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HMBA depolymerizes microtubules, activates mitotic checkpoints and induces mitotic block in MCF-7 cells by binding at the colchicine site in tubulin

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ABSTRACT

10-[(3-Hydroxy-4-methoxybenzylidene)]-9(10H)-anthracenone (HMBA), a synthetic compound, has been reported to have a potent antitumor activity. In this study, we found that HMBA depolymerized microtubules in MCF-7 cells and produced aberrant spindles in the MCF-7 cells. It also reduced the distance between the centrosomes and activated the mitotic checkpoint proteins BubR1 and Mad2. Further, HMBA inhibited the progression of MCF-7 cells in mitosis and induced apoptotic cell death involving p53 pathway. *In vitro*, HMBA bound to purified brain tubulin with a dissociation constant of 4.1 ± 0.9 μM. It inhibited microtubule assembly and increased the GTP hydrolysis rate of microtubule assembly. The compound did not alter the binding of 2′(or 3′)-*O*-(trinitrophenyl) guanosine 5′-triphosphate (TNP–GTP), a fluorescent analogue of GTP, to tubulin suggesting that it did not inhibit the binding of GTP to tubulin. However, we obtained evidence indicating that HMBA perturbed the conformation of the GTP binding site in tubulin. In addition, an analysis of the modified Dixon plot suggested that HMBA competitively inhibited the binding of colchicine to tubulin. A computational analysis of the binding of HMBA to tubulin supported the finding that HMBA shared its binding site with colchicine in tubulin and indicated that the binding of HMBA to tubulin was primarily stabilized through hydrogen bonding.

1. Introduction

HMBA (Fig. 1) was used in several phase I and one phase II clinical trials against different human cancers in the 1980s and 1990s. It has shown solid tumor regression in several phase I trials when administered to patients with treatment-refractory or unresponsive tumors [1–3]. However, the efficacy was limited due to side-effects e.g. neurotoxicity or thrombocytopenia. In a phase II clinical trial involving patients suffering from myelodysplastic syndrome or acute myelogenous leukemia, HMBA caused a partial or complete amelioration of symptoms in 9 out of 41 patients [4]. Apart from anticancer activity, HMBA has also shown promise in treating HIV infections [5]. HMBA treatment of latent CD4+ T cells from recovering patients carrying HIV-1 showed clearance of the latent virus.

In spite of its relative success in clinical trials, its mode of action was not clear. Based upon studies on cultured cells, HMBA was thought to induce differentiation in malignant cells so that they return to normalcy after differentiation [6]. It was suggested that HMBA induced differentiation in murine erythroleukemia cells by modulating protein kinase C mediated signaling pathway and by suppressing cdk4-dependent kinase activity and hypophosphorylating retinoblastoma tumor suppressor protein [7,8]. HMBA has been suggested to inhibit Akt and MAPK signaling cascade in lung cancer cells [9]. Moreover HMBA has been shown to induce apoptosis in human myeloma cells, malignant pleural mesothelioma cells and hepatocellular carcinoma cells by down-regulating Bcl-2 [10–12]. A recent study has suggested that tubulin is the primary target of HMBA in cancer cells [13]. It was shown that HMBA inhibited the binding of colchicine to tubulin, prevented microtubule polymerization *in vitro* and in K562 (leukemia) cells and induced G2/M arrest in K562 and HeLa cells [13,14]. HMBA was also found to reduce the microtubule network and to increase the level of acetylated tubulin in a ciliated protozoa *Tetrahymena pyriformis* [15]. Further, sulfonate derivatives of HMBA were also found to inhibit the proliferation of HeLa, SF 268 and NCI-H460 cells in culture and to depolymerize microtubules *in vitro* [16].

Several inhibitors of microtubules are being successfully used in clinics for the treatment of different types of cancers [17,18]. These inhibitors have been found to stall cell proliferation and induce apoptotic cell death by diverse mechanisms; several of them have also been shown to suppress the dynamic instability of microtubules by directly interacting with tubulin [19,20]. Inhibitors of microtubule assembly have generally been found to bind to

Abbreviations: IgG, immunoglobulin G; GTP, guanosine triphosphate; GDP, guanosine diphosphate; FITC, fluorescein isothiocyanate; ANS, 1-anilinonaphthalene-8-sulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; DAMA–colchicine, N-deacetyl-N-(2-mercaptoacetyl)-colchicine; PI, propidium Iodide; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide.

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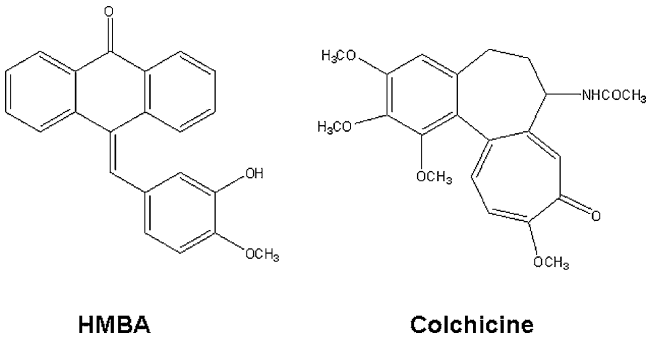

Fig. 1. Structures of HMBA and colchicine are shown.

tubulin in one of its two well-characterized sites, namely the vinblastine site or the colchicine binding site. However, the interaction of HMBA to tubulin, its binding site on tubulin and its antiproliferative mechanism of action are far from clear. In this study, we sought to unravel the mode of interaction of HMBA with tubulin and to determine the mechanism of antiproliferative action of HMBA using MCF-7 cells, a human breast cancer cell line.

Using both computational and experimental approaches, we have shown that HMBA binds to tubulin in the colchicine site and presented a mechanism through which it interacts with tubulin and inhibits microtubule polymerization. We have also provided significant insights into the mechanism through which HMBA activates mitotic checkpoint, inhibits mitosis and induces apoptosis in MCF-7 cells.

2. Materials and methods

2.1. Antibodies and compounds

Mouse monoclonal anti-α tubulin IgG, rabbit monoclonal γ-tubulin, FITC-labeled anti-rabbit IgG conjugate, bovine serum albumin (BSA), Hoechst 33258 were purchased from Sigma, MO, USA. Anti mouse IgG-Alexa 568 antibody and TNP–GTP were purchased from Molecular Probes, CA, USA. Mouse anti-p53, p21 antibodies and Annexin V-Propidium Iodide (PI) Apoptosis Detection Kit was purchased from Santa Cruz Biotechnology, CA, USA. Mouse anti-BubR1 and Mad2 antibodies were obtained from BD Biosciences, CA, USA. HMBA was purchased from Calbiochem, NJ, USA. Fetal bovine serum (FBS) was obtained from Biowest, Nuaille', France. All other reagents were of analytical grade and obtained from Sigma, MO, USA and Himedia, Mumbai, India.

2.2. Tubulin isolation

Microtubule proteins were isolated from goat brain by two cycles of assembly–disassembly process in the presence of 4 M glycerol, 5 mM $MgCl_2$, 1 mM EGTA and 0.5 mM GTP [21]. Pure tubulin (MAPs-free) was isolated from goat brains by two cycles of polymerization and depolymerization using 1 M monosodium glutamate buffer for assembly [22]. The composition of the assembly buffer was 1 M monosodium glutamate, 10% DMSO and 0.5 mM GTP. Depolymerization buffer contained 50 mM Pipes, pH 6.8, 1 mM EGTA, 3 mM $MgCl_2$ and 0.1 mM GTP. The protein was stored at −80 °C until further use. The protein concentration was determined by the method of Bradford using bovine serum albumin as standard [23].

2.3. Determination of the dissociation constant of tubulin and HMBA interaction

Tubulin (2 μM) in 25 mM Pipes, pH 6.8 was incubated in the absence and presence of different concentrations of HMBA (0.5–20 μM) for 30 min at 25 °C. The fluorescence emission spectra (310–360 nm) were monitored using 295 nm as the excitation wavelength. To minimize the inner filter effect, a cuvette of 0.3 cm path length was used. The inner filter effect was corrected using the formula:

$$F_{\text{corrected}} = F_{\text{observed}} \times \text{antilog}\left[\frac{A_{\text{ex}} + A_{\text{em}}}{2}\right]$$

where A_{ex} and A_{em} are the absorbance at the excitation and emission wavelengths and F_{observed} and $F_{\text{corrected}}$ are the observed and corrected fluorescence intensities, respectively. The dissociation constant (K_d) was calculated by fitting the fluorescence data in the following equation:

$$\Delta F = \frac{\Delta F_{\max} L}{K_d + L}$$

where ΔF and $\Delta F_{\max}$ represent change in the fluorescence intensity of the tubulin upon binding to HMBA and the maximum change in the fluorescence intensity of tubulin when it is fully bound with HMBA and L is the concentration of HMBA. ΔF was calculated by subtracting the fluorescence intensity of tubulin in the absence of HMBA from that in the presence of different concentrations of HMBA. The $\Delta F_{\max}$ was estimated using the Graph Pad Prism 5 software (Graph Pad Software, CA, USA).

2.4. Effect of HMBA on the fluorescence of tubulin–ANS complex

Tubulin (2 μM) in 25 mM Pipes buffer (pH 6.8) containing 1 mM EGTA, 3 mM $MgCl_2$ (PEM) was incubated in the absence and presence of different concentrations of HMBA at 25 °C for 10 min. Then, 20 or 260 μM ANS was added to the reaction mixtures and incubated for an additional 20 min. The fluorescence intensity was measured at 470 nm using 380 nm as the excitation wavelength. Experiment was repeated four times.

2.5. Competition between colchicine and HMBA for binding to tubulin

Tubulin (5 μM) was incubated without or with different concentrations of HMBA in PEM buffer in dark for 30 min at 25 °C. Then, colchicine (10 μM) was added to the reaction mixtures and incubated at 37 °C for an additional 1 h. Similarly, respective control solutions containing DMSO, colchicine (10 μM), HMBA (5, 10 and 25 μM) were incubated under identical conditions. The fluorescence spectrum of tubulin–colchicine complex was monitored using 340 nm as the excitation wavelength and the fluorescence intensity was noted at 430 nm.

2.6. Modified Dixon plot

Different concentrations of HMBA were mixed with tubulin along with a fixed concentration of colchicine. For one set, tubulin (5 μM) was mixed with different concentrations (0, 1, 2, 5, 10 and 15 μM) of HMBA and a fixed concentration of colchicine (5 μM) was added to the reaction mixtures. Similarly, two more sets were prepared with 15 and 20 μM colchicine. The mixtures were incubated at 37 °C for 1 h and the fluorescence of tubulin–colchicine complex was measured using 340 nm and 430 nm as excitation and emission wavelengths, respectively. The experiment was repeated thrice. $1/F$ (F = fluorescence intensity) versus HMBA concentration was plotted and inhibitory constant (K_i) was calculated using the modified Dixon plot [24,25].