

Identification of S-aryl dithiocarbamate derived novel antiproliferative compound exhibiting tubulin bundling

4.1 Abstract

The cell division machinery involves MT nucleating centrosomes and the bipolar arrangement of spindle MT. It is essential for proper DNA segregation between the two daughter cells at the end of cell division. This division machinery is tightly controlled during the cell cycle and any kind of errors in the mitotic machinery could lead to cancer. Anticancer drugs targeting MTs have been widely used in chemotherapeutics. However, many cancer types develop resistance to these drugs. It is therefore imperative to identify novel chemical scaffolds which provide flexibility for designing new drugs for chemotherapeutics. This part of my work involves the identification of an S-aryl dithiocarbamates-based chemical moiety exhibiting antiproliferative activity in cancer cell lines. Our collaborators established a new synthetic route involving transition-metal-free, one-pot three-component annulation between cyclic and acyclic aliphatic amines, diaryliodonium triflates, and carbon disulfide which resulted in efficient and convenient access to diverse categories of S-secondary alkyl and S-aryl dithiocarbamates. Our *in silico* analysis revealed good pharmacophore and drug-like properties of the synthesized compounds. Further, screening the synthesized series of N-Boc piperazine containing S-aryl dithiocarbamates led us to the identification of compound 1 as a potent antiproliferative agent in cervical, lung, and breast cancer cell lines. Compound 1 shows the best inhibitory activity against cervical cancer cells, HeLa with an IC_{50} of $0.432 \pm 0.138 \mu M$, and lung cancer cells, A549 with an IC_{50} of $0.447 \pm 0.051 \mu M$, at 72 hours post-treatment using MTT assay. The HeLa cells treated with compound 1 lead to cell cycle arrest at the G_2/M phase of the

cell cycle and increase cell apoptosis *via* upregulating the p53-p21 signaling pathway. Interestingly, compound 1 treated cells demonstrated a tubulin bundling phenotype which is a characteristic of MT-stabilizing anticancer drugs like paclitaxel. The molecular docking studies revealed a comparable affinity of compound 1 in the β -tubulin taxol binding pocket, establishing it as a promising candidate for anticancer drug discovery.

4.2 Introduction

Cancer cells have characteristic high division rates than normal cells, conventional chemotherapeutics exploit their property to target DNA replication and cell division machinery. Anticancer drugs such as taxane and colchicine target the dynamic behavior of MTs to cause MT stabilization and destabilization, respectively [Jordan and Wilson 2004]. Notably, paclitaxel, a member of the taxane class of drugs, has emerged as a cornerstone in the treatment of various types of cancers [Sharifi-Rad et al. 2021]. It exerts its anticancer effects through a well-defined mechanism of action. As a MT-stabilizing agent, paclitaxel binds to the beta subunit of tubulin, a key component of MTs, preventing their depolymerization and promoting their assembly into stable structures [Field et al. 2013; Ballatore et al. 2012]. This leads to the formation of abnormal and non-functional MT bundles, which disrupt normal cell division and mitosis. Consequently, paclitaxel induces cell cycle arrest at the G₂/M phase and triggers apoptosis in cancer cells [Choi and Yoo 2012; Khing et al. 2021; Ballatore et al. 2012]. Additionally, paclitaxel inhibits tumor angiogenesis by interfering with MT dynamics in endothelial cells, thereby reducing the blood supply to the tumor [Pasquier et al. 2005]. This multifaceted mechanism of action contributes to the potent anticancer activity of paclitaxel against a wide range of cancer types. Despite the effectiveness of paclitaxel as an anticancer drug, the development of resistance remains a significant challenge in its clinical use. Several mechanisms have been identified to underlie paclitaxel resistance in cancer cells. One of the primary mechanisms is alterations in the expression or function of β -tubulin, the target of paclitaxel [Huzil et al. 2007]. Mutations in the β -tubulin gene can reduce the affinity of paclitaxel for its binding site, leading to decreased drug binding and reduced efficacy [Monzó et al. 1999]. Over-expression of drug efflux transporters, such as P-glycoprotein (P-gp), can actively pump out paclitaxel from cancer cells, limiting its intracellular concentration and therapeutic effect [Karthika et al. 2022; Pilotto Heming et al. 2022; Xue and Liang 2012; Amin 2013]. Other mechanisms include alterations in apoptotic pathways, upregulation of anti-apoptotic proteins (e.g., Bcl-2), activation of survival signaling pathways (e.g., PI3K/Akt), and enhanced DNA repair mechanisms [Mohammad et al. 2015;

Yang et al. 2019]. Tumor microenvironment factors, such as hypoxia and tumor-associated fibroblasts, can also contribute to paclitaxel resistance [Senthebane et al. 2017; Yang et al. 2019]. Additionally, it is associated with various adverse effects, including bone marrow suppression, peripheral neuropathy, and hair loss [Saggam et al. 2022; Scripture et al. 2006]. Hence, the identification of chemical entities with novel structures and biological activity targeting specific molecular pathways or factors in cancer cells is imperative to the field of anticancer drug discovery [Ma et al. 2021].

Dithiocarbamates belong to a versatile class of compounds with organo-sulfur moieties having diverse applications in the field of polymer, material, and, biological sciences [Odularu and Ajibade 2019; Mayadunne et al. 1999; Demartino et al. 2017] (Fig. 4.1).

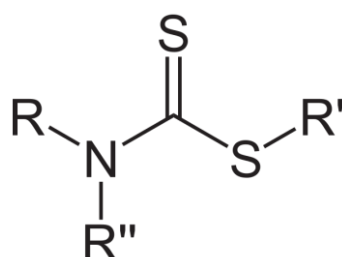


Figure 4.1: Chemical structure of dithiocarbamates ($\text{R}_2\text{NC}(\text{S})\text{SR}$). (Adapted from PubChem CID 3037131).

Its use was first reported by Debus in the year 1850. Structurally, dithiocarbamates ($\text{R}_2\text{NCS-2R}$) consist of a broad class of 1,1-dithioligand monoacids [Meco et al. 1994; Buac et al. 2012; Topping and Jones 1988]. The ability of the dithiocarbamate C-S moiety to interact with metals gives an important biological value as a lot of proteins, and enzymes, have metallic composition centers. These can act as chelators of metals, destabilizing the structure of these molecules and inhibiting their action [Oliveira et al. 2019]. They impart a plethora of biological effects including anti-fungal [Monti et al. 2012; Rafin et al. 2000], anti-bacterial [Ayman et al. 2019; Jiang et al. 2020], anti-inflammatory [El Ashry et al. 2012], metal chelating ability [Ronconi et al. 2006; Shiri et al. 2016] and also acts as inhibitors of Pyruvate dehydrogenase kinase 1 (PDK1) and Monoacylglycerol lipase (MAGL) [Liu et al. 2017; Ronconi et al. 2006; Shiri et al. 2016]. Due to access to intracellular components, dithiocarbamates have been proved to be effective anti-*Trypanosoma Cruzi* drugs by inhibiting the enzyme superoxide dismutase which is an important enzyme in the oxidative system of many protozoa [Rodrigues et al. 1995]. Additionally, the amine present in this structure can interact with different substituents modulating cellular responses with specific targets. Further, it produces reactive oxygen species (ROS) conferring an important biological activity for the

dithiocarbamates [de Koning et al. 2011]. Hence, dithiocarbamates act as an attractive scaffold in the design and development of medicinally active compounds, discussed below.

- Brassinin was the first dithiocarbamate motif containing indole phytoalexin, isolated from Chinese cabbage *Brassica campestris* with anticancer activities [Mehta et al. 1995]. 1-Methoxybrassinin showed the most potent anti-proliferative activity in Caco-2 cells with IC_{50} of $8.2 \pm 1.2 \mu\text{mol/L}$. Brassinin elevates levels of p21 and p27 via inhibiting the phosphatidylinositol-3-kinase signaling pathway causing G_1 cell cycle arrest. It also induces apoptosis by suppressing PI3Kakt/mTOR/S6K1 signaling cascade [Chripkova et al. 2014].
- Another novel dithiocarbamic acid derivative TM208 (III) was developed, showing effective *in vitro* anticancer activities [Hou et al. 2006].
- Wang *et al.* reported a new family of butenolides-containing dithiocarbamates and their *in vitro* cytotoxic activity [Wang et al. 2011]. Dithiocarbamates with piperazine and pyrrolidine as a secondary amine exhibited significant and selective cytotoxicity on the HeLa cancer cell line, with an IC_{50} of $0.77 \pm 0.3 \mu\text{M}$ and 2.63 ± 0.2 , respectively.
- Pyrrolo[2,1-c][1,4]benzodiazepines (PBDs) obtained from *Streptomyces* species are known for their potent antibiotic and antitumor activities. It interacts with the minor groove of DNA by forming a covalent amine bond between the C11-position of the central B-ring and the N2-amino group of guanine [Thurston and Bose 1994; Barkley et al. 1986].
- The β -carboline alkaloids show anticancer activity through various mechanisms like DNA intercalation, inhibiting DNA topoisomerase I and II, CDK, MK-2, PLK1 and kinesin Eg5. β -carbolines are considered to be DNA intercalators with the property to stack between DNA base pairs, attributed to their planar aromatic structures. The derivative compounds exhibited significant cytotoxicity suggesting that the inclusion of the dithiocarbamate motif has an essential role in cytotoxic activity [Murata et al. 2004; Wink 1987; Haribabu et al. 2022].
- Emetine alkaloid obtained from *P. Ipecacuanha* exerts good anti-cancer activity [Siddiqui et al. 1973]. Akinboye *et al.*, synthesized emetine-containing dithiocarbamate ester derivatives and found that the majority of dithiocarbamate ester analogues had appreciable potency in all prostate cancer cell lines with IC_{50} in the ranging from 1.312 to 5.201 μM with lower toxicity [Akinboye et al. 2015].

- Chalcone is an important class of natural products with significant anticancer properties as illustrated against EC-109, SK-N-SH and MGC-803 cancer cell lines [Shankaraiah et al. 2017].
- A study reported the synthesis of different artemisinin-piperazine-dithiocarbamate derivatives active against the human prostate and liver cancer cell lines with low toxicity [Yu et al. 2019]. Artemisinin and its derivatives such as artesunate, artemisone and dihydroartemisinin (DHA) show good anti-cancer activity [Jung et al. 2003].
- Coumarin-1,2,3-triazole-dithiocarbamates as an LSD1 inhibitor was potentially used with an IC₅₀ value of 0.39 μ M. The SAR-analysis expressed that, the presence of the tert-butyloxy carbonyl moiety on piperazine and one carbon length between triazole and phenyl/coumarin showed optimum activity [Ye et al. 2014].
- Another related study on dithiocarbamate scaffolds containing 2(5H)-furanone-piperazine (VI) reported compounds with IC₅₀ < 1 μ M [Wei et al. 2018].

Therefore, dithiocarbamates linked to biologically active heterocycles have exhibited diverse pharmacological profiles including strong anticancer activities. These derivatives exert their action by targeting various enzymes like HDAC, LSD1, carbonic anhydrases, FAK, etc. These biological activities of dithiocarbamate derivatives have inspired the designing of novel molecules with improved biological display. However, the majority of these identified dithiocarbamate-based anticancer compounds contain S-alkyl dithiocarbamate derivative moieties. So far, none of the studies report systematic analyses of the anticancer properties of S-secondary alkyl or S-aryl dithiocarbamates-based derivatives. Our collaborators established a highly efficient synthetic route involving one-pot, three-component, metal-free coupling of diaryliodonium salts, cyclic and acyclic aliphatic amines, and carbon disulfide to access a variety of structurally and electronically variable S-aryl dithiocarbamates [Parida et al. 2021]. *In silico* analyses of these compounds demonstrated that the synthesized compounds have good drug-likeness and ADMET properties. This motivated us to further screen and characterize the anticancer properties of the synthesized dithiocarbamate compounds and their derivatives.

4.3 Materials and Methods

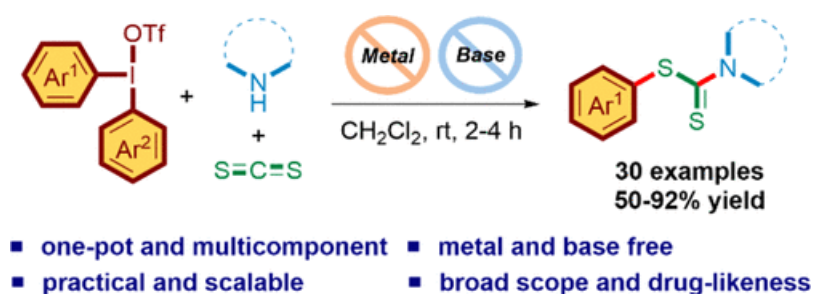
4.3.1 Synthesis of chemical library

An efficient metal-free one-pot multicomponent coupling between p-quinone methides, carbon disulfide and amines provided biologically relevant S-secondary alkyl dithiocarbamates (Scheme 1).



Scheme 1: Multicomponent method involving p-Quinone Methides and synthesis of Diarylmethane Dithiocarbamates. (Adapted with permission from Parida et.al. 2022).

A similar coupling between diaryliodonium salts, carbon disulfide, and amines was used to synthesize biologically relevant S-aryl dithiocarbamates efficiently. Diphenyliodonium triflate was reacted with an array of electronically and structurally diverse amines. A range of cyclic aliphatic amines, such as piperidine, pyrrolidine, morpholine, N-Boc-protected piperazine, tetrahydroisoquinoline, and azepane that are diverse with regard to ring size and heteroatoms, participated in the transformation providing the corresponding products in moderate to excellent yields (50%–92% yields) (Scheme 2).



Scheme 2: Multicomponent method for the synthesis of Aryl Dithiocarbamates. (Adapted with permission from Parida et.al. 2021).

4.3.2 Cell culture and reagents

The cell lines were cultured in DMEM medium (Himedia, #AL111) supplemented with 10% FBS (Sigma, #12103C), 2 mM antibiotics (penicillin and streptomycin) solution (Sigma, #P4333), and L-glutamine (Sigma, #G7513) in a humidified incubator with 5% CO₂ at 37 °C.

4.3.3 MTT Assay

Approximately 3000 cells were seeded per well in a 96-well tissue culture plate (Tarsons, #980040) and allowed to settle overnight. The next day, the compound was added at 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 μ M concentrations. Each concentration was performed in triplicates. DMSO was added to three wells as a control, and three wells were left untreated, which acted as a background control. The cells were treated for 72 hours at 37 °C in a 5% CO₂ atmosphere. Then, 10 μ l of MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Invitrogen, #M6494) stock solution prepared in PBS at 5 mg/ml was added to each well in 100 μ l serum-free medium. The plate was incubated at 37 °C in humidified incubator chamber for 4 hours. Acidic isopropanol (100 μ l) was added to each well and mixed thoroughly in a shaker to dissolve the purple formazan crystals. The absorbance was measured using a microplate reader (Biotek Synergy H1) at a wavelength of 570 nm. The results were analyzed in Origin using a dose-response curve on a Windows 10 PC and plotted in Microsoft excel.

4.3.4 Clonogenic Assay

The cells were seeded in 60 mm cell culture dishes at the density of 300 cells per plate in triplicates for each concentration. The next day, DMSO alone (control) and an appropriate concentration of compound (IC_{50} and $2*IC_{50}$ doses) were added at 37 °C for 72 hours. The media was renewed in culture dishes every 2 days till isolated colonies were visible, which took about 2 weeks. The colonies were fixed using acetic acid: methanol (1:7 v/v) solution for 5 minutes. After fixation, colonies were stained with 0.5% crystal violet in 25% (v/v) methanol at room temperature for 2 hours. Excess dye was removed by washing with water, and the culture dish was dried at room temperature. The images were captured using a gel documentation system (Aplegen Omega Lum G). The colonies were counted manually under a light microscope, and only colonies which contained > 50 cells/colony were scored. The graph was plotted in Microsoft excel, which represents the relative number of colonies (%) as compared to the DMSO control.

4.3.5 Cell cycle analyses by DAPI staining

Cells were seeded at 30-40% density on poly D-lysine coated coverslips in a 12-well cell culture plate. The next day, DMSO (control) and compound (IC_{50} and $2*IC_{50}$ doses) were

added to respective wells for 72 hours. The coverslips were fixed in 4% (v/v) paraformaldehyde solution prepared in 1x PBS for 10 minutes at room temperature. This was followed by permeabilization with 0.1% Triton-X solution prepared in 1x PBS for 5 minutes at room temperature. The coverslips were incubated in DAPI solution (0.5 µg/ml) prepared in 1x PBS. The coverslips were mounted on a glass slide with the help of an anti-fade solution (DABCO). The fluorescent images were acquired using the 20X objective lens of the epi- fluorescence microscope (Olympus IX3). The DAPI signal from these images was subsequently quantified by an automated image analysis tool (DAPI integrated intensity) [Roukos et al. 2015] using Cell Profiler v 4.1.31.

4.3.6 Annexin-V/PI-based apoptotic assay

Cells were seeded at 30-40% density on poly D-lysine coated coverslips in a 12-well cell culture plate. The next day, DMSO (control) and compound (IC₅₀ and 2*IC₅₀ doses) were added to respective wells for 72 hours. The coverslip was washed thrice with ice-cold 1x PBS solution. 500 µl of 1x Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 1.5 mM CaCl₂ at pH 7.4) containing 5 µl of 1 mg/ml FITC-Conjugated Annexin-V (Invitrogen, #A13199) and 5 µl of 100 µg/ml propidium iodide, PI (Sigma, #P4170) was added to each well-containing coverslips. The plate was incubated in the dark at room temperature for 15 minutes. Each well was washed with 1x Annexin binding buffer. The coverslips were fixed in 2% (v/v) paraformaldehyde solution prepared in 1x Annexin binding buffer on ice for 10 minutes. The coverslips were washed with 1x PBS and mounted on glass slides using DABCO anti-fade solution. Images were acquired using a 60X objective lens of an epifluorescence microscope. The cells were scored as apoptotic, necrotic, or viable by visualizing Annexin-V staining at the outer membrane and PI in the nucleus using ImageJ v 1.53. The cells were considered viable when a very weak to no Annexin-V signal was visible on the outer membrane, and no PI signal was visible in the nucleus. The cells were considered apoptotic when a strong Annexin-V signal is visible on the outer membrane and PI is not visible in the nucleus. Necrotic cells show a strong PI signal in the nucleus and also a strong Annexin-V signal at the outer membrane.

4.3.7 Cell cycle analyses by flow cytometry

Cells were seeded in 60 mm culture plates at 60-70% confluency. The next day, they were treated with DMSO (control) or compound 1 (IC₅₀ and 2*IC₅₀ doses) for 72 hours. The

cells were collected in a 15 mL tube and fixed with 70% ice-cold ethanol at -20° C. The tube was centrifuged at 2000 rpm for 5 minutes at 4 °C. The cell pellet was washed once with 1x PBS solution and resuspended in 500 µl of 1 mg/ml PI staining solution prepared in water at room temperature in the dark for 30 minutes. The DNA content of the cells was analyzed using a flow cytometer (BD FACS Diva v 8.0.2), and the percentage of cells in different phases of the cell cycle was calculated using ModFit LT v 5.0. The plot was generated in Microsoft excel.

4.3.8 Western Blotting

Cells were seeded at 60-70% confluency in 60 mm plates and then treated with DMSO (control) or compound 1 (IC₅₀ and 2*IC₅₀ doses) for 72 hours at 37 °C. Subsequently, the cell pellet was collected and lysed with 2X laemmli lysis buffer to extract the total protein. Comparable volumes of total protein extract were loaded on 10% SDS-PAGE gels and transferred to a 0.2 µm nitrocellulose membrane (Biorad). The membrane was blocked in 5% BSA in 1x PBST (0.05% tween-20) for 2 hours at room temperature with shaking. This was followed by overnight incubation of membrane at 4°C with the relevant primary antibody against mouse p21 (CST, #2946 T, 1:1,000), rabbit cyclinB1 (CST, #4138S, 1:1,000), mouse phosphorylated-p53 (CST, #9286T, 1:1,000), mouse p53 (CST, #2524S, 1:1,000) and mouse β-actin (Santa-Cruz bio, #SC-47778, 1:2,500). The membrane was washed thrice with 1x PBST for 10 minutes each wash at room temperature under shaking conditions. This was followed by a 1-hour treatment with HRP-conjugated relevant secondary antibody at 1:10,000 [anti-mouse (CST, #7076S) and anti-rabbit (CST, #7074S)] dilution in the blocking solution at room temperature with shaking. The membrane was washed four times with 1x PBS, followed by the last wash with 1x PBS. The blot was developed using Clarity™ Western Enhanced Chemiluminescent Substrate (Bio-Rad Laboratories, Inc., #1705060) and visualized by a gel documentation system.

4.3.9 Microtubule bundling assay

Cells were plated at a confluency of 50-60% on poly-D lysine-coated coverslips and treated with DMSO (control), Paclitaxel (Spectrochem, #011615) at 0.5 µM and compound 1 (0.5 and 1.0 µM concentrations) for 24 hours. The coverslips were washed thrice with 1x PBS and permeabilized using 0.1% Triton-X in 1x PBS for 2 minutes. This was followed by fixation with 4% (v/v) paraformaldehyde solution in 1x PBS for 10 minutes at room

temperature. The fixing solution was removed, and the coverslip was washed using 1x PBS. 1% BSA in PBST was used for blocking the coverslips for 1 hour. Anti- α - tubulin antibody (Sigma, #T6074, 1:200) was added and left overnight at 4°C. The next day, the coverslips were washed with 1x PBS and incubated with anti-mouse IgG TRITC (Sigma, #SAB3701020, 1:500) in the blocking solution for 1 hour. The nuclei were stained by incubating with 0.5 μ g/ml DAPI solution in 1x PBS. The coverslip was mounted on a glass slide using DABCO anti-fade solution. The images were acquired using a 100X objective lens of epifluorescence microscopy. The images were analyzed in ImageJ v 1.53 using plug-in LPX [Higaki 2017] which provides i-skewness of the tubulin fluoresce intensity distribution.

4.3.10 *In vivo* tubulin fractionation assay

HeLa cells were treated with DMSO (1 μ M), Paclitaxel (0.5 μ M) and Compound 1 (1 μ M and 5 μ M) for 48 hours. The cells were washed with 1X PBS and collected by centrifugation at 2,500 rpm for 10 minutes. The cells were lysed in 250 μ l of microtubule stabilization buffer (20 mmol/l Tris-Cl pH 6.8, 0.14 mol/l NaCl, 0.5% NP-40, 1 mmol/l MgCl₂, 2 mmol/l EGTA). The insoluble (pellet) and soluble (supernatant) fractions were separated by centrifugation at 13,000 $\times$ g for 30 minutes at 4°C. The supernatant was mixed with 2X laemmli sample buffer. The pellet with polymerized tubulin was resuspended in water, followed by one freeze/thaw cycle and finally resuspended in 2X laemmli sample buffer. Equal volumes of soluble and insoluble fractions were loaded for western blotting and analyzed using an anti- α -tubulin antibody.

4.3.11 Molecular docking

The Patch dock webserver v 3.4 [Schneidman-Duhovny et al. 2005] was employed to predict the atomic contact energy and interaction interface area of receptor tubulin (PDB ID: 6I2I) with ligands (compound 1 and paclitaxel). The clustering RMSD was set to 2.0 and complex type to the protein-small ligand. The top-scoring model was analyzed for binding of the ligands to the taxol binding pocket and visualized using UCSF Chimera version 1.14. The receptor-ligand interaction map was plotted using LigPlot + v.2.2.4.

4.4 Results and Discussion

4.4.1 Anti-proliferative activity of S-secondary alkyl dithiocarbamates in lung adenocarcinoma cells

Due to the diverse biological activities of dithiocarbamates as well as p-quinone methides and their derivatives, we investigated the anti-proliferative activity of a representative set of synthesized dithiocarbamates on the human lung adenocarcinoma cancer cell line, A549, using the MTT assay (Fig. 4.2).

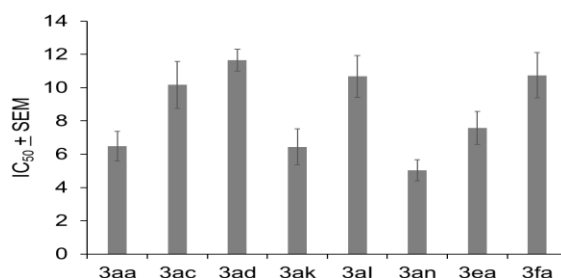


Figure 4.2: Bar graph representing a comparison of the inhibitory activity of the selected compounds. (Adapted with permission from Jaiswal et.al. 2022).

We reported the half-maximal inhibitory concentration (IC_{50}) value of all the tested compounds (Table 4.1). Notably, all of the tested compounds demonstrated promising anti-cancer activity, and in the case of compounds 3 aa, 3 ak, 3 an and 3 ea, an IC_{50} value less than 10 μ M was observed. Importantly, all these four compounds demonstrated anti-proliferative activity comparable to the reported activity of anti-cancer drugs Doxorubicin and Etoposide in the A549 cell line [Kanintronkul et al. 2011; Kashkin et al. 2010]. These findings explicitly underline the importance of the developed method, enabling straightforward synthesis of novel S-diaryl methane dithiocarbamates that could serve as a promising scaffold for designing effective anti-cancer drugs.

Table 4.1: Selected examples of identified antiproliferative drugs. SEM: Standard error of the mean, R^2 : Coefficient of determination. (Adapted with permission from Parida et.al. 2022).

Entry	Compound	$IC_{50} \pm SEM (\mu M)$	R^2
1	3aa	6.48 ± 0.89	0.97
2	3ac	10.17 ± 1.41	0.97
3	3ad	11.65 ± 0.67	0.94
4	3ak	6.44 ± 1.08	0.94
5	3al	10.68 ± 1.26	0.88
6	3an	5.03 ± 0.64	0.98
7	3ea	7.58 ± 1.00	0.97
8	3fa	10.75 ± 1.36	0.92

4.4.2 Antiproliferative activity of S-aryl dithiocarbamates satisfy drug-likeness filters and ADMET analysis

A transition-metal-free one-pot three-component annulation between diaryliodonium triflates, cyclic and acyclic aliphatic amines, and carbon disulfide providing convenient and efficient access to biologically relevant S-aryl dithiocarbamates was developed in collaboration [Parida et al. 2021]. The *in silico* analysis revealed that the majority of the compounds had drug-likeness and lead-like pharmacokinetic properties. The compounds were found to be in good compliance (75%–100%) with different drug-likeness filters (Lipinski, Ghose, Veber, Egan, and Muegge) (Fig. 4.3 and Tables 4.2-4.4).

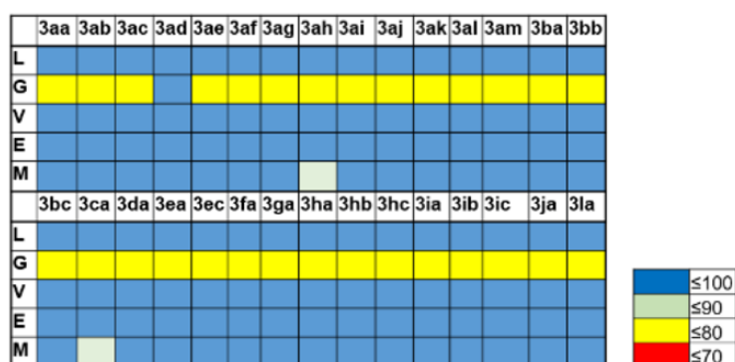


Figure 4. 3: *In silico* analysis of drug-likeness prediction . Color map indicating percentage compliance of each drug for L=Lipinski, G=Ghose, V=Veber, E=Egan, and M=Muegge drug-likeness filters .(Adapted with permission from Parida et.al. 2021).

The compliance percentage for different drug-likeness filters Lipinski's (Pfizer), Ghose (Amgen), Veber (GSK), Egan (pharmacia) and Muegge (Bayer) was calculated (Table 4.2).

Table 4.2: Drug-likeness percentage compliance for Lipinski (Pfizer), Ghose (Amgen), Veber (GSK), Egan (pharmacia) and Muegge (Bayer) filters. (Adapted with permission from Parida et.al. 2021).

Compound Name	Lipinski (Pfizer)	Ghose	Veber (GSK)	Egan (pharmacia)	Muegge (Bayer)
3aa	100	75	100	100	100
3ab	100	75	100	100	100
3ac	100	75	100	100	100
3ad	100	100	100	100	100
3ae	100	75	100	100	100
3af	100	75	100	100	100
3ag	100	75	100	100	100
3ah	100	75	100	100	89
3ai	100	75	100	100	100
3aj	100	75	100	100	100
3ak	100	75	100	100	100
3al	100	75	100	100	100
3am	100	75	100	100	100
3ba	100	75	100	100	100
3bb	100	75	100	100	100
3bc	100	75	100	100	100
3ca	100	75	100	100	89
3da	100	75	100	100	100
3ea	100	75	100	100	100
3ec	100	75	100	100	100
3fa	100	75	100	100	100
3ga	100	75	100	100	100
3ha	100	75	100	100	100
3hb	100	75	100	100	100
3hc	100	75	100	100	100
3ia	100	75	100	100	100
3ib	100	75	100	100	100
3ic	100	75	100	100	100
3ja	100	75	100	100	100
3la	100	75	100	100	100

The SMILES notation of the compounds (Table 4.3) (was utilized to extract physiochemical properties from the SwissADME and the admetSAR platforms (Table 4.4).

Table 4.3: SMILES notation for the listed compounds. (Adapted with permission from Parida et.al. 2021).

Compound Name	SMILES
3aa	<chem>S=C(N1CCCCC1)Sc1ccccc1</chem>
3ab	<chem>S=C(N1CCCCC1)Sc1ccccc1</chem>
3ac	<chem>S=C(N1CCOCC1)Sc1ccccc1</chem>
3ad	<chem>S=C(N1CCN(CC1)C(=O)OC(C)(C)C)Sc1ccccc1</chem>
3ae	<chem>S=C(N1CCc2c(C1)cccc2)Sc1ccccc1</chem>
3af	<chem>S=C(N1CCCCC1)Sc1ccccc1</chem>
3ag	<chem>CCN(C(=S)Sc1ccccc1)CC</chem>
3ah	<chem>CCCCN(C(=S)Sc1ccccc1)CCCC</chem>
3ai	<chem>CN(C(=S)Sc1ccccc1)Cc1ccccc1</chem>
3aj	<chem>S=C(Sc1ccccc1)NCc1ccccc1</chem>
3ak	<chem>C=CCNC(=S)Sc1ccccc1</chem>
3al	<chem>S=C(N[C@H](c1ccccc1)C)Sc1ccccc1</chem>
3am	<chem>COC(=O)C1CCCN1C(=S)Sc1ccccc1</chem>
3ba	<chem>S=C(N1CCCCC1)Sc1ccc(cc1)C</chem>
3bb	<chem>S=C(N1CCCCC1)Sc1ccc(cc1)C</chem>
3bc	<chem>S=C(N1CCOCC1)Sc1ccc(cc1)C</chem>
3ca	<chem>S=C(N1CCCCC1)Sc1ccc(cc1)C(C)(C)C</chem>
3da	<chem>S=C(N1CCCCC1)Sc1cc(C)ccc1C</chem>
3ea	<chem>S=C(N1CCCCC1)Sc1c(C)cc(cc1C)C</chem>
3ec	<chem>S=C(N1CCOCC1)Sc1c(C)cc(cc1C)C</chem>
3fa	<chem>COc1ccc(cc1)SC(=S)N1CCCCC1</chem>
3ga	<chem>S=C(N1CCCCC1)Sc1ccc(cc1)F</chem>
3ha	<chem>S=C(N1CCCCC1)Sc1ccc(cc1)Cl</chem>
3hb	<chem>S=C(N1CCCCC1)Sc1ccc(cc1)Cl</chem>
3hc	<chem>S=C(N1CCOCC1)Sc1ccc(cc1)Cl</chem>
3ia	<chem>S=C(N1CCCCC1)Sc1ccc(cc1)Br</chem>
3ib	<chem>S=C(N1CCCCC1)Sc1ccc(cc1)Br</chem>
3ic	<chem>S=C(N1CCOCC1)Sc1ccc(cc1)Br</chem>
3ja	<chem>Clc1ccccc1SC(=S)N1CCCCC1</chem>
3la	<chem>S=C(N1CCCCC1)Sc1ccc(cc1)N(=O)=O</chem>

Table 4.4: Physiochemical properties of the compounds from SwissADME and admetSAR . (Adapted with permission from Parida et.al. 2021).

Compound Name	MW (g/mol)	nAtoms	nRings	nCarbon	nHetero atoms	Rotatable Bonds	H-Bond Acceptor (HBA)	H-Bond Donor (HBD)	Molecular refractivity (MR)	TPSA (Å sq.)	XlogP	WlogP	MLogP
3aa	237.38	15	2	12	3	3	2	0	74.69	60.63	3.67	3.17	2.85
3ab	223.36	14	2	11	3	3	2	0	69.88	60.63	3.31	2.78	2.57
3ac	239.36	15	2	11	4	3	3	0	70.96	69.86	2.45	2.01	1.64
3ad	338.49	22	2	16	6	6	4	0	102.44	90.17	3.48	2.85	2.36
3ae	285.43	19	3	16	3	3	2	0	89.72	60.63	4.35	3.59	3.64
3af	251.41	16	2	13	3	3	2	0	79.49	60.63	4.03	3.56	3.12
3ag	225.37	14	1	11	3	5	2	0	68.08	60.63	3.56	3.41	2.96
3ah	281.48	18	1	15	3	9	2	0	87.31	60.63	5.33	4.97	4.03
3ai	273.42	18	2	15	3	5	2	0	82.95	60.63	4.32	4.04	3.78
3aj	259.39	17	2	14	3	5	2	1	78.05	69.42	4.14	3.7	3.53
3ak	209.33	13	1	10	3	5	2	1	62.7	69.42	3.29	2.84	2.58
3al	273.42	18	2	15	3	5	2	1	82.86	69.42	4.54	4.09	3.78
3am	281.39	18	2	13	5	5	4	0	80.78	86.93	3.27	2.32	2.04
3ba	251.41	16	2	13	3	3	2	0	79.65	60.63	4.03	3.48	3.12
3bb	237.38	15	2	12	3	3	2	0	74.84	60.63	3.68	3.09	2.85
3bc	253.38	16	2	12	4	3	3	0	75.93	69.86	2.82	2.32	1.92
3ca	293.49	19	2	16	3	4	2	0	93.96	60.63	5.34	4.47	3.89
3da	265.44	17	2	14	3	3	2	0	84.62	60.63	4.4	3.79	3.38
3ea	279.46	18	2	15	3	3	2	0	89.58	60.63	4.76	4.09	3.64
3ec	281.44	18	2	14	4	3	3	0	85.86	69.86	3.55	2.94	2.46
3fa	267.41	17	2	13	4	4	3	0	81.18	69.86	3.64	3.18	2.46
3ga	255.37	16	2	12	4	3	2	0	74.64	60.63	3.77	3.73	3.25
3ha	271.83	16	2	12	4	3	2	0	79.7	60.63	4.3	3.82	3.39
3hb	257.8	15	2	11	4	3	2	0	74.89	60.63	3.94	3.43	3.12
3hc	273.8	16	2	11	5	3	3	0	75.97	69.86	3.08	2.67	2.19
3ia	316.28	16	2	12	4	3	2	0	82.39	60.63	4.36	3.93	3.52
3ib	302.25	15	2	11	4	3	2	0	77.58	60.63	4	3.54	3.25
3ic	318.25	16	2	11	5	3	3	0	78.66	69.86	3.14	2.78	2.33
3ja	271.83	16	2	12	4	3	2	0	79.7	60.63	4.3	3.82	3.39
3la	282.38	18	2	12	6	4	4	0	83.51	106.45	3.5	3.6	2.39

All compounds showed a good range of average ADMET score (0.76-0.84) with regards to human intestinal absorption, blood-brain barrier penetration, Caco-2 permeability, Ames mutagenicity, carcinogenicity, and acute oral toxicity class (Table 4.5).

Table 4.5: The admetSAR tool provides pharmacokinetic properties of compounds under the different criteria of absorption, distribution, metabolism, excretion, and toxicity. Accordingly, the predicted values for human intestinal absorption, blood-brain barrier penetration, Caco-2 permeability, Ames mutagenicity, carcinogenicity, and acute oral toxicity class were used to calculate the average ADMET-score of the synthesized compounds. (Adapted with permission from Parida et.al. 2021).

Compound Name	Human intestinal absorption	Caco-2 Permeability	Blood Brain Barrier	Carcinogenicity	Ames Mutagenesis	Acute Oral Toxicity	Average score	Standard deviation
3aa	0.9771	0.8392	0.9918	0.8714	0.8	0.5134	0.83215	0.1736226
3ab	0.9771	0.9071	0.99	0.8714	0.78	0.4341	0.826616667	0.206913
3ac	0.9896	0.8905	0.9877	0.6571	0.75	0.4955	0.795066667	0.1974155
3ad	0.9917	0.8371	0.9803	0.6571	0.78	0.5918	0.806333333	0.164049
3ae	0.9858	0.8564	0.9938	0.9571	0.75	0.5482	0.84855	0.174185
3af	0.9771	0.7689	0.988	0.8714	0.8	0.5206	0.821	0.172158
3ag	0.994	0.9524	0.9907	0.6571	0.59	0.6613	0.807583333	0.1900744
3ah	0.9896	0.9337	0.9896	0.5286	0.74	0.5886	0.795016667	0.2057041
3ai	0.9851	0.8629	0.9901	0.5571	0.72	0.6633	0.796416667	0.17797
3aj	0.9792	0.8601	0.9902	0.5429	0.52	0.6952	0.7646	0.2096975
3ak	0.9805	0.8853	0.9892	0.5571	0.73	0.6487	0.798466667	0.1802393
3al	0.9925	0.8153	0.99	0.6429	0.79	0.8449	0.845933333	0.1323084
3am	0.9803	0.9078	0.9769	0.7857	0.76	0.4743	0.814166667	0.190842
3ba	0.9903	0.8409	0.9921	0.7286	0.71	0.6048	0.811116667	0.1583343
3bb	0.9903	0.8645	0.9904	0.7286	0.68	0.5146	0.794733333	0.1883673
3bc	0.9949	0.8617	0.9875	0.6571	0.68	0.4473	0.771416667	0.215065
3ca	0.991	0.8697	0.9895	0.6429	0.82	0.7113	0.8374	0.1426919
3da	0.9903	0.8485	0.9911	0.7286	0.69	0.6449	0.815566667	0.1515821
3ea	0.9903	0.7256	0.9911	0.7286	0.73	0.6739	0.806583333	0.1441581
3ec	0.9949	0.7488	0.9875	0.6571	0.71	0.5965	0.782466667	0.1696183
3fa	0.988	0.8748	0.9833	0.8857	0.72	0.5819	0.83895	0.1592152
3ga	0.9803	0.8244	0.9907	0.8714	0.58	0.5607	0.80125	0.1898542
3ha	0.9731	0.8162	0.9905	0.8429	0.83	0.586	0.839783333	0.1452972
3hb	0.9731	0.8707	0.9865	0.8429	0.83	0.5181	0.836883333	0.1695819
3hc	0.9884	0.8527	0.9869	0.6429	0.77	0.4617	0.783766667	0.2057424
3ia	0.971	0.8295	0.9911	0.8714	0.77	0.5411	0.829016667	0.1640767
3ib	0.917	0.8614	0.9874	0.8714	0.75	0.4736	0.810133333	0.1822616
3ic	0.985	0.8398	0.9876	0.6857	0.76	0.4455	0.783933333	0.2048359
3ja	0.9731	0.7906	0.9905	0.8429	0.85	0.586	0.83885	0.1465761
3la	0.959	0.7064	0.9786	0.8	0.81	0.5776	0.805266667	0.1518784

The *in silico* study helped us to categorize the synthesized S-aryl dithiocarbamates into best (100% compliance with all the five filters), good (<100% compliance in one of the five filters), and average compounds (<100% compliance in two of the five filters) based on their ability to satisfy the five drug-likeness filters [Muegge et al. 2001; Veber et al. 2002; Ghose et al. 1999; Egan et al. 2000; Lipinski et al. 1997]. To validate their biological relevance as an antiproliferative compound, five representative compounds were selected from each of the aforementioned categories, namely, compound 1 (best category), compounds 2, and, 3 (good category), and, compounds 4, and, 5 (average category). The human lung adenocarcinoma cells A549 were treated with varying concentrations of respective compounds for 72 hours, which was followed by a colorimetric cell viability-based MTT assay. The drug-response curve of the assay was used to calculate the IC₅₀ value of these compounds. As shown in (Fig. 4.4), compound 1 having an IC₅₀ value of 0.447 ± 0.051 μM emerged as the most potent

compound as compared to compounds 2 ($IC_{50} = 7.931 \pm 0.577 \mu M$), 3 ($IC_{50} = 2.518 \pm 0.038 \mu M$), 4 ($IC_{50} = 9.131 \pm 0.107 \mu M$) and 5 ($IC_{50} = 9.756 \pm 0.156 \mu M$).

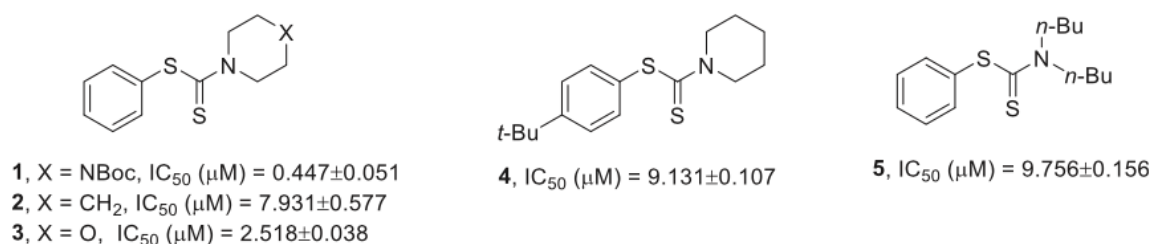


Figure 4.4: Validation of potency of representative S-aryl dithiocarbamates in the MTT assay. The IC_{50} was calculated from at least two independent experimental measurements. The values are reported as the average $\pm$ Standard Error of the Mean. (Adapted with permission from Jaiswal et.al. 2022).

Compound 1 (3ad) had one of the best drug-likeness properties in the *in silico* analysis and also proved to be the most potent compound in the cell viability-based MTT assay. Furthermore, N-Boc protected piperazine moiety (compound 1, $IC_{50} = 0.447 \pm 0.051 \mu M$) on aryl dithiocarbamate was essential to the observed high cytotoxicity, as replacing the same with either piperidine (compound 2, $IC_{50} = 7.931 \pm 0.577 \mu M$) or morpholine (compound 3, $IC_{50} = 2.518 \pm 0.038 \mu M$) plummeted the potency. Hence, the compound 1 chemical scaffold was further used for structure-activity relationship studies.

4.4.3 Structure-activity relationship of the selected S-aryl dithiocarbamate moiety

Since the N-Boc-protected piperazine moiety was crucial to the observed high antiproliferative potency, the substitution pattern on the aryl ring was varied to construct a library of electronically and structurally diverse S-aryl dithiocarbamates. The targeted dithiocarbamates (compound 1, 20–31 in Table 4.6) were prepared through a one-pot, metal-free, three-component coupling between diaryliodonium triflates, carbon disulfide, and N-Boc protected piperazine to furnish the corresponding products in moderate to good yields [Parida et al. 2021] (Fig. 4.5).

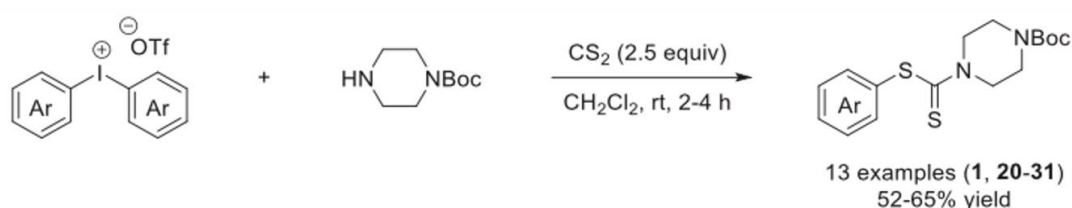
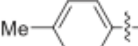
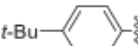
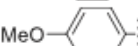
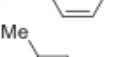
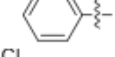
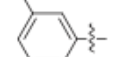
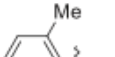
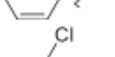
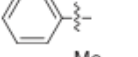


Figure 4.5: Synthesis of S-aryl dithiocarbamates (1, 20–31). (Adapted with permission from Jaiswal et.al. 2022).

The antiproliferative activity of synthesized analogues was evaluated against lung cancer cell line A549 using an MTT assay (Table 4.6).

Table 4.6: Chemical structures of analogues 1, 20–31 and their antiproliferative activities in the MTT assay . (Adapted with permission from Jaiswal et.al. 2022).

entry	compd.	Ar	IC ₅₀ ± SEM (μM)	R ²
1	1		0.447 ± 0.051	0.92
2	20		2.105 ± 0.53	0.879
3	21		0.769 ± 0.165	0.92
4	22		0.712 ± 0.192	0.856
5	23		1.557 ± 0.376	0.851
6	24		1.066 ± 0.247	0.927
7	25		1.815 ± 0.326	0.938
8	26		0.914 ± 0.011	0.893
9	27		0.634 ± 0.128	0.952
10	28		2.269 ± 0.076	0.917
11	29		1.556 ± 0.076	0.885
12	30		0.539 ± 0.058	0.927
13	31		1.455 ± 0.327	0.907

Our results demonstrated that introducing a methyl group (compound 20) at the para-position of the phenyl ring led to a five-fold decrease in their potency (entry 2 vs entry 1). The introduction of other electron-rich functionalities, such as tertiary-butyl (compound 21), and methoxy (compound 22), restored the potency to some extent but remained less potent than the parent compound 1 (entries 3–4). Electron-deficient functional groups like fluorine (compound 23), chlorine (compound 24), and bromine (compound 25) at the para-position imparted similar levels of efficacy and showed marginal effects as compared to electron- rich substituted analogues (entries 5–7). A comparison of the IC₅₀ value of these compounds (entries 2–7) revealed that the electronic variation did not have a profound

effect on the potency of these compounds. Since these electronic variations were all at the para-position, it was interesting to test the role of positional variation on the inhibitory activity of the parent scaffold. Installation of electron-rich (Methyl, compound 26) and electron-withdrawing (Chloro, compound 27) functionality at the meta-position had a slightly beneficial effect as compared to the corresponding para-congeners and IC_{50} values of $0.914 \pm 0.011 \mu\text{M}$ and $0.634 \pm 0.128 \mu\text{M}$ were obtained, respectively (entries 8–9). On the other hand, placing either the methyl (compound 28, $IC_{50} = 2.269 \pm 0.076 \mu\text{M}$) or chloro (compound 29, $IC_{50} = 1.556 \pm 0.076 \mu\text{M}$) group at the ortho-position furnished inferior results in comparison to para- and meta-analogs (entries 10–11). Furthermore, di-methyl (compound 30, $IC_{50} = 0.539 \pm 0.058 \mu\text{M}$) and tri-methyl (compound 31, $IC_{50} = 1.455 \pm 0.327 \mu\text{M}$) substitution pattern on the phenyl ring also failed to improve the potency (entries 12–13). Overall, the SAR studies indicated that although electronic variation had marginal effects on the potency of the compounds, the positional variation had a more pronounced effect. The meta-substitution pattern was most potent with ortho-variation being the least preferred. Nevertheless, the unsubstituted parent dithiocarbamate (compound 1) remained the most optimal and potent compound, hence was taken forward to further gain mechanistic insights into its antiproliferative effect in cancer cells.

4.4.4 Antiproliferative activity of compound 1 on different cancer types

To compare the anticancer activity of compound 1, four different human cancer cell lines, i.e., A549 (lung cancer), MCF7 (breast cancer), MDA-MB-231 (triple-negative breast cancer), and HeLa (cervical cancer) were used. As shown (Fig 4.6 a and b), compound 1 had comparable antiproliferative activity in lung and cervical cancer cell lines with IC_{50} of $0.447 \pm 0.051 \mu\text{M}$ and $0.432 \pm 0.138 \mu\text{M}$, respectively.

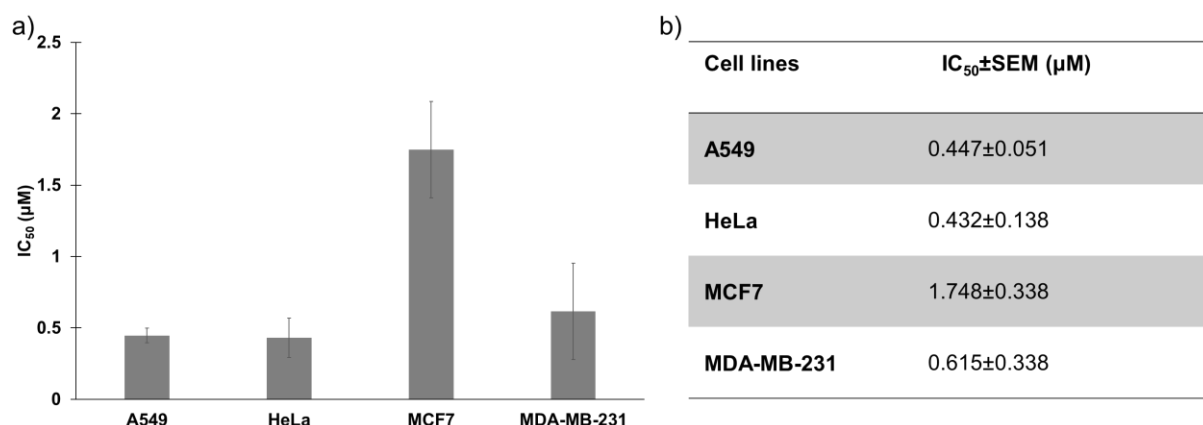


Figure 4. 6: a) Bar graph comparing IC₅₀ value of compound 1 in different cancer cell lines, A549, HeLa, MCF7, and MDA-MB-231. Error bars represent the standard error of the mean (SEM). b) The table provides the average IC₅₀ ± SEM. (Adapted with permission from Jaiswal et.al. 2022).

However, it demonstrated relatively less potency in breast cancer cell lines, MDA-MB-231 (IC₅₀ = 0.615 ± 0.338 μM) and MCF7 (IC₅₀ = 1.748 ± 0.338 μM). This shows that the compound has varying potency depending on the cancer type. HeLa cells were used for further analysis as compound 1 showed the least IC₅₀ value in the cell line. We corroborated the findings by performing the clonogenic assay in HeLa cells, which provides an overall readout of the cell survival and their ability to reproduce to form colonies. Compound 1 was able to reduce the number of colonies in a dose-dependent manner (Fig. 4.7 a and b).

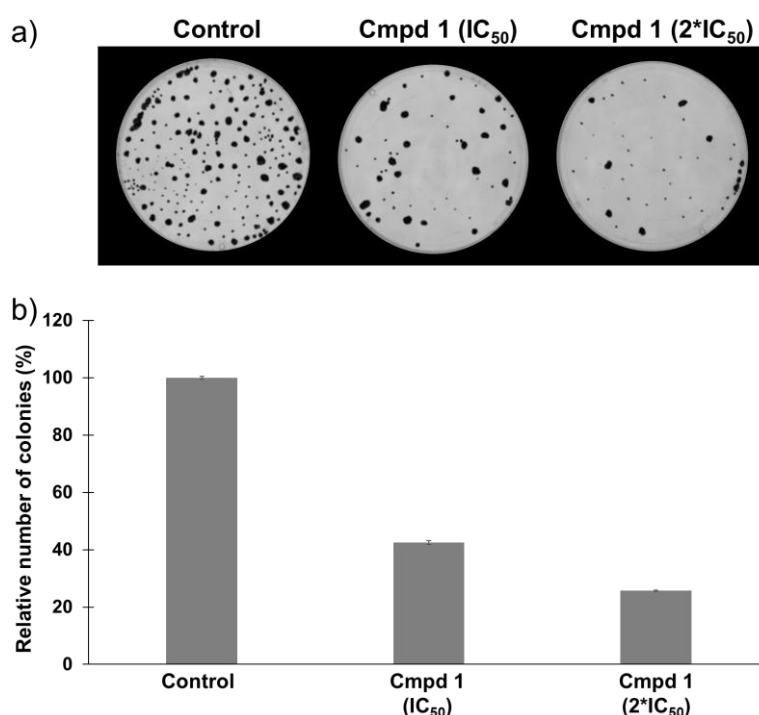


Figure 4.7: Clonogenic Assay of compound 1. a) Representative image of the clonogenic assay in HeLa cells treated with DMSO (control), compound (Cmpd) 1 at IC₅₀ (0.432 μM) & 2*IC₅₀ (0.864 μM) doses. b) Bar graph showing the relative number of colonies (%) formed by HeLa cells after treatment with compound 1 at the IC₅₀ and 2*IC₅₀ doses compared to the control. (Adapted with permission from Jaiswal et.al. 2022).

4.4.5 Compound 1 induces the p53-dependent G₂/M cell cycle arrest and apoptosis in cervical cancer cells

Cell viability is a function of the rate of cell division and cell death. For initial analysis DAPI stained nucleus of cells in fluorescence microscopy images were quantified to score for different cell cycle stages (G₁, S, and G₂/M phases) [Roukos et al. 2015]. We observed in DMSO (control) treated cells that the majority of cell populations were in the G₁ phase however, treatment with compound 1 (IC₅₀ and 2*IC₅₀ doses) resulted in a significant shift of cell cycle profile towards the G₂/M phase (Fig. 4.8 a and b).

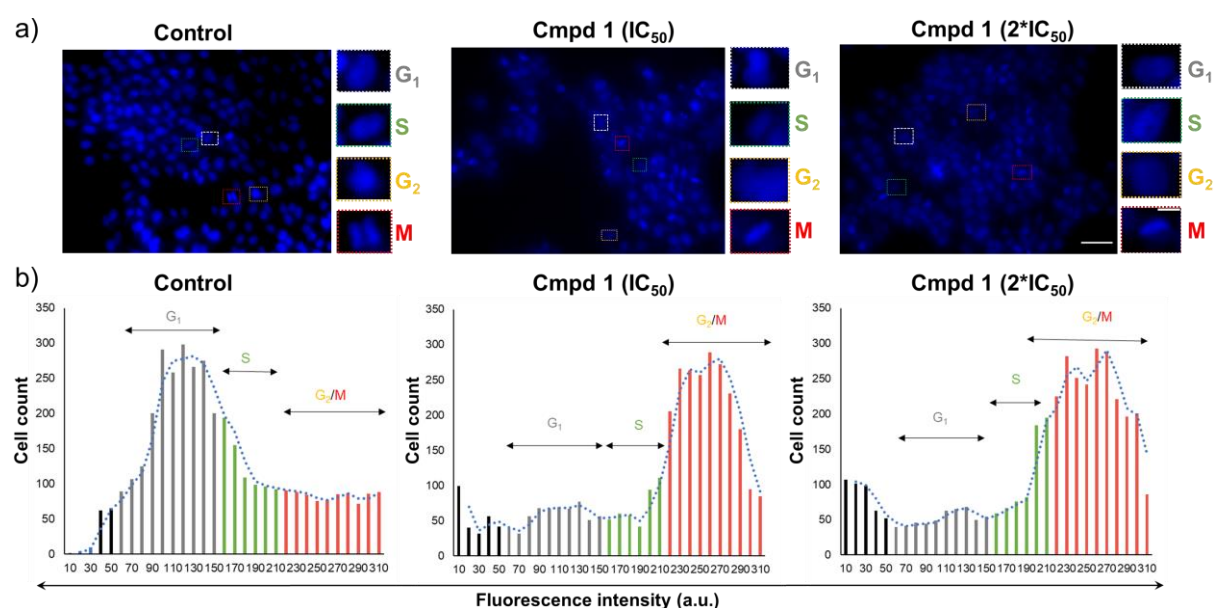


Figure 4.8: Compound 1 exhibits cell cycle arrest as shown by Fluorescence Microscopy. a) Fluorescence images representing DAPI stained nucleus in HeLa cells treated with DMSO (control), and compound (Cmpd) 1 at IC₅₀ (0.432 μ M) and 2*IC₅₀ (0.864 μ M) doses. The scale bar is 50 μ m. The small adjacent box shows a magnified view of a representative DAPI-stained nucleus categorized in the indicated cell cycle stages. The scale bar is 10 μ m. b) Bar graph representing cell count vs. DAPI fluorescence intensity (a.u.: arbitrary units) in the three stated conditions. The DAPI fluorescence intensity was used to categorize cell populations in different cell cycle stages, i.e., G₁ (grey bars), S (green bars), and G₂/M phases (orange-red bars). (Adapted with permission from Jaiswal et.al. 2022).

Next, the above observation was verified by performing flow cytometry analysis to check the population of cells in different stages of the cell cycle. The flow cytometry analysis (Fig. 4.9 a and b) showed a ~ 4-5-fold increase in the percentage of G₂/M phase cells and a 4-fold increase in the apoptotic cell population after compound 1 treatment when compared to the DMSO control.

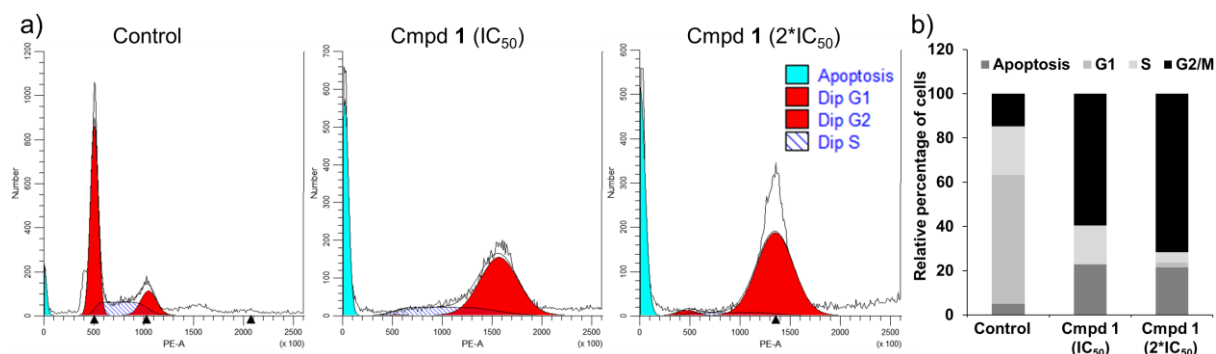


Figure 4.9: Flow cytometric analysis of compound 1. a) Cell cycle profile of the HeLa cells treated with the DMSO (control) and compound (Cmpd) 1 at IC₅₀ (0.432 μ M) and 2*IC₅₀ (0.864 μ M) doses. b) Bar graph representing the relative percentage of cell population in the different cell cycle (G2/M, S, and G1) stages and apoptotic, as analyzed by the flow cytometry in (a). (Adapted with permission from Jaiswal et.al. 2022).

The prolonged cell cycle arrest could induce apoptosis [Orth et al. 2012]. Thus, we analyzed FITC-conjugated Annexin-V, a marker for apoptosis that stains the membrane of apoptotic cells and propidium iodide, which stains nuclei of dead cells in fluorescence microscopy to score the population of apoptotic cells under different conditions. The data (Fig. 4.10 and b) confirmed ~ 3-4-fold increase in the apoptotic/necrotic cell percentage after treatment with compound 1 as compared to the DMSO control.

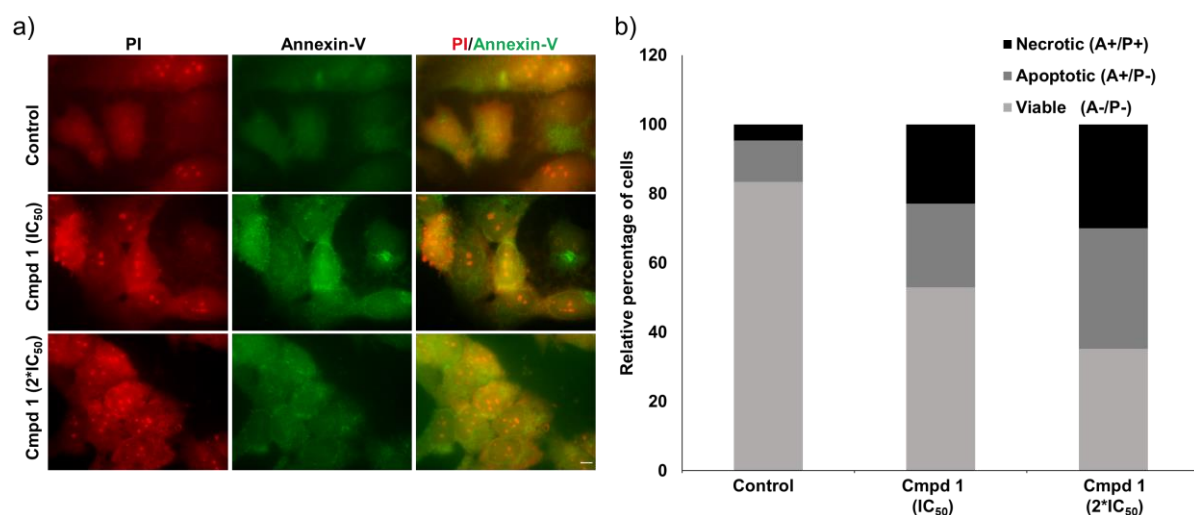


Figure 4.10: Compound 1 causes an increase in an apoptotic cell population. a) Representative image of HeLa cells after treatment with the three stated conditions showing propidium iodide (PI) and FITC-conjugated Annexin-V fluorescence signal. The scale bar is 10 μ m. b) Bar graph representing the relative percentage of viable, apoptotic, and necrotic cell populations as quantified by scoring for the presence (+) or the absence (-) of AnnexinV (A) and propidium iodide (P) fluorescence signal. (Adapted with permission from Jaiswal et.al. 2022).

In order to further confirm that compound 1 indeed causes cell cycle arrest in the G₂/M stage, we analyzed the protein levels of G₂/M specific cyclin B1 [Gavet and Pines 2010]. The western blot analysis (Fig. 4.11) confirmed that the cyclin B1 levels were increasing in a dose-dependent manner in the compound 1 treated cells as compared to the DMSO control.

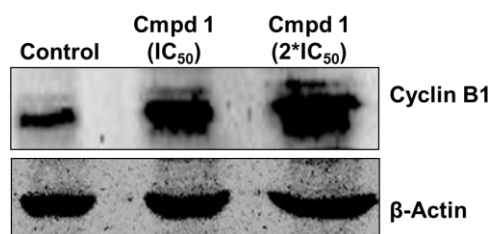


Figure 4.11: Western blot showing the Cyclin B1 levels in HeLa cells treated with the three stated conditions. β-actin was used as a loading control. (Adapted with permission from Jaiswal et.al. 2022).

It is known that the G₂/M arrest can be induced by the p53-p21 signaling pathway [Chen 2016]. We could also show an increase in the protein levels of p53 protein levels, which is in its active phosphorylated form (Fig. 4.12 a and b) as well as the p53 downstream regulator p21 was also elevated in compound 1 treated cells as compared to DMSO control cells.

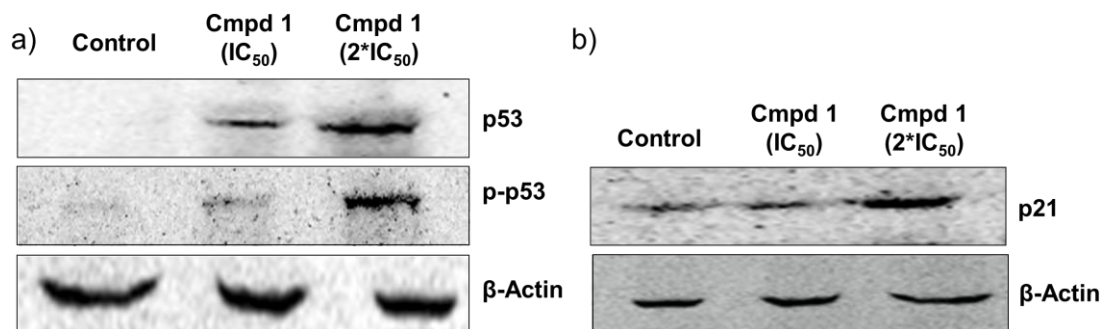


Figure 4.12: Western blot showing the p53 and p21 levels in HeLa cells. a) Western blot showing levels of p53, phosphorylated p53 (p-p53), and b) p21 proteins in the HeLa cells treated with the three stated conditions. β-actin was used as a loading control. (Adapted with permission from Jaiswal et.al. 2022).

4.4.6 Compound 1 induces microtubule bundling and docks at the taxol-binding pocket of the β-tubulin protein.

Microtubule dynamics play an important role during cell division. Compounds that induce stabilization or destabilization of MTs are known to induce G₂/M phase cell cycle

arrest [Jordan and Wilson 2004]. Fluorescence microscopy was used to analyse MT organization in the HeLa cells. We observed MT bundling in compound 1 treated cells (Fig. 4.13 a and b). Since, MT bundling is a characteristic phenotype of drugs that induce MT stabilization, for example, paclitaxel, we compared the compound 1 phenotype with cells treated with paclitaxel. The quantification of tubulin fluorescence signal showed average i-skewness, which is an indicator of tubulin fluorescence signal distribution in the cell. The result confirmed that compound 1 exhibits a similar phenotype as paclitaxel.

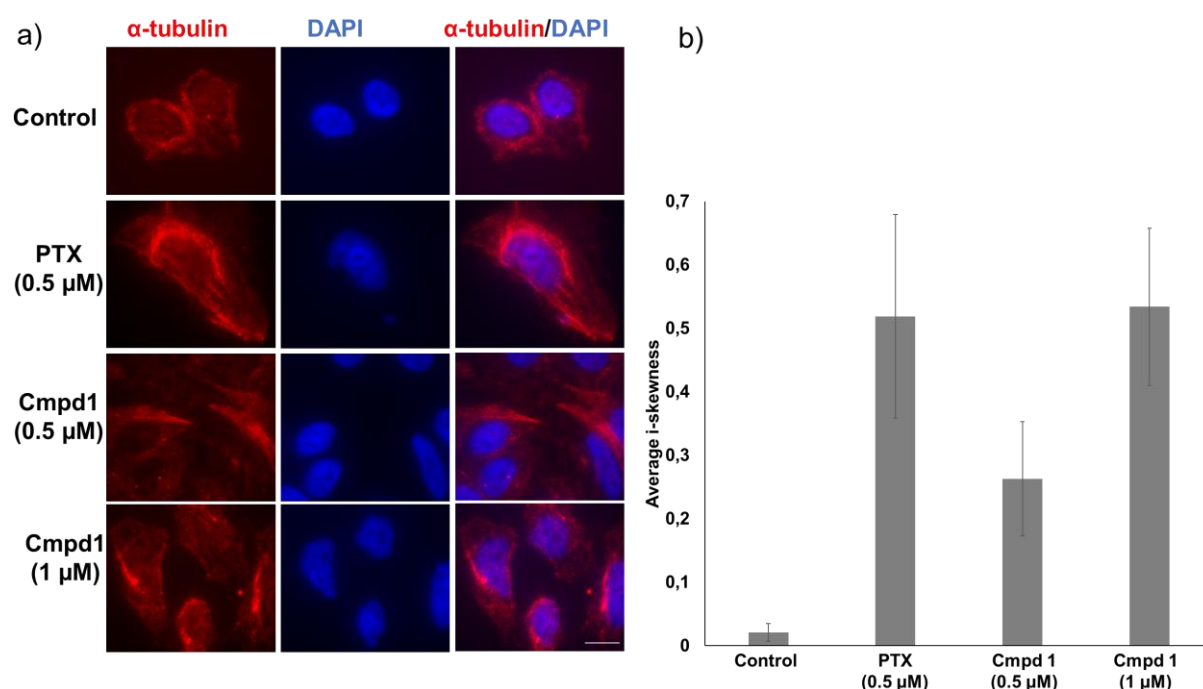


Figure 4.13: Compound 1 exhibits tubulin bundling phenotype. a) Fluorescence images representing microtubule bundling in HeLa cells treated with paclitaxel (0.5 μ M) and dose-dependent effect of compound 1 (cmpd 1) at 0.5 μ M and 1 μ M concentrations. The scale bar is 10 μ m. b) Bar graph representing average i-skewness of tubulin signal in the experiment (a) for the stated conditions. The error bars represent the standard deviation. (Adapted with permission from Jaiswal et.al. 2022).

Paclitaxel is known to enhance the pool of polymerized MTs visible in the cell pellet as compared to free tubulin in the supernatant of the *in vivo* cellular fractions [Giannakakou et al. 1997]. The effect of compound 1 on tubulin pools in pellet and supernatant was analyzed and compared using the same assay. It was observed that the compound 1 treated cells, just like paclitaxel showed an enhanced level of tubulin in the cell pellet as compared to the supernatant (Fig. 4.14). The percentage of polymerized tubulin in compound 1 treated cells was found to increase in a concentration-dependent manner.

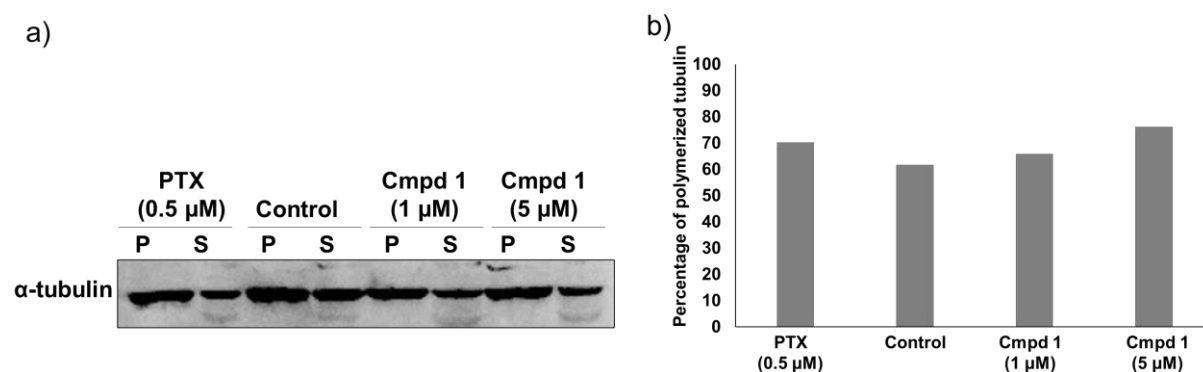


Figure 4.14: Microtubule fractionation assay. a) Western blot showing α -tubulin signal in the supernatant (S) and pellet (P) fractions of cells treated with paclitaxel (PTX), control (DMSO), compound 1 (Cmpd 1) at indicated concentrations for 48 hrs. c) The box plot representing the percentage of polymerized tubulin calculated from the quantitative measurement of tubulin signal in pellet divided by total tubulin signal (pellet + soluble) for indicated conditions in experiment (a). (Adapted with permission from Jaiswal et.al. 2022).

Additionally, paclitaxel is known to cause its effect on MTs by binding to the β -tubulin protein. This intrigued us to explore the binding affinity of compound 1 to the same protein pocket. Molecular docking (blind mode) of compound 1 with α/β -tubulin structure (PDB ID: 6I2I) revealed it binds to the same pocket as paclitaxel (Fig. 4.15). The atomic contact energy (ACE) score, which is considered an estimate of the change in desolvation energy of the protein and ligand going from the unbound to complex formation state for compound 1 was -213.21 kcal/mol, which was close to the ACE score for paclitaxel (-224.54 kcal/mol). Close analysis of the interface area of paclitaxel in the β -tubulin pocket validated previously reported [Kellogg et al. 2017], interactions, where paclitaxel forms extensive van-der waals interactions between its 3'-benzoyl group and N-acyl group with Histidine 229 of β -tubulin.

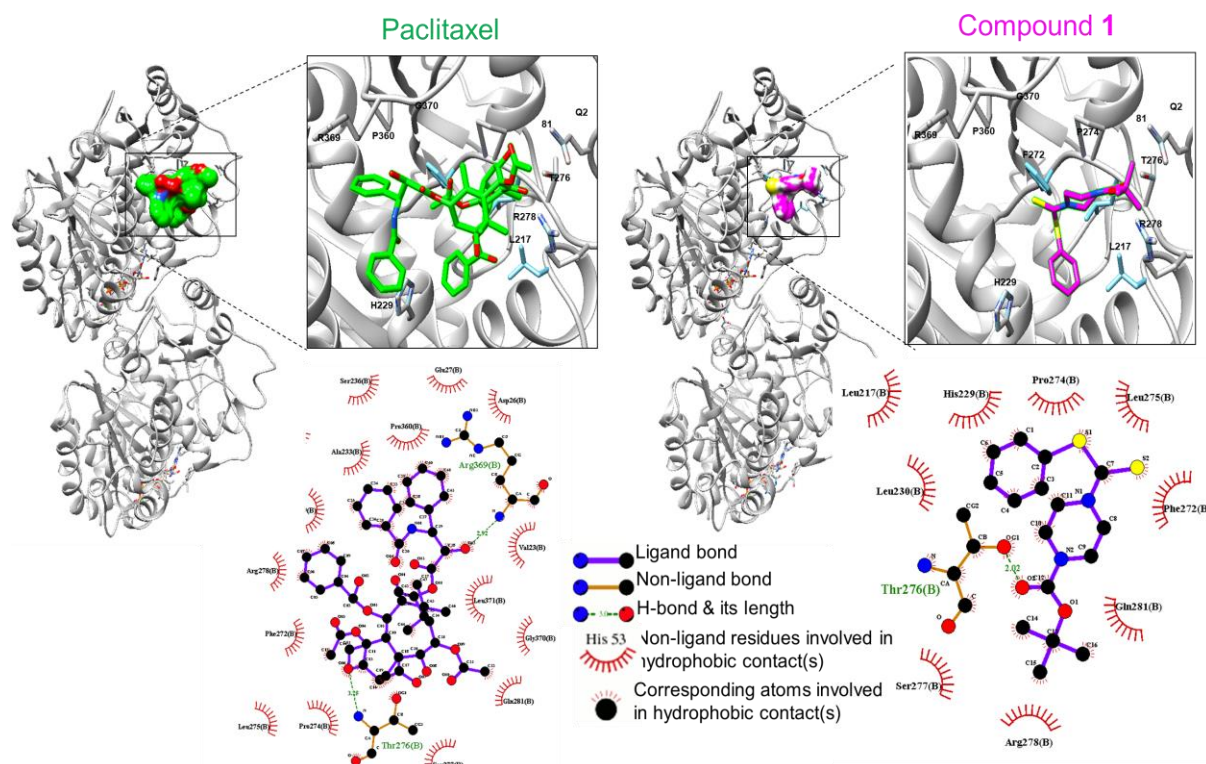


Figure 4.15: Comparing molecular docking of paclitaxel and compound 1 in the taxol-binding pocket of β -tubulin (in the highlighted boxes). The lower panel compares the interaction map of residues surrounding paclitaxel and compound 1. (Adapted with permission from Jaiswal et.al. 2022).

The 2'-OH group of paclitaxel forms a hydrogen bond (2.92 Å) with the carbonyl of Arginine 369, which is important for its binding affinity. NH-group of Threonine 276 (strand 7 of β -tubulin) forms a hydrogen bond (3.25 Å) with the oxetane ring of paclitaxel, an important group for its stabilizing activity. Interestingly, docking analysis revealed that the same Threonine 276 of β -tubulin is involved in hydrogen bonding (2.02 Å) with the N- Boc group of compound 1. Furthermore, the interaction map of compound 1 revealed that the atoms in the S-aryl ring could form hydrophobic contacts with the Histidine 229 in the β -tubulin paclitaxel binding pocket. This provides an exciting lead toward the possible target of compound 1, which requires further investigation.

4.5 Conclusion

In this work, we validated the biological activity of newly synthesized S-secondary alkyl and S-aryl dithiocarbamate-derived compounds which also satisfy all drug-likeness filters. This makes them a promising candidate for drug discovery. Using the structure-activity relationship, we could show that the electronic variations in the S-aryl ring do not have any drastic effect on the potency of these compounds as antiproliferative agents in the

MTT assay. However, positional variations of the functional groups showed a preference for the meta-positioned substitutions as compared to the ortho- or para-positioned substitutions. Despite our efforts, the original scaffold of compound 1 had the best potency in the MTT assay. The flow cytometry and fluorescence microscopy revealed that compound 1 induces G₂/M phase cell cycle arrest and apoptosis in cancerous cells. This is found to be mediated via the p53-p21 signaling pathways as we could confirm a dose-dependent increase in the expression of these regulatory molecules. Interestingly, fluorescence microscopy revealed MT bundling in compound 1 treated cells, which can result in cellular stress. The phenotype is comparable to the MT-stabilizing anticancer drug paclitaxel. Finally, molecular docking of compound 1 revealed that it binds to the taxol-binding pocket of the β -tubulin protein with comparable atomic contact energy (ACE), to paclitaxel. In a nutshell, we identified S-aryl dithiocarbamate derived novel scaffold having antiproliferative activity in cancer cells and our work sheds light on the mechanism of action. We also identified a novel role of this class of compounds in affecting tubulin dynamics by inducing MT bundling, making them a promising scaffold for designing new anticancer drugs (Fig. 4.16).

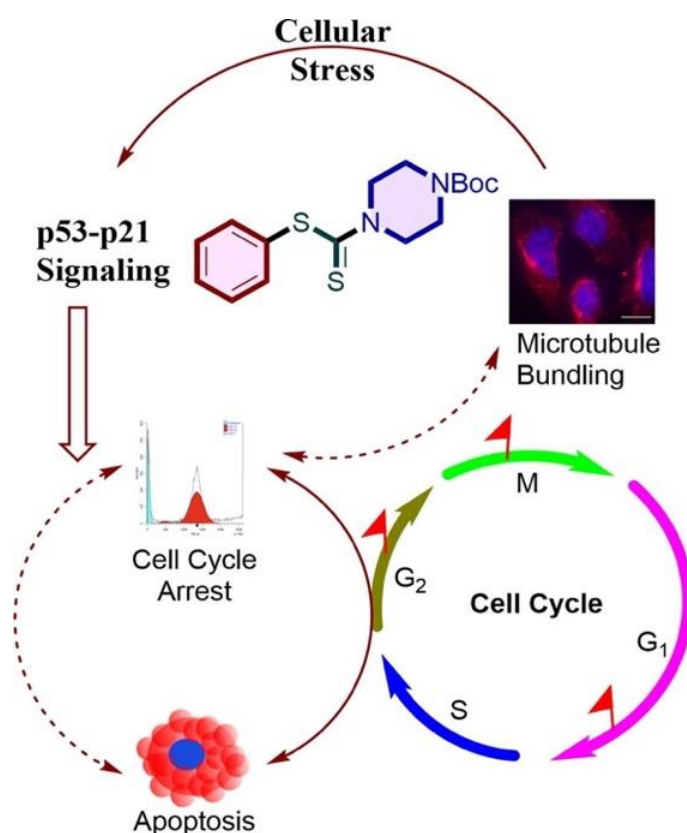


Figure 4.16: Schematic representing the effects of synthesized S-aryl dithiocarbamate in HeLa cells. (Adapted with permission from Jaiswal et.al. 2022).

4.6 Scientific doctoral publications related to the chapter

- **Jaiswal S.**, Parida S.,K., Singh P, Murarka S. Development of S-Aryl Dithiocarbamate Derived Novel Antiproliferative Compound exhibiting Tubulin bundling. *Bioorganic and Medicinal Chemistry*, 2022, 68, 116874.
- Parida S.,K., **Jaiswal S.**, Singh P, Murarka S. Multicomponent synthesis of biologically relevant S- aryl dithiocarbamates using diaryliodonium salts. *Organic Letters*, 2021, 23, 6401–6406.
- Parida S. K., Hota S. K., **Jaiswal S.**, Singh P. and Murarka S. Multicomponent Synthesis of Biologically Relevant S-Diarylmethane Dithiocarbamates Using p-Quinone Methides. *Advance Synthesis & Catalysis*, doi: 10.1002/adsc.202200029. (*very important publication*)

