent of inhibition.

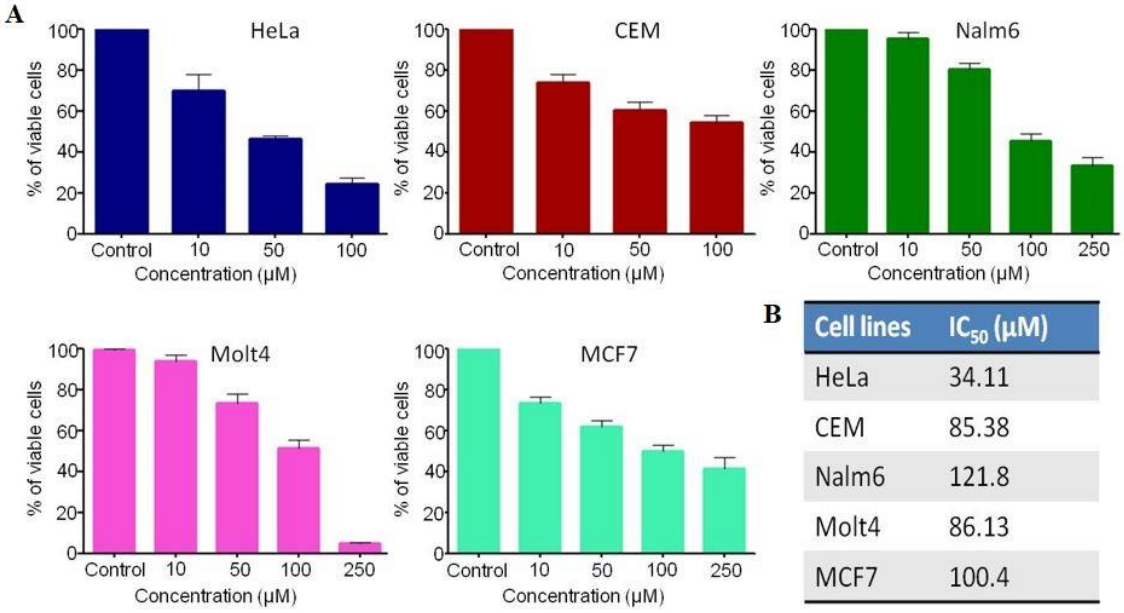

Figure 3: Evaluation of cytotoxicity induced by WS-SCR7 in cancer cell lines. **A)** Bar graphs representing cytotoxicity of WS-SCR7 (10, 50, 100 and 250 µM) after 48 h of treatment in various cancer cell lines, including HeLa, CEM, Nalm6, Molt4 and MCF7. **B)** Table representing IC_{50} values (in µM) of WS-SCR7 in various cancer cell lines at 48 h.

Cytotoxicity of WS-SCR7 was evaluated in various cancer cell lines (HeLa, CEM, Nalm6, Molt4 and MCF7) by incubating with increasing concentrations (10, 50, 100 and 250 µM), and IC_{50} values were determined (Figures 3A & 3B). Among the cell lines tested, cervical cancer cell line, HeLa showed the highest sensitivity, with an IC_{50} of 34 µM (Figures 3A & 3B), which was comparable to that of SCR7.

However, WS-SCR7 induced cytotoxicity was comparatively less in the case of other cell lines (Figures 3A & 3B).

In order to investigate the effect of WS-SCR7 on cell cycle progression in cancer cells, Molt4 was treated with increasing concentrations of the inhibitor (0, 90 and 180 μM) (Figure 4A). Results revealed an increase in the SubG1 population upon treatment with WS-SCR7 in a dose-dependent manner. A subtle S-phase arrest was also observed (Figure 4A). The effect of WS-SCR7 on mitochondrial membrane potential was evaluated by JC-1 assay in Nalm6 (Figure 4B) at different concentrations (0, 50 and 100 μM). 2, 4-DNP, an uncoupler was used as positive control and showed more than 50% depolarisation of mitochondria. A shift from red to green population in WS-SCR7 treated samples suggested an increase in depolarised mitochondrial membrane potential in a dose-dependent manner (Figure 4B). Overall, WS-SCR7 can inhibit end joining in Ligase-IV dependent manner similar to SCR7 and induce cell death in cancer cells.

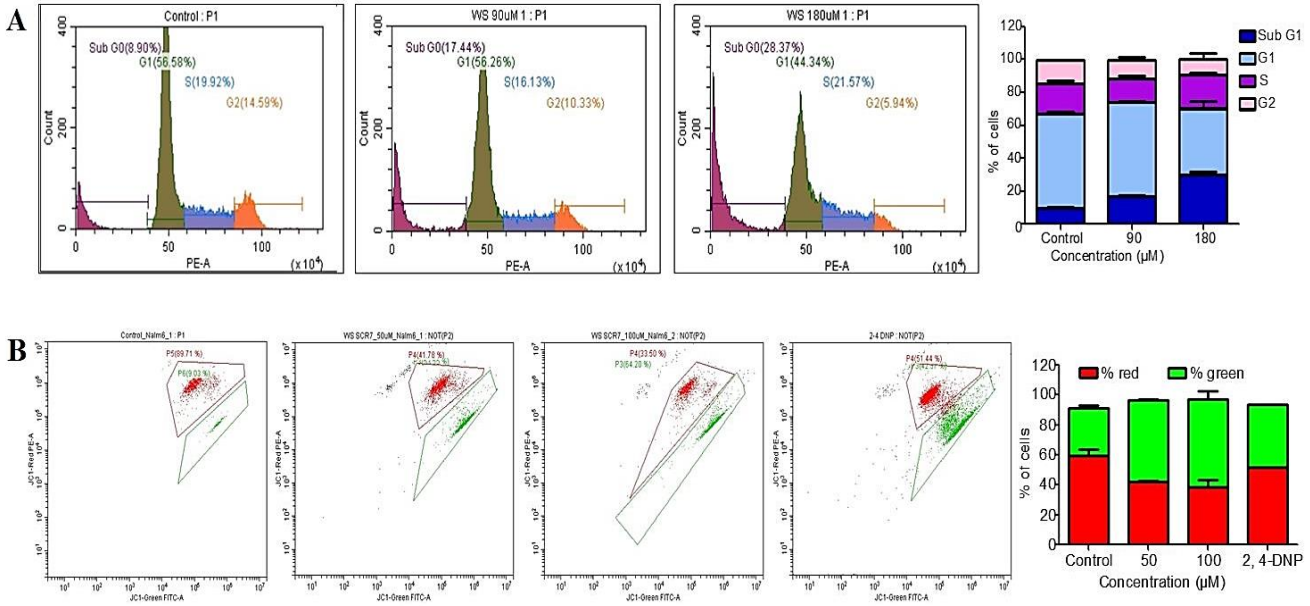

Figure 4: Evaluation of effect of WS-SCR7 in cancer cell lines. **A)** Analysis of cell cycle progression in Molt4 cells after treatment with WS-SCR7 in a dose-dependent manner (0, 90, 180 μM). Percentage of cells in each stage of cell cycle following treatment with WS-SCR7 for 48 h is represented as bar graphs. **B)** JC-1 assay for detecting loss of mitochondrial membrane potential in Nalm6 cells post-treatment with WS-SCR7 in a dose-dependent manner (0, 50 and 100 μM for 48 h). 2, 4-DNP (4 mM) serves as positive control. Bar graphs depict percentage of green cells undergoing loss in membrane potential to that of red cells.

DNA repair inhibitors are considered as attractive cancer therapeutic agents owing to the upregulation of several repair proteins in cancer. Being one of the major DSB repair pathways in mammals, the upregulation of NHEJ has been shown to provide resistance to various cancer cells [43]. Reports suggest upregulation of KU and DNA-PKcs in breast, gastric, lung and esophageal cancers, and polymorphisms in Ligase IV and XRCC4 [4, 44-46]. Current literature in the field offers very few reports on the characterization of inhibitors targeting Ligase IV, a crucial protein involved in resealing of breaks at the last step of the NHEJ pathway. In this line, SCR7 and its forms are considered potent NHEJ inhibitors that can work in a Ligase IV dependent manner [14, 15]. The abrogation of NHEJ led to increased cytotoxicity and tumor regression both inside the cell lines and *in vivo*. Further, a water-soluble version of SCR7-pyrazine (Na-SCR7-P) was synthesized and characterized. Na-SCR7-P inhibited end joining of all three Ligases and inhibited tumor progression in mice [38].

In the present study we have investigated the potential of water-soluble version of SCR7 in inhibition of end joining compared to its parental form that is DMSO soluble. Interestingly we found that WS-SCR7 can abrogate end joining mediated by Ligase IV/XRCC4 at a concentration similar to that of SCR7. Although no inhibition was observed with respect to Ligase I mediated joining, WS-SCR7 inhibited Ligase III/XRCC1 at higher concentration, which was 3-fold higher than that for Ligase IV. We also observed that similar to DMSO soluble version, WS-SCR7 induced cytotoxicity in several cancer cell lines and could lead to the depolarisation of mitochondria suggesting activation of the intrinsic pathway of apoptosis. Consistently, an increase in the SubG1 population was also obtained, which along with observed ability of inhibition of NHEJ suggests the accumulation of DSBs leading to apoptosis.

Acknowledgements

We thank Sumedha Dahal and other members of the SCR laboratory for comments on the manuscript. We thank Supriya Vartak for help in cell culture.

Funding

The work was funded by grants from CEFIPRA (IFC/5203-4/2015/131), DAE (21/01/2016-BRNS/35074), DBT (BT/PR13458/COE/34/33/2015), IISc-DBT partnership programme

[DBT/BF/PR/INS/2011-12/IISc] to SCR. UR is supported by Senior Research fellowship (SRF) from CSIR, India.

Conflicts of Interest

None.

Author Contributions

SCR coordinated the study and provided the reagents; SCR, UR and MN designed the experiments, interpreted results and wrote the manuscript; UR and RP conducted the experiments; Chemical synthesis and characterization of the inhibitor were done by AEJ, UK and HAS.

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