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A Facile and Effective Screening Method for p21^{WAF1} Promoter Activators from Microbial Metabolites

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We have developed a novel p21^{WAF1} promoter activator screening system based on rapid and facile luciferase activity assay of a model cell system (H1299/tsp53-luc cells), a stable luciferase expression cell line established by transfecting H1299/tsp53 cells with a reporter gene construct pWWP-Luc-BSD. This plasmid was constructed by subcloning the 2.4 kb p21^{WAF1} promoter and a 2.6 kb of luciferase cDNA fragment activated by the p21^{WAF1} promoter into a pMAM2-BSD expression vector containing the blasticidin S deaminase gene (BSD). A BSD-resistant clone H1299/tsp53-luc#4, showing the highest response to p53 activation (by temperature shift from 37°C to 32°C) by luciferase production, was used for screening microbial culture broths. Among approximately 1200 screened samples, trichostatin A related compounds and a new compound, lucilactaene, were isolated. This provides an effective and facile screening system for p21^{WAF1} promoter activators which should be of considerable value in the rapid identification of new anticancer agents.

p21^{WAF1} was first cloned and identified as a potent cyclin-dependent kinase inhibitor[1,2]. It blocks cell growth in response to DNA damage in a p53-dependent or independent manner[3,4] and also blocks DNA replication by inhibiting PCNA (proliferation cell nuclear antigen) activity[5,6]. p21^{WAF1} plays a critical role not only in cell cycle control but also in terminal differentiation and apoptosis. Ectopic expression of p21^{WAF1} has been shown to promote differentiation of the megakaryoblastic leukemia cell line CMK[7] and the myelomonocytic cell line U937[8]. Expression of p21^{WAF1} is also involved in myogenesis[9], ceramide-induced apoptosis in hepatocarcinoma cells[10,11] and retinoic acid-induced apoptosis in Hep3B[12].

On the other hand, p53, a major negative regulator of cell growth, which has been found to be mutated or deleted in more than half of human tumors[13,14], exerts its cell growth inhibitory effect mainly by upregulating p21^{WAF1} [3,15,16]. Overexpression of p21^{WAF1} can stop some p53-deficient cell cycle progression at the G1 phase[17,18]. Furthermore, a number of agents have been proved to be p21^{WAF1} promoter activators, for example, TGF-β[19], butyrate[20], lovastatin[21], Ca^{2+} [22] and histone deacetylase inhibitor trichostatin A (TSA)[23,24] induce p21^{WAF1} expression through activating the Sp1-3 site in the p21^{WAF1} promoter. The p21^{WAF1} promoter was activated *via* an AP2 consensus binding site and cell growth was arrested after treatment of cells with phorbol ester (TPA) or okadaic acid (OA) in K562 human leukemia cells[25].

Taken together, p21^{WAF1} promoter activators may provide a potent therapeutic approach, and substances upregulating p21^{WAF1} expression are potential agents in cancer chemotherapy. Here, we report a reliable and rapid screening system being used to isolate p21^{WAF1} promoter activators from microbial metabolites. Besides several known compounds including tricostatin A (TSA) and its derivatives[26], a new compound, lucilactaene, which induced p21^{WAF1} promoter activation and G1 cell cycle arrest of H1299/tsp53 cells[27], was isolated using this screening system. The precise mode of action and the molecular target of lucilactaene is under further investigation.

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Materials and Methods

Materials

The wild $p21^{WAF1}$ promoter luciferase reporter construct pWWP-Luc was kindly provided by Dr. G. L. FIRESTONE (U.C. Berkeley, CA)[28]. The pMAM2-BSD expression vector which contains the blasticidin S deaminase gene was provided by Dr. I. YAMAGUCHI (RIKEN, Japan). The luciferase assay reagents were purchased from Promega (Madison, WI). Transfection reagent LIPOFECTAMINE PLUS™ was obtained from Life Technologies, Inc. (Rockville, MD)

Cell Culture

The cell line H1299/tsp53 was the gift of Dr. C. J. NORBURY (ICRF, U.K.). H1299/tsp53 cells were routinely cultured in RPMI1640 (GIBCO BRL, Rockville, MD) supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. 200 μg/ml of G418 was added to the medium to maintain the normal expression of tsp53 which is inactive at 37°C and is fully activated when the temperature is shifted to 32°C.

Reporter Plasmid Constructs

The construct pWWP-Luc was obtained by cloning a 2.4 kb $p21^{WAF1}$ promoter region and a 2.6 kb *Bam*H1 luciferase cassette into the *Hind*III and *Xho*I sites of pBluescript KS(+). In order to obtain a stable expression cell line, 2.6 kb of luciferase cDNA which was linked into the 3′ of the 2.4 kb of $p21^{WAF1}$ promoter was excised from the pWWP-Luc construct at the *Eco*RI and *Kpn*I sites, and subsequently this fragment was subcloned into the pMAM2-BSD expression vector encoding blasticidin S deaminase. This construct, which was designated as pWWP-Luc-BSD, was confirmed by sequencing to verify the correct reading frame. This plasmid DNA was purified with Quantum Prep Plasmid (Bio-Rad, Hercules, CA) before being used for transfection.

Stable Transfection

Stable transfection was performed by using the LIPOFECTAMINE PLUS™ reagent as directed by the manufacturer. H1299/tsp53 cells were distributed into 60 mm dishes at a density of 1×10^5/ml to achieve 50~70% confluency after overnight incubation at 37°C. Two μg of pWWP-Luc-BSD was used for transfection. Forty-eight hours after transfection, the cells were replated at one-third density in RPMI1640 medium containing 10% of FCS with 8 μg/ml of blasticidin S. The antibiotic-containing medium was changed every 3~5 days. Two weeks later, 10 isolated colonies were selectively removed with cloning cylinders into 35 mm dishes for further culture. The integration of the reporter gene into the host genomic DNA and its expression were confirmed by PCR using the genomic DNA as template and luciferase activity assay, respectively. The primer was designed according to the reading frame sequence of luciferase gene. Luc-sense is (5′-GCC ATT CTA TCC TCT AGA GGA ATG GAA CCG-3′) and Luc-antisense is (5′-AGC TGA TGT AGT CTC AGT GAG CC-3′). Clone#4, then, was selected for the screening due to its relatively higher response to p53 activation.

Screening Microbial Culture Broths by Luciferase Activity Assay

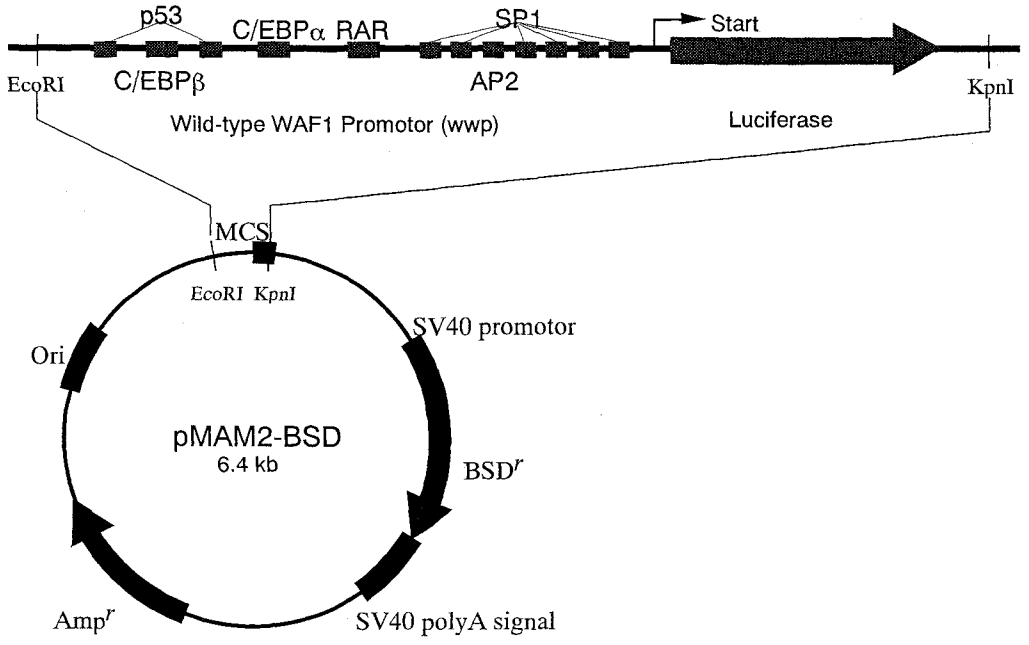
Culture broths of microorganisms, mainly actinomycetes and fungi, were diluted in 50% methanol. Stable luciferase expression clone#4 (named H1299/tsp53-Luc) was distributed into a light protective 96-well plate at a density of 5×10^3/100 μl/well 16 hours prior to treatment. For sample treatment, 1 μl of a ready-to-use broth or authentic drugs were added to each well and incubated at 37°C for 24 hours. Methanol (0.5%) and tricostatin A (500 ng/ml) were used as negative and positive controls, respectively. Luciferase activity was measured with a luminometer according to the protocol provided by Promega (Madison, WI). Briefly, the cells were washed with phosphate-buffered saline twice and the plate was subsequently loaded into a 1420 Multilabel Counter (Amersham Pharmacia Biotech, NJ). Twenty μl of lysis buffer were automatically injected into each well and shaken for 3 seconds. Three minutes later, 80 μl of luciferase substrate buffer was added to each sample and luminescence was measured as relative light units.

Results

Establishment of a Stable Cell Line Expressing a $p21^{WAF1}$ Promoter-induced Reporter Gene

The constrct pWWP-Luc-BSD shown in Fig. 1 was transfected into human lung carcinoma cells H1299/tsp53 which overexpress a temperature-sensitive mutant p53 protein. After a two-weeks cultivation, ten BSD-resistant clones were selected. A 900 bp band consistent with the length of luciferase PCR product was detected in all of 10 clones, suggesting that the selected clones had integrated the transfected *WWP-Luc* fragment into genomic DNA (data not shown). Next, we analyzed the p53-dependent activity of the luciferase gene by incubating cells at 32°C,

Fig. 1. The schematic diagram of pWWP-Luc-BSD construct.



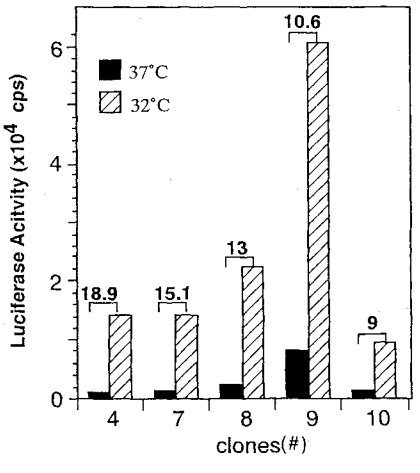
The *WWP-Luc* fragment (a 2.6 kb of luciferase cDNA linked at 3′ of 2.4 kb of $p21^{WAF1}$ promoter) was excised from WWP-Luc-pBluescript vector and subcloned at *Kpn*I and *Eco*RI sites of pMAM2-BSD vector.

which forms active p53 protein, and the p53-independent expression at 37°C, which contains inactive p53 protein, respectively. As shown in Fig. 2, the five tested clones, which express relatively high luciferase activity among the 10 selected clones, showed a p53-enhanced luciferase activity. Clone#9 showed the highest activity of luciferase at 32°C but the p53 independent luciferase activity at 37°C was also high. On the other hand, clone#4 showed nearly 20-fold higher expression at 32°C than at 37°C, therefore this clone was used for the next screening.

Screening for $p21^{WAF1}$ Promotor Activators in Microbial Culture Broths

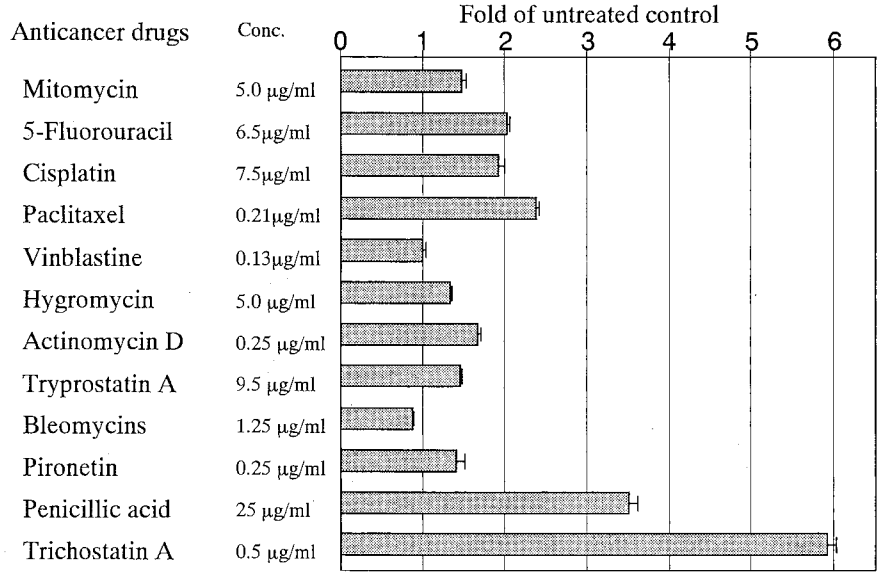
Using clone#4, we first checked its feasibility as a reliable screening model using known drugs. More than ten known antibiotics and chemotherapeutic agents such as 5-fluorouracil, mitomycin C, paclitaxel, vinblastine, cisplatin, penicillic acid and TSA were used as test drugs. As shown in Fig. 3, among the tested agents, TSA showed a significant induction of luciferase activity as expected; it is known that TSA induces $p21^{WAF1}$ transcription by increasing histone acetylation. Besides TSA, penicillic acid,

Fig. 2. p53-Dependent luciferase activity detected by temperature shift from 37°C to 32°C.



The clone#4 showed the highest response to p53 activation and this clone was used for screening. Results are the average of two independent experiments.

Fig. 3. Feasibility test of the screening system by authentic drugs.



Clone#4 cells were treated with authentic drugs and incubated at 37°C for 24 hours. The luciferase activity is shown as fold-increase relative to that of untreated cells. Results are the mean of triplicate determinations±S.E. from a representative experiment.

a differentiation inducer, also showed significant luciferase induction. DNA or tubulin attacking reagents slightly activated luciferase activity at the optimal concentrations. The luciferase activity of clone#4 in response to TSA did not change during cell passage (more than 3 months' culture, data not shown), indicating the stability of this novel recombinant cell line. These results suggested that the clone#4 cell line provided a stable and feasible screening system.

Subsequently , approximately 1200 samples prepared from the microbial culture broths were screened using the clone#4 cells at 37°C for 24 hours followed by luciferase activity assay. Among them, four samples, RK98-A15, RK98-A35, RK98-A74, and RK97-94 showed similar high ability to activate the reporter gene compared with the positive control agent TSA. After further purification and structure determination, TSA and its derivatives were isolated from sample RK98-A74[26]. In addition, a new compound, lucilactaene, was isolated from sample RK97-94[27]. These results added further data to confirm that this screening system provides an effective and reliable approach for rapid screening of biologically active compounds with potential chemotherapy candidates.

Discussion

$p21^{WAF1}$ was first identified as a potent cyclin-dependent kinase (CDK) inhibitor[1,2]. Subsequently, $p21^{WAF1}$ was identified as a direct transcriptional target of p53 which is involved in maintaining genomic integrity and the most frequently seen genetic mutations in human cancer[3,15,16]. Transfection of the $p21^{WAF1}$ gene expression vector into p53-deficient cells can inhibit cell overgrowth and stop the cell cycle at the G1 phase[16,17]. Thus, it is quite reasonable to assume that inducing $p21^{WAF1}$ expression may provide a novel strategy for controlling the overgrowth of p53-defective tumor cells. Furthermore, the induction of $p21^{WAF1}$ preferred cell cycle arrest to apoptosis, thus reducing dramatically the side effects of traditional anticancer drugs by inducing normal cell apoptosis at the same time.

Our present cell system has the following three characteristics. First, the established cell line H1299/tsp53-luc clone#4 is stable, since both the luciferase activity and its response to TSA and authentic samples did not change during cell passage (data not shown), indicating the feasibility of this screen system; second, this assay system

is based on the measurement of luciferase activity which can be automatically performed by a luminometer, so it is quantitated by a highly sensitive, rapid and easy-to-perform assay; third, most importantly, through this screen system, TSA, its derivatives and a new cell cycle inhibitor, lucilactaene, were isolated, validating the approach for the isolation of active and promising compounds.

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