Deoxynyobomycin Is a Selective Anti-Tumor Agent Inducing Apoptosis and Inhibiting Topoisomerase I

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Deoxynyobomycin was identified as an inducer of p21WAF1 gene following screening using a reporter, p21/luciferase. The present study examined its anti-proliferative effect on human tumor cell lines. Deoxynyobomycin selectively inhibited growth of human osteoblastic sarcoma Saos-2, gastric cancer TMK-1, and monocytic leukemia THP-1 cells, but did not affect survival of normal human fibroblasts at doses up to 5 μg/mL. Results from an assay system using a panel of 39 human cancer cell lines indicated that deoxynyobomycin has selective cytotoxic activity against lung carcinoma cell lines. Deoxynyobomycin induced apoptosis in Saos-2, TMK-1, and THP-1 cells as revealed by DNA fragmentation and TUNEL assays. It inhibited topoisomerase I but not topoisomerase II. These results suggest that deoxynyobomycin may be useful in cancer chemotherapy.

Key words p21/WAF1; deoxynyobomycin; apoptosis; topoisomerase I

The p21/WAF gene is a target for a tumor suppressor p53, and encodes an inhibitor of cyclin-dependent protein kinase, thereby negatively regulating cell cycle progression. Its expression is induced by a variety of signals including cellular senescence, radiation, cancer chemotherapeutic agents, and cytokines, in both a p53-dependent and -independent manner. Therefore, activation of p21/WAF1 is a sensitive ways of detecting biologically active substances.

From this background, it is expected that substances that cause induction of these cdk inhibitors will be potential anti-cancer agents, and the p21 gene seemed the most promising target for screening such active substances. We have established screening systems to isolate active substances that increase the expression of p21 gene, and identified several known antibiotics. In these systems, human osteosarcoma (Saos-2) or gastric carcinoma (TMK-1) cells were stably transformed with luciferase reporter under transcriptional control of human p21 enhancers, and culture fluids of Streptomyces were tested. We previously reported the identification of biologically active substances from this screening using transcriptional activation of p21/WAF1 promoter and a reporter, luciferase. During the course of such screening, deoxynyobomycin was found to activate the p21/WAF1 promoter, and exhibited cell type-specific cytotoxicity in several human cell lines.

Deoxynyobomycin is a derivative of nybomycin which was first chemically prepared, and then found as a fermentation product of Streptomyces hyalinum. Although more potent than nybomycin, its clinical use has been hampered by its solubility. In the present study, we examined cytotoxic specificity of deoxynyobomycin using several human cancer cell lines, and investigated its mode of action.

Materials and Methods

Luciferase Assay Stable transformants of human osteoblastic cells (Saos-2) were isolated by transfection of plasmids, pRc/CMV that confers G418-resistance and a reporter (WWP/Luc), with selection in G418-containing medium as described previously using clone #22. Cells were grown in 96-well plastic plates (1×10^4 cells/well), and incubated in the presence of drugs for 24 h. Luciferase activity was determined using a luciferase assay kit (Promega, Madison, WI) according to the manufacturer’s protocol. All the assays were carried out at least three times. Deoxynyobomycin was purified from culture filtrates of Streptomyces sp. MJ530-NF11 by ourselves using butylacetate extraction, silica gel chromatography, and crystallization from ethyl acetate.

Apoptosis Assay DNA fragmentation was assayed by agarose gel electrophoresis. After treatment with the drug, cells were harvested, washed in PBS and lysed in lysis buffer (10 mm Tris—HCl, pH 8.0, 100 mm NaCl, 25 mm EDTA, 0.5% SDS, and 0.5 mg/ml DNase-free proteinase K). After incubation at 55 °C for 5 h, lysates were extracted with phenol/chloroform, chloroform/isoamyl alcohol, and DNA was precipitated with isopropanol. The pellets were dissolved in TE, incubated in the presence of RNase (40 μg/ml) for 1 h at 37 °C, and DNA precipitated by addition of ethanol. Five micrograms DNA was run on a 2% agarose gel, and stained with ethidium bromide.

TUNEL (terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling) assay was performed using an in situ cell death detection kit (Boehringer Mannheim GmbH, Mannheim) according to the manufacture’s protocol. Briefly, cells were trypsinized, washed twice in PBS, and fixed in 2% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature. After washing in PBS, cells were resuspended in 0.1% sodium citrate for 2 min on ice, and washed in PBS. Cells were then, resuspended in 50 μl TUNEL reaction mixture, and incubated at 37 °C for 1 h in a humidified chamber. Cells were placed on slides, mounted, and viewed under a fluorescence microscope.

Determination of Cytotoxicity in Cultured Cell Lines Cell viability was assessed by tetrazolium (MTT) assay. Cells (2×10^4/well) were cultured in 96-well plastic plates, and incubated with a drug for indicated times. Ten microliters MTT solution (5 mg/ml in PBS) was added to 100 μl culture
medium, and incubated for 4 h at 37°C to generate a blue reaction product. The reaction product was extracted with 100 μl isopropanol containing 40 mM HCl and the absorbance at 570 nm determined.\textsuperscript{19,20}

**A Human Cell Line-Panel for Screening Anticancer Drugs** This assay was performed using the anticancer drug screening model developed at the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. The screening panel consisted of 39 human tumor cell lines representing five breast, six central nervous system, five colon, seven lung, one melanoma, five ovarian, two renal, six stomach, and two prostate cancer cell lines. Cellular viability was assessed by protein binding staining using sulforhodamine B (SRB) assay. Cells were plated in 96-well plates (1×10^4 cells/well) and, after 24 h, they were treated with the drug for 48 h. At the end of incubation, cells were fixed in situ by adding 50 μl cold 50% trichloroacetic acid (final concentration of 10%), and incubated for 60 min at 4°C. Cells were washed three times with deionized water and air-dried. 100 μl SRB solution (0.4% in acetic acid) was added to each well, and incubated for 10 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid, and plates were air-dried. Bound dye was solubilized with Tris buffer, and the optical density was determined on a microplate reader at 515 nm. The concentration of drug needed to inhibit proliferation of tumor cells varied, and produced a characteristic pattern called a “finger print” (mean graph). This fingerprint was analyzed by computer using a data-base program.\textsuperscript{19,20}

**Topoisomerase Activity** The activity of topoisomerase I and II was assayed by DNA-relaxation and -decatenation assays, respectively. For the relaxation assay, topoisomerase I (fetal bovine thymus, Takara Shuzo Co., 1 unit) and pBR322 DNA (0.5 μg) were incubated in 20 μl reaction mixture (10 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM MgCl₂, 1 mM ATP, 0.5 mM dithiothreitol, 0.5 mM EDTA, 15 μg/ml BSA)

![Graph](image)

**Fig. 1.** Induction of p21-Luciferase Gene by Deoxyxynobomycin
Saos\textsuperscript{2} cells were treated with deoxyxynobomycin for 24 h at the indicated doses, and luciferase activity was measured.

![Graph](image)

**Fig. 2.** Selective Growth Inhibition of Human Cell Lines by Deoxyxynobomycin
Cells were plated into 96-well plastic plates, and treated with deoxyxynobomycin at various doses of deoxyxynobomycin for 1 to 4 d. The percentage of viable cells was measured by the MTT method.
containing various concentrations of the drug at 37°C for 1 h. For the decatenation assay, topoisomerase II, purified from L1210 cells as described with some modification (1 unit), and kinetoplast DNA (0.5 μg) were incubated in 20 μl reaction mixture at 37°C for 1 h. Samples were run on a 2% agarose gel for the relaxation assay or 1% agarose gel for the decatenation assay.  

RESULTS AND DISCUSSION

Induction of p21/WAF1 Promoter Activity A stable cell line of Saos-2 (Saos#22) was established by transfection of p21/WAF1/luciferase and neomycin-resistant pRc/CMV plasmids as reported previously. From screening of approximately 1500 culture fluids of Streptomyces, we found several active substances that exhibited p21/WAF1/luciferase activity, and one of them was identified as deoxynystomycin. It activated p21/WAF1/luciferase in a dose-dependent manner (Fig. 1).

Cytotoxic Effect of Deoxynystomycin on Cultured Cell Lines Since the isolation of deoxynystomycin, its biological effects on mammalian cells have not been reported. In our previous paper, some of the substances that induced p21/WAF1/luciferase were shown to be anti-cancer drugs, such as Actinomycin or Bleomycin analogues. We, therefore, examined the cytotoxic effect of deoxynystomycin on

![Graph](image)

Fig. 3. Results of the Cytotoxicity Assay Using a Panel of Human Tumor Cell Lines

The mean graph was produced by computer processing of the GL50 as described in Materials and Methods. The logGL50 for each cell line is indicated. Bars extending to the right indicate sensitivity to deoxynystomycin, and those extending to the left indicate resistance to it. The scale is a logarithmic one. MG-MIG is the mean of the logGL50 values for 39 cell lines. Delta is the logarithm of the difference between the MG-MID and the logGL50 of the most sensitive cell line. Range is the logarithm of the difference between the logGL50 of the most resistant cell line and logGL50 of the most sensitive one. Br, CNS, Co, Lu, Me, Os, Re, Sl, and xP indicate breast, central nervous system, colon, lung, melanoma, ovarian, renal, stomach, and prostate cancer cell lines, respectively.
human osteosarcoma Saos-2, gastric carcinoma TMK-1, monocytic leukemia THP-1 and normal diploid fibroblast TIG-3 cells. Deoxyxobymycin was significantly cytotoxic to TMK-1 and THP-1 at doses that induced p21/WAF1 promoter. Saos-2 cells were less sensitive, but normal fibroblasts TIG-3 was resistant to the cytotoxic effect of deoxyxobymycin at doses up to 2.5 μg/ml (Fig. 2).

**Differential Growth Inhibition in a Panel of Human Cancer Cell Lines** We next examined the effects of deoxyxobymycin on *in vitro* growth of 39 human cancer cell lines that included various types of tumors using a panel developed in the Cancer Chemotherapy Center. Results of the comparison shown in Fig. 3 indicate that deoxyxobymycin is selectively toxic to breast carcinoma BSY-1 and lung carcinoma NCI-H522 and DMS114.

**Induction of Apoptosis by Deoxyxobymycin** As deoxyxobymycin was cytotoxic to some types of tumor cells, its mode of action was examined. One typical example is induction of apoptosis. In Fig. 4, it can be seen that deoxyxobymycin induced DNA fragmentation and increased TUNEL-positive cells in sensitive cells (TMK-1) but not in resistant TIG-3 cells. The fractions of TUNEL-positive cells treated with the drug under the condition described in the figure legend was about 70%. Deoxyxobymycin also induced apoptosis in Saos-2 and THP-1 cells (data not shown).

**Inhibition of Topoisomerase I** To identify a biochemical target for deoxyxobymycin, we examined its effects on several enzymes that are known to be inhibited by chemotherapeutic agents. Among them, topoisomerase I was found to be selectively inhibited in an *in vitro* assay (Fig. 5), whereas topoisomerase II activity was not affected at the doses used (data not shown). In Fig. 5, the inhibition of topoisomerase I by deoxyxobymycin seemed to be saturated in assays at 37°C, but dose-dependent inhibition was observed at 25°C.

Induction of apoptosis by inhibitors of topoisomerase I, such as NU/ICRF 505 and camptothecin, has been reported, and these drugs are also tumor-specific inhibitors. The results presented in the present communication indicate that deoxyxobymycin is a candidate anti-cancer drug.

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