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Indirubin, a bis-indole alkaloid binds to tubulin and exhibits antimitotic activity against HeLa cells in synergism with vinblastine

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ABSTRACT

Indirubin, a bis-indole alkaloid used in traditional Chinese medicine has shown remarkable anticancer activity against chronic myelocytic leukemia. The present work was aimed to decipher the underlying molecular mechanisms responsible for its anticancer attributes. Our findings suggest that indirubin inhibited the proliferation of HeLa cells with an IC_{50} of 40 μM and induced a mitotic block. At concentrations higher than its IC_{50}, indirubin exerted a moderate depolymerizing effect on the interphase microtubular network and spindle microtubules in HeLa cells. Studies with goat brain tubulin indicated that indirubin bound to tubulin at a single site with a dissociation constant of 26 ± 3 μM and inhibited the *in vitro* polymerization of tubulin into microtubules in the presence of glutamate as well as microtubule-associated proteins. Molecular docking analysis and molecular dynamics simulation studies indicate that indirubin stably binds to tubulin at the interface of the α-β tubulin heterodimer. Further, indirubin stabilized the binding of colchicine on tubulin and promoted the cysteine residue modification by 5,5′-dithiobis-2-nitrobenzoic acid, indicating towards alteration of tubulin conformation upon binding. In addition, we found that indirubin synergistically enhanced the anti-mitotic and anti-proliferative activity of vinblastine, a known microtubule-targeted agent. Collectively our studies indicate that perturbation of microtubule polymerization dynamics could be one of the possible mechanisms behind the anticancer activities of indirubin.

1. Introduction

Indigo naturalis is an herbal formulation of the Chinese medicine obtained from the leaves of plants such as *Baphicacanthus cusia*, *Polygonum tinctorium*, *Isatis indigotica*, and *Indigofera tinctoria.* It is traditionally used for the treatment of liver ailments, fever, chest pain, mouth ulcers, mumps, inflammation of throat and larynx, hemoptysis, eczema, psoriasis, and gingivitis [1]. Danggui Longhui Wan, a traditional Chinese recipe comprising of *Indigo naturalis* along with ten other herbal mixtures was found to exert a significant therapeutic effect on patients who have chronic myelocytic leukemia (CML) [2]. Later, the antileukemic activity of the recipe was attributed to indirubin, an isomer of indigo [2].

Several reports have indicated that indirubin has significant antiviral, antifungal, and anti-inflammatory effects [3–5]. Indirubin (INR) and its derivatives have shown anticancer effects on different cancer cell lines like PC3, HepG2, HCT116, HeLa, A549, NCI-H358, MDA-MB-468, and MDA-MB-435 [2,6–9]. Indirubin has been reported to modulate cellular targets like aryl hydrocarbon receptor [10,11], glycogen synthase kinase-3 [12], glycogen phosphorylase b [13], c-Jun N-terminal kinase [14], the Stat3 transcription factor [15], and DNA synthesis [2]. Inhibition of several kinases involved in cell division was reasoned for the possible G2/M phase arrest induced by indirubin and its analogues in cancer cells [2,7,8]. It was predicted that the antileukemic effects of indirubin and its structural analogue N-methyl-isoindigo could be partly due to inhibition of microtubule polymerization [2]. However, the biochemical and biophysical interactions of indirubin with tubulin were not investigated thoroughly. Interestingly, a lot of natural and synthetic indole and bisindole compounds have been shown to bind to tubulin and inhibit its polymerization into microtubules [16,17]. Hence, in this paper, we have studied the effect of indirubin on the organization of interphase and mitotic microtubules and characterized its binding interactions with goat brain tubulin isolated *in vitro*.

A large number of tubulin targeted drugs like vinblastine, vincristine, and paclitaxel, have been approved for anticancer therapy [18,19]. Tubulin is a heterodimer consisting of α and β-monomeric subunits. Tubulin heterodimers are highly dynamic and polymerize to form microtubules which are essential for various cellular functions like intracellular vesicle transport, cell migration, and fabrication of the

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spindle apparatus during segregation of the sister chromatids and cytokinesis [18,19]. Tubulin harbours three well-characterized drug binding sites like the colchicine binding site, the paclitaxel binding site, and the *Vinca* alkaloid binding site. The colchicine binding site is present at the interface of the α-β subunit where colchicinoids, podophyllotoxin, combretastatin, and curacin A bind [18,19]. The paclitaxel binding site is located at the β-tubulin, and the *Vinca* alkaloids binding site is located in the N-terminal region of the β-tubulin subunit [19].

In the present study, we have investigated the antiproliferative effects of indirubin in the light of its inhibitory effects on the functioning of microtubules in cells and *in vitro*. It perturbed the organization of the interphase microtubule network and mitotic spindles without causing significant depolymerization of the microtubules at the IC_{50}. The experimental evidence indicated that indirubin bound to tubulin at a unique binding site at the interface of the α-β tubulin heterodimer. Binding of indirubin inhibited the polymerization of tubulin into microtubules and altered the conformation of tubulin structure.

Results from the clinical trials conducted upon CML patients suggested that the toxicity of indirubin was low and the side effects were confined to mild abdominal pain, nausea, and vomiting [2,20–22]. Since, indirubin was found to be well tolerated in animals with little or no bone marrow toxicity [2,20,23], we studied the effects of indirubin in combination with vinblastine for possible application in combination therapy. Our results indicated a strong synergism between indirubin and vinblastine in blocking the HeLa cells at mitosis and inhibiting their proliferation. Together, the results suggest that the antiproliferative effects of indirubin could be partly through its inhibitory effects on tubulin and combination of indirubin with other anticancer drugs might provide a therapeutic advantage for the treatment of cancer.

2. Experimental procedures

2.1. Chemicals

Indirubin was purchased from TCI Chemicals (India) Pvt. Ltd. Vinblastine, 1-anilinonaphthalene-8-sulfonic acid (ANS), podophyllotoxin, colchicine, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), sulforhodamine B, (SRB), Hoechst 33342, guanosine 5′-triphosphate (GTP), EGTA, $MgCl_2$, piperazine-*N,N′*-bis (2- ethanesulfonic acid) (PIPES), mouse monoclonal anti-α-tubulin IgG and FITC conjugated anti-mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 568 conjugated goat anti-mouse IgG and fetal bovine serum (FBS) were purchased from Molecular Probes, Invitrogen, CA, USA. Minimal essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), cell culture tested antibiotic solution, phosphate-buffered saline (PBS), were purchased from HiMedia, Mumbai, India. All other reagents used in the study were of analytical grade.

2.2. Cell culture and cytotoxicity assay

Human cervical cancer cell line (HeLa) and mouse fibroblast cells (L929) were obtained from the National Centre for Cell Science, (NCCS) Pune, India. HeLa and L929 cells were grown in MEM, and DMEM respectively supplemented with 10% (v/v) FBS, sodium bicarbonate and 1% antibiotic solution (100 units of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin B per mL). Cells were maintained in 25 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For cytotoxic assays, HeLa cells were seeded at a density of 0.5×10^5 cells/mL in 96-well tissue culture plates and incubated in a CO_2 incubator. A stock solution of INR was made in 100% DMSO, and the concentration of DMSO was maintained at 0.1% in all the cell culture experiments. After 24 h of seeding the cells, the medium was removed, and fresh medium containing vehicle (0.1% DMSO) or different concentrations of INR (0–100 μM) or different concentrations of cisplatin (0–80 μM) were added, and the cells were further incubated for 24 h. The cytotoxicity of INR (0–200 μM) on mouse fibroblast cells (L929) was determined in a similar manner using DMEM as the culture medium. The inhibition of cell proliferation was determined by the standard sulforhodamine B (SRB) assay as described previously [24,25] by measuring the absorbance of bound sulforhodamine B at 560 nm using a 96-well plate reader. The percentage inhibition of proliferation was calculated by using the formula:

% Inhibition of proliferation = 100 – {(Mean OD of the treated sample – Mean OD of Zero Control)/(Mean OD of control – Mean OD of Zero Control)} × 100

2.3. Mitotic index and immunofluorescence

To calculate the mitotic index, HeLa cells were grown on poly-l-lysine coated glass coverslips in 24-well tissue culture plates and treated with different concentrations of INR for 24 h. The cells were then fixed with 3.7% (v/v) formaldehyde in PBS for 30 min at 37 °C and washed with PBS. Cells were then permeabilized with ice-cold methanol for 20 min at −20 °C and stained with Hoechst 33342 staining solution. The coverslips were then washed with PBS and mounted on glass slides containing 1,4-diazabicyclo[2.2.2]octane (DABCO) as an anti-quenching agent. The cells were then observed under an inverted fluorescence microscope (Nikon ECLIPSE Ti-E, Japan) and the number of mitotic and interphase cells were counted. Mitotic index was calculated as the percentage of cells blocked at mitosis compared to the total number of cells [24–27]. At least 1000 cells were scored for each concentration of INR and the experiment was repeated three times.

HeLa cells (0.5×10^5 cells/ml) were incubated with either a vehicle (0.1% DMSO) or different concentrations of INR for 24 h to study the effects of INR on the organization of microtubules and DNA. After incubation, the cells were fixed with 3.7% formaldehyde for 30 min at 37 °C and then washed with PBS. Immunostaining was performed by using antibody specific for α-tubulin, as described previously [24–27]. DNA was stained using Hoechst 33342. Immunofluorescence imaging was performed using Nikon Eclipse T*i*-E microscope and processed by using the software ImageJ (NIH, USA).

2.4. Cell migration assay

HeLa cells (1×10^5 cells/mL) were grown in 35 mm cell culture dishes. At 90% confluence, a wound was made using a sterile micropipette tip [25]. The floating cells were removed immediately after wounding, and the medium was changed with a fresh one containing 0, 40 or 80 μM INR. Cells were observed at 0, 24, 48, and 72 h intervals. Fresh medium was replaced with the old medium at the corresponding intervals. The bright field images of the wounds were recorded using the inverted microscope. Percentage migration was calculated by using the formula:

%Migration = [1-(width of scratch at specific time point 't'/width of the scratch at zero time, 't_0')] × 100

The data were analyzed using one-tailed student's *t*-test to assess the statistical significance between control and the treatment groups. A statistically significant difference was considered to be present at $P \le 0.05$.

2.5. Purification of goat brain tubulin

Microtubule-associated protein (MAP) free tubulin was isolated from goat brains by two cycles of polymerization and depolymerization. Depolymerization of tubulin polymers was carried out in a Dounce homogenizer in cold as described earlier [25]. MAP-rich tubulin was isolated using 4 M glycerol as the inducer for polymerization [25,28,29]. The protein samples were stored in aliquots at −80 °C until further use. All the experiments with tubulin were performed in PEM

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