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Structure-activity profiles of eleutherobin analogs and their cross-resistance in Taxol-resistant cell lines

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Abstract *Purpose*: Eleutherobin, a natural product, is an antimitotic agent that promotes the polymerization of stable microtubules. Although its mechanism of action is similar to that of Taxol, its structure is distinct. A structure-activity profile of synthetic eleutherobin derivatives that have modifications at C3, C8 and C15 was undertaken to define the structural requirements for microtubule stabilization and cross-resistance in Taxol-resistant cell lines. *Methods*: The biological activity of five eleutherobin analogs was assessed using three techniques; (1) cytotoxicity and drug-resistance in three paired Taxol-sensitive and -resistant cell lines; (2) polymerization of microtubule protein in vitro in the absence of GTP and (3) induction of microtubule bundle formation in NIH3T3 cells. *Results*: Eleutherobin had an IC_{50} value comparable to that of Taxol, whereas neo-eleutherobin, which has a carbohydrate domain that is enantiomeric with that of the parent compound, was less cytotoxic and had 69% of the maximum microtubule polymerization ability of eleutherobin. Both of these compounds exhibited cross-resistance in *MDR1*-expressing cell lines. Removal or replacement of the C15 sugar moiety resulted in reduced microtubule polymerization and cytotoxicity compared to eleutherobin and loss of cross-resistance in the cell lines SKVLB and J7-T3-1.6, both of which express high levels of P-glycoprotein. By contrast, removal of the urocanic acid group at C8 resulted in virtually complete abrogation of biological activity. The compound lost its ability to polymerize microtubules, and its cytotoxicity was reduced by a minimum of 2000-fold in lung carcinoma A549 cells. *Conclusions*: Removal or modification of the sugar moiety alters the cytotoxic potency of eleutherobin and its pattern of cross-resistance in Taxol-resistant cells, although such compounds retain a small percentage of the microtubule-stabilizing activity of eleutherobin. The *N*(1)-methylurocanic acid moiety of eleutherobin, or perhaps some other substituent at the C8 position, is essential for Taxol-like activity. These findings will be important for the future design and the synthesis of new and more potent eleutherobin derivatives.

Key words Eleutherobin · Microtubules · Taxol · Structure-activity · Drug-resistance

Introduction

Microtubules are important components of the eukaryotic cytoskeleton that are essential for separation of the duplicated chromosome pairs during mitosis. They also have significant functions in interphase cells that include intracellular transport, maintenance of cell shape, locomotion, and transmission of signals between cell surface receptors and nuclear effectors [8].

The functional diversity of the microtubule system is exemplified by the mechanisms of action of the antitumor drug Taxol, isolated in 1971 from the Pacific yew tree, *Taxus brevifolia* [30]. The primary target of Taxol is the tubulin/microtubule system. The drug interacts with the *N*-terminal 31 amino acids [21], and amino acids 217–231 [22] of the β-tubulin subunit. Taxol promotes hyperstabilization of microtubules that are resistant to depolymerization by either calcium or cold [26], conditions that depolymerize normal microtubules. This re-

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sults in microtubule bundling and arrest of cells in mitosis [25], eventually leading to cell death. It has been demonstrated in HeLa cells that low concentrations of Taxol suppress microtubule dynamics and thereby interrupt normal mitosis in the absence of an increase in polymerized tubulin [12]. Evidence indicates that Taxol also modulates specific intracellular signaling events including the induction of tumor necrosis factor alpha (TNF-α) [1, 4, 7], and increased tyrosine phosphorylation of proteins, including MAP kinase and Raf-1 kinase [28, 32]. Extended exposure of cells to Taxol induces DNA fragmentation, indicating that the cells undergo apoptosis [15, 29].

The approval of Taxol for the treatment of breast, ovarian and lung carcinomas has led to renewed interest in the microtubule as an important target for the development of new chemotherapeutic drugs, and this has inspired a search for novel natural compounds that mimic the activity of Taxol. One such compound, eleutherobin, was isolated from an *Eleutherobia* species of a rare marine soft coral found off the coast of western Australia, and its structure has been elucidated [14]. It has been reported that eleutherobin has a mechanism of action similar to that of Taxol in that it potentiates the assembly of stable microtubules resistant to depolymerization [16]. Due to the limited availability of natural eleutherobin, and its potential as an anticancer drug, it was rapidly synthesized in the laboratory [5, 6, 17, 19].

Although Taxol has come to have an important role in the treatment of human tumors, its toxicities plus its aqueous insolubility that have made its formulation difficult, are continuing problems that may not be associated with new compounds with Taxol-like activity. In addition, a major concern in the clinical treatment of human tumors is the acquisition of drug resistance which often leads to chemotherapeutic failure. The multidrug-resistance (MDR) phenotype is associated with the overexpression of P-glycoprotein, an energy-dependent drug efflux pump that maintains a low intracellular drug concentration [9]. Taxol is a substrate for P-glycoprotein. The degree of hydrophobicity of a given compound influences its affinity for P-glycoprotein, therefore, solubility in aqueous solvents and efficacy in drug-resistant cells are important considerations in screening for new and potent antitumor agents. The analysis of eleutherobin analogs in drug-resistant cells helps identify structural components that may modulate patterns of resistance.

In the study reported here we investigated the biological profiles of five eleutherobin derivatives. Three of the compounds have no carbohydrate domain and one lacks the C8 *N*(1)-methylurocanic acid. The objective of this study was to define a structure-activity profile for eleutherobin to determine cross-resistance patterns which may aid in the elucidation of a common pharmacophore between Taxol and eleutherobin. These studies will help in the design of more potent analogs of eleutherobin.

Materials and methods

Materials

Eleutherobin and its derivatives were synthesized as described previously [5, 6]. Taxol was obtained from the Drug Development Branch, National Cancer Institute (Bethesda, Md.). All compounds were dissolved in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.) at stock concentrations of 5 m*M* and stored at −20 °C. All control samples contained an equal volume of DMSO. GTP was obtained from Sigma Chemical Co. and dissolved in sterile distilled water at a concentration of 10 m*M* and stored at −20 °C. Microtubule protein was purified from calf brains by two cycles of temperature-dependent assembly and disassembly [31] and stored in liquid nitrogen prior to use. The antimouse β-tubulin monoclonal antibody T-4026 was obtained from Sigma Chemical Co.

Cell culture

Human non-small-cell lung carcinoma cells, A549, were maintained in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco Laboratories, Grand Island, N.Y.) at 37 °C in an atmosphere containing 7% CO_2. The Taxol-resistant cell line A549-T12 was selected for resistance to Taxol and maintained in a final concentration of 12 n*M* drug [13]. Human ovarian carcinoma cells SKOV3 and the MDR variant SKVLB (obtained from Dr. V. Ling) were maintained in α-MEM plus ribonucleotides, deoxynucleotides, 15% fetal bovine serum and 1% penicillin-streptomycin (Gibco Laboratories) at 37 °C in an atmosphere containing 5% CO_2. SKVLB cells were maintained in 1 μ*M* vinblastine (Sigma Chemical Co.). The mouse macrophage-like cell line J774.2 and its Taxol-resistant variant J7-T3-1.6 that overproduces P-glycoprotein [24], were grown in DMEM containing 20% heat-inactivated horse serum, 1% L-glutamine, 1% nonessential aminoacids and 1% penicillin-streptomycin at 37 °C in an atmosphere containing 7% CO_2. J7-T3-1.6 cells were maintained in 1.6 μ*M* Taxol.

Cytotoxicity assays

Taxol-resistant and -sensitive cell lines were seeded into 12-well plates at a density of 5×10^4 cells per well and exposed to tenfold serial dilutions of each compound (10^{-10} to 10^{-6} *M*) for 72 h after a 12-h attachment period. Cells were then trypsinized and counted (Coulter counter model ZF0031, Coulter Corp., Miami, Fl.). The IC_{50} was determined by plotting cell number against log of the drug concentration and this was used to determine the concentration of compound resulting in 50% growth inhibition compared to untreated cells. Each cell line was assayed in a minimum of three independent experiments with each compound and the results expressed as the mean IC_{50}.

Tubulin polymerization assay

The ability of each compound to polymerize tubulin was evaluated by recording the change in turbidity of microtubule protein at 350 nm for 80 min in a spectrophotometer (UVIKON, Research Instruments Int., San Diego, Calif.). Purified microtubule protein [27] was diluted in assembly buffer containing 0.1 *M* MES, 1 m*M* EGTA, 0.5 m*M* $MgCl_2$ and 3 *M* glycerol, pH 6.6, to a final volume of 1 ml and a final concentration of 1 mg. All compounds were evaluated at a concentration of 10 μ*M* at 37 °C in the absence of GTP. GTP was used at a final concentration of 1 m*M* in the control assays. Microtubule stability was assessed by the addition of 10 m*M* Ca^{++}. The changes in absorption at 350 nm for 10 min following the addition of each compound were monitored to calculate initial slopes from the linear portion of each curve in order to compare the activity of each compound (relative to the activity of

Taxol which was assigned a value of 100%). Maximum tubulin polymerization was assessed at 60 min.

Immunofluorescence

NIH 3T3 cells were grown to subconfluency on glass coverslips in 35-mm plastic petri dishes. Cells were exposed to 2 μ*M* Taxol and each of the experimental compounds for 5.5 h at 37 °C, rinsed twice in PBS and extracted with 0.5% Triton-X-100 in microtubule stabilizing buffer (PEM; 100 m*M* PIPES, 2 m*M* EGTA and 2 m*M* $MgCl_2$, pH 6.8) for 4 min. Following a wash in PEM, the cells were fixed for 40 min at room temperature in 3% formaldehyde in PEM followed by incubation in 0.1 *M* glycine in PBS for 10 min and washing (four times) in PEM containing 5 μ*M* EGTA. Following blocking in 20% normal goat serum (NGS) for 30 min at room temperature, cells were incubated for 1 h at 37 °C with a 1:100 dilution of β-tubulin mAb diluted in 10% NGS, washed twice in 1% BSA, once in PBS and three times in 1% BSA. Cells were then incubated in a 1:200 dilution of the secondary fluorophore antibody, IndocarbocyanineCy3 (Jackson Immunoresearch Laboratories, Pa.) in 20% NGS for 20 min at room temperature. Finally cells were washed three times in 1% BSA and the dried slides mounted in 30% glycerol in PBS containing β-phenylene diamine (1 mg/ml). Fixed slides were examined using a Zeiss Axioskop microscope.

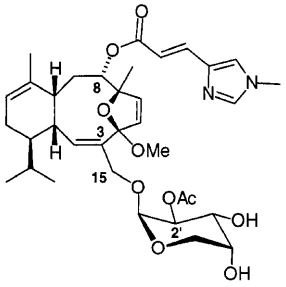
Results

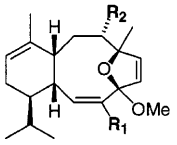
Cytotoxicity

The cytotoxic efficacy of eleutherobin and its derivatives (Fig. 1) was evaluated in two drug-sensitive human cancer cell lines, A549 and SKOV3, a murine macrophage-like cell line J774.2, and their Taxol-resistant counterparts. The results are summarized in Table 1 in order of decreasing potency. A549-T12 cells are sevenfold resistant to Taxol, do not express *MDR1*, and therefore P-glycoprotein is excluded as a mechanism of resistance. Although the mechanism of resistance has not been fully defined for A549-T12, an investigation of the mRNA expression of β-tubulin isotypes has revealed an altered ratio of distinct isotypes in these cells [13]. The Taxol-resistant cell line SKVLB overexpresses high levels of P-glycoprotein [3], as does the Taxol-resistant cell line J7-T3-1.6 [24]. In sensitive cell lines and the *MDR1*-expressing resistant cells, eleutherobin was less potent than Taxol. Neo-eleutherobin was less active than eleutherobin, although the only difference between the two agents is the enantiomeric relationship in the carbohydrate (2′-acetylarabinose) sector. Neither eleutherobin nor neo-eleutherobin displayed cross-resistance in the Taxol-resistant A549-T12 cell line, suggesting that the mechanism of resistance in this cell line is specific to Taxol. In contrast, both compounds demonstrated high resistance in SKVLB and J7-T3-1.6 cells, both of which express high levels of *MDR1*. These results suggest that both eleutherobin and neo-eleutherobin, like Taxol, are excellent substrates for P-glycoprotein.

The compounds SKBII.294 and 296, which lack the carbohydrate domain, were less cytotoxic than either eleutherobin or neo-eleutherobin. Compound SKBII.294 was more cytotoxic than SKBII.296 in the two human cell lines, indicating that subtle alterations at the C3 position influence the overall activity of these compounds in some cell lines. In contrast, SKBII.294 and 296 were poor substrates for P-glycoprotein in both SKULB and J7-T3-1.6 cells suggesting that the carbohydrate domain may have a role in the interaction of these compounds with P-glycoprotein. Compound SKBII.298, which is structurally similar to SKBII.294 but lacks the C15 *O*-acetyl group, was considerably less cytotoxic (in the micromolar range), where tested. Compound SKBIII.13 in which the *N*(1)-methylurocanic acid moiety has been removed was basically inactive in all tests.

Eleutherobin





	R_1	R_2
Neo-Eleutherobin	CH_2-L-β-2'-O-acetylarabinose glycoside	*N*(1)-methylurocanic acid ester
SKBII.294	CH_2OAc	*N*(1)-methylurocanic acid ester
SKBII.296	H	*N*(1)-methylurocanic acid ester
SKBII.298	CH_2OH	*N*(1)-methylurocanic acid ester
SKBIII.13	CH_2-D-β-arabinose glycoside	OH

Fig. 1 Chemical structures of eleutherobin and analogs

The effect of eleutherobin and derivatives on tubulin polymerization

The ability of each compound to polymerize and/or stabilize microtubules was assessed (Fig. 2). To investigate whether eleutherobin and its derivatives shared the microtubule-stabilizing properties of Taxol, 10 m*M*

Table 1 Cytotoxicity of eleutherobin and its derivatives in Taxol-sensitive and Taxol-resistant cell lines. Cytotoxicity was determined by counting cells after 72 h drug exposure. Each value represents the mean IC_{50} (n*M*) derived from at least three independent experiments. Values in parentheses are the -fold resistance of the compound relative to the parental cell line (*ND* Not determined)

Drug	Lung carcinoma A549		Ovarian carcinoma SKOV3		Murine macrophage-like J774.2	
	Sensitive	Resistant[a] (A549-T12)	Sensitive	Resistance (SKVLB)	Sensitive	Resistant (J7-T3-1.6)
Taxol	2	14.2 (7.1)	3	> 5000 (≥1666)	20	3200 (160)
Eleutherobin	6.2	12.2 (1.9)	9.5	> 10000 (≥1052)	200	> 10000 (≥50)
Neo-eleutherobin	125	146 (1.2)	134	> 10000 (≥74.6)	1513	> 10000 (≥6.6)
SKBII.294	152	162 (1.1)	307	1036 (3.4)	6309	7000 (1.1)
SKBII.296	400	4000 (10)	1626	1350 (0.8)	1778	3200 (1.8)
SKBII.298	1059	ND	> 5000	ND	ND	ND
SKBIII.13	4150	ND	> 5000	ND	ND	ND

[a] Cells were maintained in 12 n*M* Taxol during cross-resistance experiments

$CaCl_2$ was added to each sample after maximum microtubule polymerization occurred, and the effects on turbidity recorded spectrophotometrically. A positive control was included in which 1 m*M* GTP was used to confirm the ability of GTP-stabilized microtubules to undergo Ca^{++}-induced depolymerization.

Eleutherobin (10 μ*M*), was slightly less active than Taxol at 37 °C, exhibiting 94% of the polymerization activity of 10 μ*M* Taxol, based on maximum tubulin polymerization at 60 min. The drug, like Taxol stabilized microtubules against Ca^{++}-induced depolymerization. The addition of Ca^{++} to Taxol- and eleutherobin-polymerized microtubules resulted in a small increase in turbidity which may have been due to some Ca^{++} precipitation. Neo-eleutherobin (10 μ*M*) exhibited 69% of the microtubule polymerization activity of the parent compound eleutherobin, and the microtubules formed also resisted Ca^{++}-induced depolymerization. The activities of the compounds SKBII.294 and 296 were identical in the tubulin polymerization assay. This was seen both for the initial rate of polymerization (Fig. 3) and for the extent of polymerization (Fig. 2). Both compounds retained 54% of the activity of eleutherobin, whereas SKBII.298 retained only 36%. It should be noted that compounds SKBII.294, 296 and 298 had reduced solubility compared to eleutherobin. This resulted in a small increase in turbidity at 350 nm when each of these compounds was added to the microtubule protein in MES buffer. This problem was resolved by measuring the small absorbance of each compound in MES buffer alone at 350 nm and subtracting this baseline value from subsequent tubulin polymerization curves.

Compound SKBIII.13, bearing an intact sugar but lacking the urocanic acid moiety, was inactive in tubulin polymerization assays. The ability of this compound to stabilize microtubules formed in the presence of GTP was examined. After a period of 15 min, 10 m*M* Ca^{++} was added and the change in turbidity recorded. Compound SKBIII.13 was unable to stabilize GTP-polymerized microtubules against Ca^{++}-induced depolymerization (data not shown).

The polymerization activity of these compounds was also assessed by determining the slope of the linear portion of each curve at A_{350nm} (t = 10 min), and expressing this relative to Taxol (which was assigned a value of 100%) (Fig. 3). The order of activity of eleutherobin and derivatives using this method, corroborates the results obtained from cytotoxicity studies.

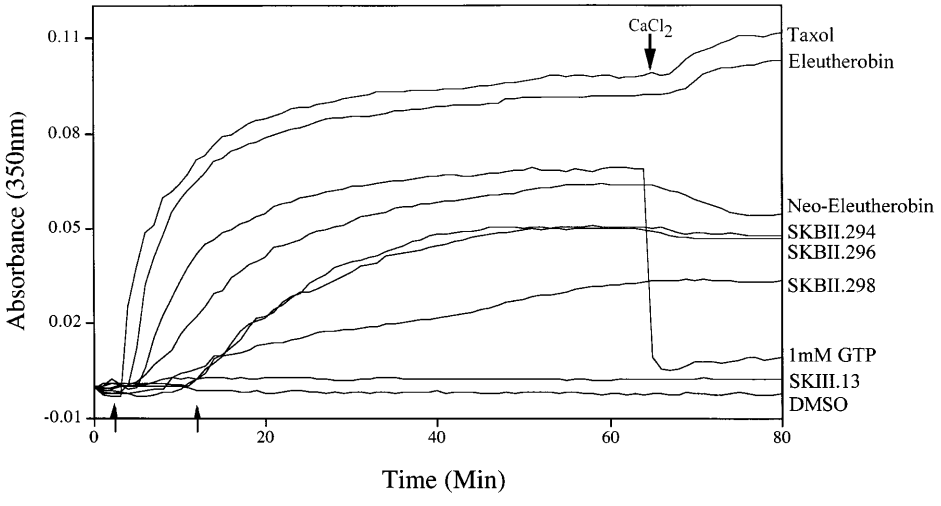


Fig. 2 Polymerization of brain tubulin in the presence of eleutherobin and related analogs. Tubulin (1 mg/ml) was polymerized at 37 °C with 10 μ*M* of each compound. No GTP was present. Taxol was added at 3 min, as indicated by the *arrow*. Each compound was added at 1–2 min intervals with the addition of the last compound, SKBII.296 at 13 min as indicated by the *arrow*. $CaCl_2$ (10 m*M*) was added to each cuvette at the time indicated by the *large arrow*

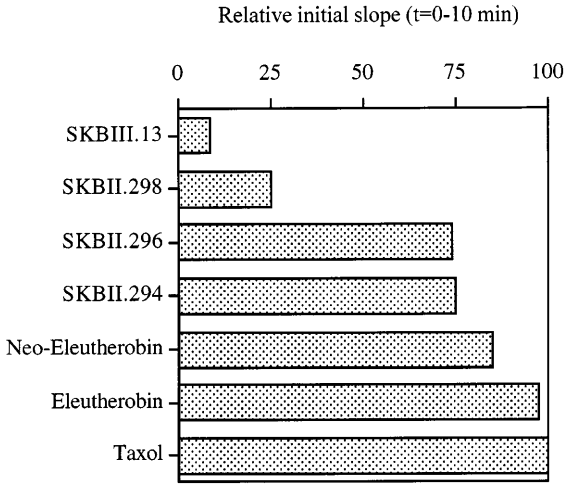


Fig. 3 Relative polymerization activity of eleutherobin and its derivatives. Values represent the slope (for 10 min) of each curve, following the addition of each compound, at A_{350nm} (Fig. 2) relative to Taxol

Microtubule bundle formation in cultured cells

Figure 4 illustrates the effects of eleutherobin and its derivatives on the morphology of cellular microtubules following exposure of cells to 2 μ*M* of each compound for 5.5 h at 37 °C. The extensive microtubule bundling observed in eleutherobin-treated cells (Fig. 4C), was identical to that seen in Taxol-treated cells (Fig. 4B). Neo-eleutherobin treatment (Fig. 4D) also resulted in bundle formation. In this case, however, the extent of bundling was reduced compared to Fig. 4B, C. Compounds SKBII.294 (Fig. 4E), SKBII.296 and SKBII.298 (not shown) induced less bundle formation than neo-eleutherobin, and the quantity was significantly reduced when compared to either eleutherobin or Taxol. Cells exposed to SKBIII.13 (Fig. 4F) displayed the same morphology, a fine microtubule network without bundle formation, as control cells treated with 0.1% DMSO only (Fig. 4A). Multiple asters that are known to be present in mitotic cells treated with Taxol [23], were also seen in eleutherobin and neo-eleutherobin-treated cells (data not shown). The number of mitotic asters correlated with the extent of microtubule bundling in interphase cells.

Discussion

This report describes the structure-activity profiles of five eleutherobin analogs based on their cytotoxicity, and their ability to polymerize and stabilize microtubules in the absence of GTP and to induce microtubule bundle formation in cells. In addition, their cross-resistance in three Taxol-resistant cell lines whose resistance arises from different mechanisms was examined.

Neo-eleutherobin, an analog that bears a carbohydrate domain (L-β-2′-*O*-acetyl arabinose) enantiomeric with that of the natural product, was less active than eleutherobin in all of the parameters examined. Therefore, the nature of the sugar moiety, while not absolutely critical for activity, definitely influences the potency of the compound. In Taxol-resistant SKVLB and J7-T3-1.6 cells which overproduce P-glycoprotein, both eleutherobin and neo-eleutherobin demonstrated cross- resistance, suggesting that they are substrates for P-glycoprotein. However, neither of these compounds showed cross-resistance to Taxol-resistant A549 cells which do not display the MDR phenotype. It has recently been shown that eleutherobin demonstrates cross-resistance in an *MDR1*-expressing cell line derived from human colon carcinoma HCT116 cells [16].

The compounds SKBII.294, 296 and 298 are derivatives of eleutherobin which lack the sugar moiety at the C15 position. Both SKBII.294 and 296 exhibited 54% of the microtubule polymerization activity of eleutherobin and reduced bundle formation in cultured cells. Cytotoxicity assays indicated that SKBII.294 was more cytotoxic than SKBII.296 in the human cell lines tested. Compound SKBII.298 with a hydroxymethyl group at the C3 position, was the least active compound in this subgroup. While these studies were being completed, compounds with the same structures as SKBII.294 and 298 were reported to have equal activity in tubulin polymerization assays [20].

Surprisingly, compounds SKBII.294 and 296 appear to be considerably less attractive substrates for P-glycoprotein compared to eleutherobin and neo-eleutherobin, suggesting that removal of the sugar or its replacement with an *O*-acetyl at C15 results in compounds that are essentially not cross-resistant to a cell line that produces high levels of P-glycoprotein. This result was unexpected since one might predict that loss of the sugar moiety would confer increased hydrophobicity and therefore enhance affinity for P-glycoprotein. The sugar moiety may be essential for maintaining a conformation of the drug that allows it to interact with P-glycoprotein. Although SKBII.296 demonstrated no cross-resistance to SKVLB cells that express high levels of P-glycoprotein, it manifested considerable cross-resistance to the Taxol-resistant cell line not expressing P-glycoprotein. These findings indicate the importance of the glycoside side chain of eleutherobin in modulating drug resistance.

The SKBIII.13 derivative differs from the other compounds in that it bears a free hydroxy group rather that an *N*(1)-methylurocanic acid ester at the C8 position. This compound is biologically inactive. Compared to eleutherobin, its cytotoxicity is greatly reduced and it no longer has the ability to polymerize microtubules. These findings indicate that the *N*(1)-methylurocanic acid ester, or some substituent at this position, is critical for the biological activity of eleutherobin. After these studies were completed, a similar conclusion was reached in a study of sarcodictyin analogs [20]. Clearly, changes in chemical structure can result in differences in biological activity that should be taken into consideration when designing new eleutherobin analogs for clinical studies.

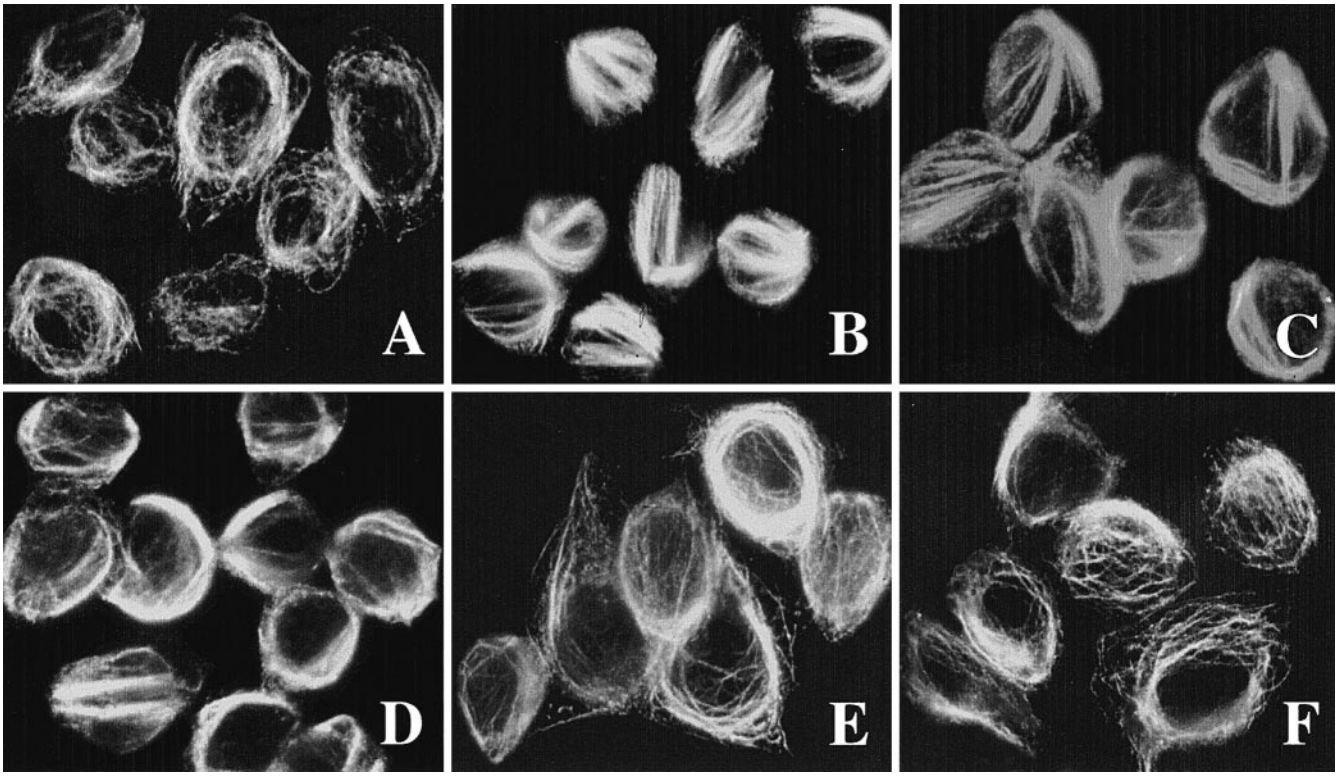


Fig. 4 Immunofluorescence images of NIH3T3 cells labeled with β-tubulin antibody following exposure to different drugs at 2 μ*M* (**A** DMSO, **B** Taxol, **C** eleutherobin, **D** neo-eleutherobin, **E** SKBII.294, **F** SKBIII.13)

In 1979, when the mechanism of action of Taxol was first determined, the polymerization of stable microtubules by a small molecule had not been previously described. Recently, in addition to eleutherobin, two other natural products, the epothilones [2] and discodermolide [10, 11], have been reported to be cytotoxic to cells and to have a very similar mechanism of action to that of Taxol. Although the antitumor activity of Taxol has been well documented in humans, it is not known if these newly discovered compounds will be useful cancer chemotherapeutic drugs. The introduction of new drugs into the clinic that may have different pharmacokinetic and pharmacodynamic properties from Taxol and also distinct toxicities and mechanisms of resistance, could prove advantageous for the cancer patient. Structure-activity studies have been reported for the epothilones [18, 27], and taken together with the data reported in this paper for the eleutherobins, may help to elucidate a common pharmacophore in these structurally distinct molecules.

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