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Napthalimidobenzamide DB-51630: a novel DNA binding agent inducing p300 gene expression and exerting a potent anti-cancer activity

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Abstract—Control of gene expression by small molecule compounds is a novel therapeutic strategy for cancer and usually it requires the presence of specific molecular recognition. The development of the compounds preferentially binding to the specific DNA sequence is one of the potential but very difficult approaches in this strategy. We designed and synthesized novel napthalimidobenzamide derivatives and analyzed their binding preferences to oligonucleotides by EtBr-displacement assay with DNA sequences, being essential fragments of the genes. To test whether these compounds modify the expression of specific genes, we analyzed the effect on the gene expression in AZ521 cells by differential display analysis using the compounds showing different characteristics in the recognition of specific DNA sequence. Among them, DB-51630, which showed approximately 350 times higher preferential binding to GC-repeats than to the AT and AA-repeating oligomers, caused the induction of a specific mRNA. The genetic sequence was identified to be the p300 gene by sequencing of the cloned cDNA. The p300 is a transcriptional co-activator protein that acts with other nuclear proteins in various cell differentiation and signal transduction pathways. This protein has intrinsic histone acetyltransferase activity and may act on chromatin directly to facilitate transcription. The increase of the amount of p300 mRNA increased after DB-51630 treatment by real time RT-PCR and Northern blot analysis. DB-51630 inhibited cell growth in various cancer cell lines at nanomolar range of concentrations, whereas p300 mRNA induction was observed at sub-nanomolar concentrations and the maximal induction occurred 8 h after DB-51630 treatment. In contrast, anti-cancer drugs such as doxorubicin, vincristine, cisplatin, etoposide, and actinomycin D did not increase p300 transcription. DB-51630 revealed potent anti-cancer activity against human solid tumor xenografts. Thus, we demonstrated the anti-cancer activity of DB-51630, which interacts with a specific DNA sequence, thereby inducing p300 gene expression and exhibited significant anti-cancer activity in human tumor xenografts. Furthermore, such compounds that bind to specific DNA sequences may not only control the expression of specific genes but also exert other mechanisms in the anti-cancer effect than those of classical DNA binding drugs.

1. Introduction

The molecular target therapy employing small molecule compounds against disease-prone or -modified proteins has been proven to be a suitable mean of therapeutic intervention. This is in contrast to the RNA-based drug development strategies, such as the targeting of specific mRNA by antisense technology, which have encountered numerous problems.[1] An alternative approach, being recently explored, is the displacement of critical transcription factors in the promoter regions of the genes involved in disease state by DNA binding compounds.[2,3] This approach requires the development of DNA binding compounds capable of recognizing specific sequences, which overlap the binding site of the relevant transcription factor.[3,4] Even if the compound of interest could bind the targeted site, the possibility of nonspecific binding cannot be ruled out. However, an obstacle may occur in the presence of the compounds exerting higher binding affinity to sequences supposedly not being a target, but being located in a gene encoding a protein essential for normal cellular function. As a result, serious toxic events may take place instead of

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therapeutic benefits. To ensure the selectivity of DNA binding agents to a target site, it is necessary to understand the relative affinity to an intended target versus other essential sequences. Therefore, it is necessary to establish a practical method for identifying the preferred DNA binding site of newly synthesized compounds in the approach of sequence-specific targeting by DNA binding compounds. Particularly, it seems to be important in the case of massive screening when the HTS technology is employed.

Many anti-cancer agents with their abilities to interact with DNA have been developed, but most of them have shown little DNA sequence binding selectivity and their clinical application was often accompanied by serious side effects resulting from the damage of normal tissues. For these reasons, the development of compounds that preferentially bind to specific DNA sequences and control specific gene expression is considered to be a promising therapeutic strategy for cancer treatment. However, an assumption must be made that both therapeutic and toxic effects result from an interaction of a compound with at least two different and unrelated DNA sequences. For instance, some therapeutic effects of doxorubicin are caused by the interaction with DNA, but the toxic effects, for example, cardio toxicity, are related to the induction of harmful reactive oxygen species.[5] The latter process is not related to a specific DNA binding of this valuable therapeutic agent, doxorubicin.

During our screening program, several novel napthalimidobenzamide derivatives exerting a preference in binding to specific DNA sequences were found. One of these compounds, DB-51630, modifies the expression of the p300 gene and has a potent anti-cancer activity. The protein p300 is a transcriptional co-activator possessing an ability to acetylate a variety of transcription factors and histones.[6,7] Histones are localized within transcriptionally active euchromatin and their interactions with p300 play a critical role in the regulation of transcription processes. A wide range of biological processes, such as the cell cycle, differentiation, and tumor growth, are regulated by p300.[8] It has the potential to activate p53 target genes and might thus act as a suppressor of tumor cell growth.[9–11]

Here we report the properties of DB-51630, whose DNA sequence preference, transactivation of p300, and other biological activities, including anti-cancer activity, may lead to the development of a promising anti-cancer agent.

2. Results and discussion

2.1. DNA sequence selectivity of naphthalimidobenzamide derivatives

The DNA binding activity of three novel naphthalimidobenzamide derivatives, shown in Figure 1, to poly dA-poly dT, poly d(A-T), poly d(G-C), poly dG-poly dC, poly d(A-G), and poly d(A-C), was determined

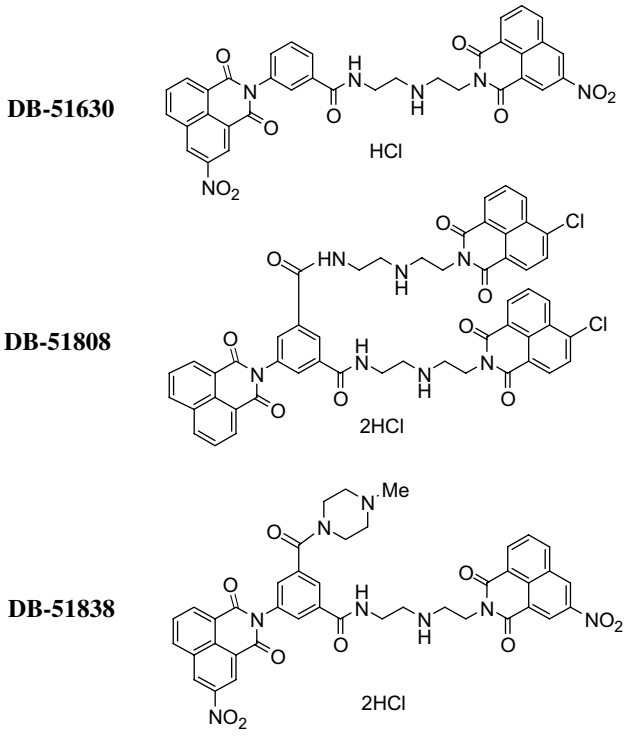

Figure 1. Chemical structures of DB-51630, 51808, and 51838.

and the corresponding IC_{50} values are shown in (Table 1). All tested compounds exerted an ability to interact with DNA. However, some differences were observed in their binding properties to poly dA-poly dT, poly d(A-T), poly d(G-C), and poly dG-poly dC oligonucleotides. The binding of DB-51630 and DB-51808 to poly d(A-T) appeared to be significantly weaker than that of DB-51838. DB-51630 and DB-51838 revealed significantly stronger affinity to poly d(A-G) than DB-51808. Further data demonstrated a very specific interaction of DB-51630 with poly dG-poly dC oligonucleotide. The strength of binding was approximately 350 times stronger than that to poly dA-poly dT and poly d(A-T). These data indicate that each of the naphthalimidobenzamide derivatives has different DNA sequence binding preferences.

The development of sequence-specific DNA binding agents has been an ongoing challenge in the field of bioorganic chemistry and molecular recognition. The minor-groove-binding bicyclic depsipeptide backbone forms the hydrogen bonds to DNA bases and has a modest sequence selectivity for poly d(G-C) rich

Table 1. DNA sequence selective binding of naphthalimidobenzamide derivatives

	DNA binding (IC_{50}, μM)					
	AT	AA	GC	GG	AG	AC
DB-51630	>50	>50	2.4	0.14	1.1	0.43
DB-51808	50	>50	3.7	1.1	18	2.1
DB-51838	3.0	26	0.95	0.43	0.73	1.8

The binding of naphthalimidobenzamide derivatives to double-stranded (ds)-DNA was monitored by using ethidium bromide displacement assay.

sites.[12–15] Similarly, the related bisintercalator triostin[16] also inhibits DNA replication and RNA synthesis,[17] and has a slight specificity toward poly d(A-T) sequences.[18] Synthetic bisintercalators were later constructed by linking two heterocycles, such as acridines,[19,20] methidium, anthracyclines,[21] and amonafide,[22] with a linker of varying lengths to maximize the bracketing of two (or more) base pairs between the intercalator sites. In general, these molecules have enhanced binding affinity to DNA but lack significant sequence specificity. The goal in development of this type of DNA binding agents is to prepare the molecules recognizing specific DNA sequences that may result in modification of specific gene expression, and in consequence leading to the desired pharmacological effects.

Many efforts were undertaken to develop DNA binding anti-cancer drugs that bind to specific DNA sequence. The suitability of that approach resulted in the synthesis of several compounds of which the DB-51630 was found to preferentially bind to GG-repeats more strongly than to AT- and AA- rich repeats.

2.2. Gene induction of p300 by DB-51630 in cancer cells

To identify the genes, which were altered by treatment with DB-51630, DB-51808, and DB-51838, the gene expression in AZ-521 cells (human gastric cancer) was determined in untreated control cells and in those pretreated with DB-51630, DB-51808, and DB-51838. This was achieved by utilizing a differential display technique.[23] After 8 h exposure of the cells, the total RNA was extracted and subjected to differential display using anchor primers in combination with primers. A single cDNA fragment was found to be upregulated by the treatment with DB-51630, as shown in the autoradiograph (Fig. 2). To identify the gene denoted in the differential display, the DNA fragment was eluted from the gel, reamplified, subcloned, and sequenced. GenBank database was subjected to the search for homologous sequences. A 98% identity was found between the sequences of the upregulated gene and the sequence of the known p300 gene (GeneBank accession no. U01877) (Fig. 3).

To confirm the upregulation of p300 transcripts in AZ-521 cells by DB-51630, Northern blot and RT-PCR analysis were performed. A Northern blot analysis of control and treated cells demonstrated a significant upregulation of the p300 transcripts by DB-51630, as shown in Figure 4. The quantification of the mRNA expressions, relative to the content of GAPDH RNA, suggested that the mRNA in the drug-treated cell was 4.5 times higher than that of control cells.

RT-PCR of total RNAs extracted from control and treated cells by using p300-specific primers also confirmed the upregulation of p300 mRNA expression following treatment with DB-51630. The quantitative analysis showed that the DB-51630 induced the expression of p300 mRNA by more than 3.5 times compared with the expression in the non-treated cell (Fig. 5A) or in the cell at the time 0 (Fig. 5B).

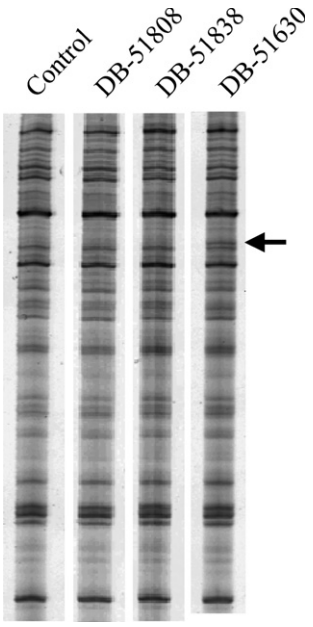

Figure 2. Autoradiography of the products of differential display electrophoresed on denaturing polyacrylamide gel. mRNA differential display reactions were performed as described in Experimental. The cDNA fragments transcribed from mRNA extracted from control and DB-51630, DB-51808, and DB-51838 treated cells at the concentration of 0.03 μM are shown. The arrow indicates the position of the band, which was then extracted and subjected to further analysis.

The specificity of p300 upregulation was confirmed in the experiments involving time- and dose-dependency of p300 mRNA induction by DB-51630, as determined by the RT-PCR analysis using p300 primers. To examine whether other genes were altered in the same manner, p21, a well known tumor suppresser gene,[24–27] was chosen as a candidate. The expression of p21 is regulated by p53, whose stability is regulated by ubiquitinations and degradation, both being under control of p300.[28] The acetylation of p53 is also controlled by p300 and promotes target gene activation by increasing the stability of the p53-p300-DNA complexes.[29] As shown in Figure 5A, p300 mRNA was upregulated by DB-51630 over a concentration of 0.0003 to 0.3 μM, however, concentration dependency was hardly observed. DB-51630 induced p300 expression at very low concentration. Presumably, there was an apparent concentration dependency, however within the range of biologically relevant concentrations the induction of p300 has already reached the plateau. In contrast to that, the p21 mRNA increased in a dose dependent manner. Significant increases in the transcripts were seen over a dose of 0.03 μM and a maximal upregulation of the p21 mRNA occurred at concentration of 0.3 μM. DB-51630 induced a time dependent expression of p300 mRNA (Fig. 5B). An increase in p300 mRNA was evident at a concentration of DB-51630 of 0.03 μM. There was an increase in the transcript initially seen within 2 h after addition of DB-51630, but the maximal upregulation of the p300 mRNA occurred at 8 h, and at 24 h the expression level was lower than that observed at 8 h. The expression of p21 mRNA was also upregulated by DB-51630 in a time-dependent manner. However, the

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Query:  503 TTNTAATGGCCTTNAACCTGACCCAAGTATGATCCGTGGCAGTGTGCCAAACCAGATGAT 444
Sbjct: 3248 TTCTAATGGCCCTCTACCTGACCCAAGTATGATCCGTGGCAGTGTGCCAAACCAGATGAT 3307

Query:  443 GCCTCGAATAACTCCACAATNTGGTTTGAATCAATTTGGCCAGATGAGCATGGCCCAGCC 384
Sbjct: 3308 GCCTCGAATAACTCCACAATCTGGTTTGAATCAATTTGGCCAGATGAGCATGGCCCAGCC 3367

Query:  383 CCNTATTGTACCCCGGCAAACCCNTCNTTTTCAGCGCCATGGACAGTTGGCTCAACCTGG 324
Sbjct: 3368 CCCTATTGTACCCCGGCAAACCCCTCCTCTTCAGCACCATGGACAGTTGGCTCAACCTGG 3427

Query:  323 AGCTCTCAACCCGCCTATGGGCTATGGGCCTCGTATGCAACAGCCTTCCAACCAGGGCCA 264
Sbjct: 3428 AGCTCTCAACCCGCCTATGGGCTATGGGCCTCGTATGCAACAGCCTTCCAACCAGGGCCA 3487

Query:  263 GTTCCTTCCTCAGACTCAGTTCCCATCACAGGGAATGAATGTAACAAATATCCCTTTGGC 204
Sbjct: 3488 GTTCCTTCCTCAGACTCAGTTCCCATCACAGGGAATGAATGTAACAAATATCCCTTTGGC 3547

Query:  203 TCCGTCCAGCGGTCAAGCTCCAGTGTCTCAAGCACAAATGTCTAGTTCTTCCTGCCCGGT 144
Sbjct: 3548 TCCGTCCAGCGGTCAAGCTCCAGTGTCTCAAGCACAAATGTCTAGTTCTTCCTGCCCGGT 3607

Query:  143 GAACTCTCCTATAATGCCTCCAGGGTCTCAGGGGAGCCACATTCACTGTCCCCAGCAT 86
Sbjct: 3608 GAACTCTCCTATAATGCCTCCAGGGTCTCAGGGGAGCCACATTCACTGTCCCCAGCTT 3665
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Figure 3. Comparison of DNA sequences from the differentially expressed mRNA clone and that reported for p300.

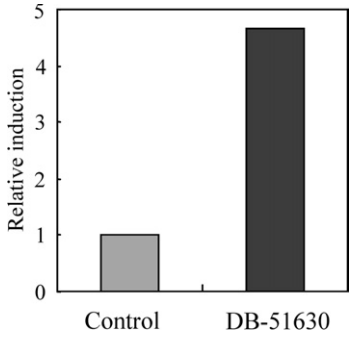

Figure 4. Relative band intensities of p300 expression in control and DB-51630-treated cells. A Northern blot analysis of mRNA extracted from AZ521 cells cultured in the absence (control) or presence of 0.03 μM DB-51630 for 8 h is shown. Band intensity was quantified by densitometry. The induction of the p300 gene in drug-treated cells was assessed by comparing the p300-GAPDH ratios in control and drug-treated cells.

maximal upregulation was observed at 24 h. As shown in Figure 6, the upregulation of p21 was not only induced by DB-51630, but was also observed after cell exposure to other anti-cancer agents such as doxorubicin (ADR), actinomycin D (actD), *cis*-diamminedichloroplatinum (CDDP), and etoposide (VP-16). However, none of these other anti-cancer agents significantly regulated the expression of p300 mRNA. These data indicate that DB-51630 induced specifically the upregulation of p300 gene transcription at non-toxic doses, while the other DNA binding anti-cancer agents did not exert such activity.

Gene suppression techniques have made extraordinary advances, most notably in the case of RNA interference (RNAi) technology.[30,31] It is still not clear how RNAi could be used as a general tool for regulation of transcriptional processes, and further as an anti-cancer drug. DNA-binding polyamides are the only cell-permeable molecules capable of recognizing specific, predetermined sequences of DNA. Furthermore, polyamide-peptide conjugates may be used as artificial transcription factors to up regulate transcription.[32–34] The most recent efforts have been devoted to the inactivation of the genes or their products given that their activity is related to pharmacological effects. However, not many studies have been undertaken to increase the activity of the genes belonging to the class of suppressor genes. An example of such an approach was reported for the induction of PTEN, a suppressor gene, by cyclooxygenase-2 (COX-2) inhibitors.[35] Gene activity induction by compounds that bind DNA in vivo has not been reported to date. DB-51630 might be one of the first discovered sequence selective DNA binding agents, which has an ability to induce gene expression.

2.3. In vitro and in vivo anti-cancer activity of DB-51630

The above presented molecular events, associated with the exposure of cancer cells to DB-51630, prompted us to examine whether those effects may contribute to anti-cancer activity, both in vitro and in vivo.

The anti- proliferating activity of DB-51630 against human gastric cancer, AZ-521, and human melanoma cell line, LOX, was analyzed. Growth inhibition of these cells by the agent was analyzed after exposure to various concentrations of DB-51630 for 72 h using the crystal violet method. DB-51630 showed a potent in vitro anti-cancer activity. The concentrations of DB-51630 that reduce the number of treated cells by one half (IC_{50}) were around 3 nM on both cell lines (Fig. 7). DB-51630 also showed a potent in vitro cytotoxicity against several other cancer cell lines originating from lung and colon (IC_{50} = 0.26 pM to 64 nM) (data not shown). These results clearly indicate that DB-51630 has a strong cytotoxic activity and is effective against several human cancer cell lines.

Human tumor xenografts are believed to be the best models for predicting drug efficacy in clinical settings. Therefore, in vivo anti-cancer activity of DB-51630 was determined against subcutaneously (s.c.)-implanted human tumor. The effects of DB-51630 on human tumor xenografts were examined by using AZ-521 and LOX human tumor xenografts. DB-51630 inhibited AZ-521 tumor growth by 50% at a dose of 10 mg/kg/day, on day

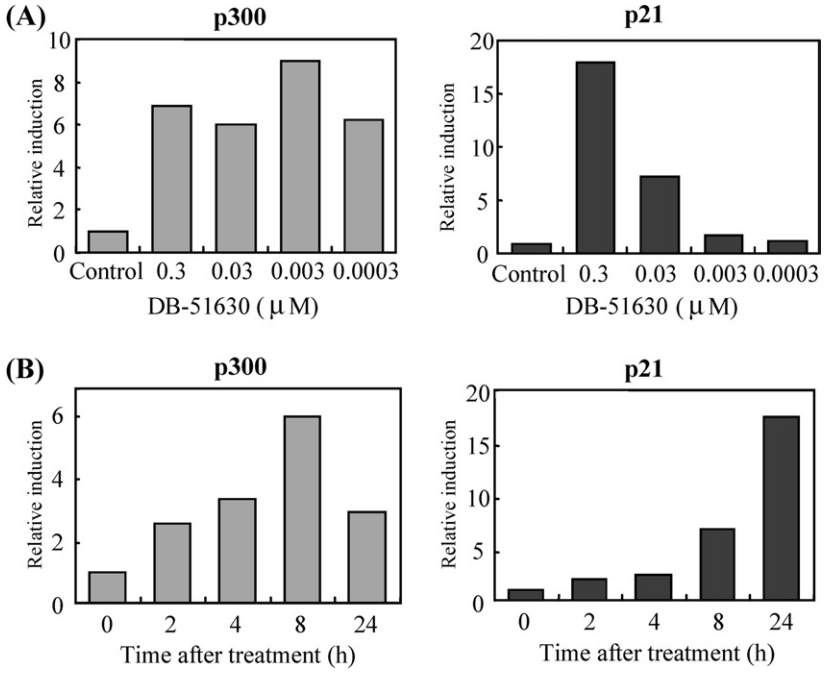

Figure 5. Dose response (A) and time course (B) of p300 and p21 mRNA expression following treatment of AZ521 cells with DB-51630. RT-PCR was used to measure the expression of p300 and p21 genes. Relative induction indicates the p300 or p21/GAPDH ratio, normalized to the vehicle control (A) or the control at 0 h (B). (A) Dose response: The cells were treated with either vehicle or DB-51630 (0.0003–0.3 μM) for 8 h. (B) Time course: The cells were treated with either vehicle or DB-51630 (0.03 μM) at indicated time intervals (in hours).

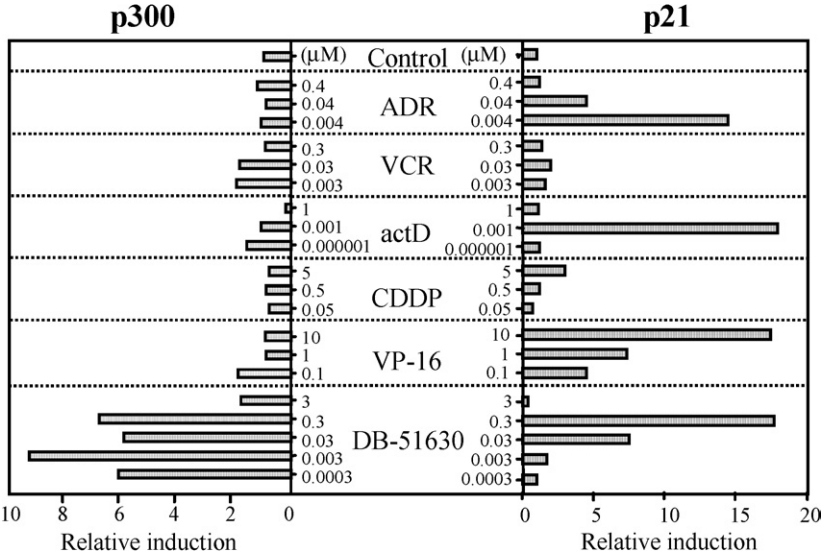

Figure 6. The induction of p300 gene during treatment of AZ-521 cells with various anti-cancer agents. AZ521 cells were treated with either vehicle or agents for 8 h. Range of doses of each agent were chosen based on in vitro cytotoxicity against AZ521 cells. Total RNAs were isolated as described in Experimental. The expression of p300 and p21 genes was analyzed by RT-PCR. Relative induction was calculated by comparison of p300/GAPDH ratios in control and drug-treated cell. Abbreviations used: ADR, doxorubicin; VCR, vincristine; actD, actinomycin D; CDDP, *cis*-diamminedichloroplatinum, VP-16, etoposide.

28 and that of LOX by 98% when given at a dose of 14.1 mg/kg/day on day 15 after tumor implantation (Table 3). In both models, there were no serious systemic side effects observed after treatment. Daily administration schedule, (qd × 5) × 2, appeared to be the most appropriate resulting in higher efficacy and lower toxicity when compared with an intermittent administration schedule.

One may expect that the strong poly d(G-C) and poly dG-poly dC preferences of DB-51630 may be responsible for anti-cancer activity. However, actinomycin D, a poly d(G-C) preference drug, appeared to be more toxic than DB-51630 (data not shown), so the different sequence preference of DB-51630 might have caused the induction of the p300 gene, whose product acts as

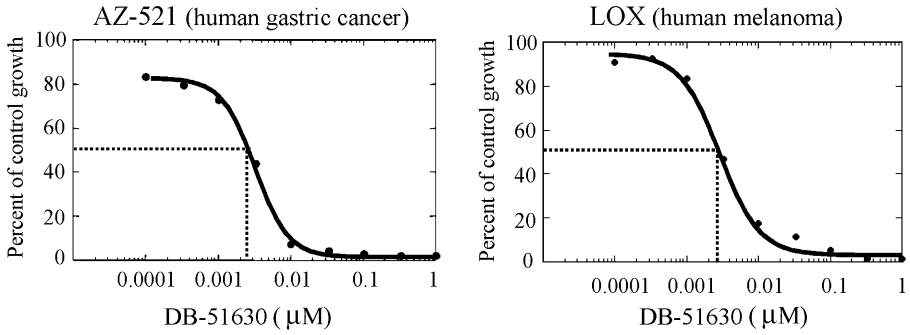

Figure 7. DB-51630 inhibits the growth of AZ-521 and LOX cancer cells. AZ-521 and LOX cells (1×10^3), seeded in a 96-well microplate, were incubated with DB-51630 for 3 days. The IC_{50} value was determined by crystal violet dye exclusion assay.

Table 2. Oligonucleotide sequences used as the primers for RT-PCR amplification

Transcript	Sequence
p300 F-Primer	5′-GACCCTCAGCTTTTAGGAATCC
p300 R-Primer	5′-TGCCGTAGCAACACAGTGTCT
p300 TaqMan-Probe	5′-ACAGTATCAGGAGCCCTGGCAGTATGTCGA
GAPDH F-primer	5′-CTTCACCACCATGGAGAAGGC
GAPDH R-primer	5′-GGCATGGACTGTGGTCATGAG
GAPDH TaqMan-Probe	5′-CCTGGCCAAGGTCATCCATGACAACTTT

Table 3. Efficacy of DB-51630 against human tumor xenografts in nude mice

Tumor	Group	Schedule	Dose (mg/kg/day)	Tumor weight (g)	T.W.I (%)
AZ-521	Control			6.986 ± 2.382	50
	DB-51630	(qd × 5) × 2	10	3.499 ± 1.953	
LOX	Control			3.167 ± 2.119	98
	DB-51630	(qd × 5) × 2	14.1	0.050 ± 0.038	

BALB/c nude mice with s.c.-implanted human tumors of the size volume between 50 and 300 mm^3 were divided into experimental groups on day 0. The mice were treated i.v. with the test agent from day 0. The mice were sacrificed on day 15 (LOX) or day 28 (AZ-521), and the tumors were removed and weighed. Anti-cancer activity was evaluated as the percentage of tumor weight inhibition (T.W.I.) compared to the mean tumor weight in the control group, according to the following formula: T.W.I. (%) = $(1 - T/C) \times 100$. T and C represent the mean tumor weight in test and control groups, respectively.

a tumor suppressor, resulting in cancer cells growth inhibitory effects.

The p300 was also identified as a cellular target of the adenoviral oncoprotein E1A.[36] E1A binds to various cellular proteins, including the Rb tumor suppressor protein family, and its gene alternations were found in various human tumors,[37–41] so the p300 was thought to be a tumor suppressor. Furthermore, p300 is a transcriptional co-activator that can acetylate a variety of transcription factors and histones.[42] The p300 protein is endowed with acetyltransferase activity not only related to histone proteins[43,44] but also involving transcription factors and the transcription apparatus.[45] The role of the p300 protein in the transcriptional process involves the interaction with transcription factors.[46] Therefore, the p300 induction by DB-51630 may cause changes in the modulation of the transcription processes. The p300 was also identified as activator of transforming growth factor (TGF)-β-dependent transcription signaling pathway.[47] Therefore, the integration of these mechanisms may cause the observed anti-cancer activity by DB51630.

3. Conclusion

DB-51630 specifically induced p300 gene expression in cancer cells at a non-toxic concentration. DB-51630 also showed a potent anti-cancer activity both in vitro and in vivo. The relationship between the DNA sequence preference of DB-51630 and its p300 gene induction is still not clear. But the data demonstrate that sequence-specific DNA binding agents, DB-51630, may control transcription of tumor-related genes and thereby inhibit tumor growth.

4. Experimental

4.1. Cell lines

KB, AZ-521, and LOX cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). These cells were cultured in RPMI-1640 (Nissui Seiyaku Co., Tokyo, Japan), containing 10% fetal bovine serum and 0.292 mg/mL L-glutamine. All cells were

cultured at 37 °C in a humidified chamber containing 5% CO_2. For in vivo experiment, AZ-521 and LOX were maintained s.c. in BALB/c nu/nu mice.

4.2. Animals

Specific pathogen-free, 6-10 weeks old, male BALB/c nu/nu mice were obtained from commercial source (Clea, Tokyo, Japan). The animals were maintained under pathogen free conditions with food and water provided ad libitum according to the internal regulation required for the in vivo drug evaluation. All experiments were carried out according to the guidelines for the Care and Use of Animals approved by the Council of the Physiological Society of Japan.

4.3. Chemicals and reagents

The test compounds, namely, DB-51630, DB-51808, and DB-51838 (Fig. 1), were synthesized by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) according to published procedures.[48] The synthesis method of these compounds will be presented in a separate publication. Other drugs were purchased from the following commercial sources: etoposide (VP-16) and *cis*-diamminedichloroplatinum (CDDP) from Nippon Kayaku Co., Ltd. (Tokyo, Japan), doxorubicin (ADR) from Kyowa Hakko Co., Ltd. (Tokyo, Japan), vincristine (VCR) from Eli Lilly, Ltd. (Tokyo, Japan), and camptothecin (CPT) and actinomycin D (actD) from Sigma–Aldrich (St. Louis, USA). Poly dA-poly dT, poly d(A-T), poly d(G-C), poly dG-poly dC, poly d(A-G), and poly d(A-C) oligonucleotides were obtained from Amersham Biosciences (NJ, USA).

4.4. Ethidium bromide displacement assay

Ethidium bromide displacement assay was carried out as described previously.[49,50] Measurements were done on a MTP-32 microplate reader (CORONA, Tokyo, Japan). The concentration of each drug that reduced fluorescence intensity by one half of the initial value (IC_{50} values) was determined and the IC_{50} value served as a determinant of binding affinity.

4.5. Differential display

Changes in gene expression were determined by differential display.[23] Briefly, AZ-521, a human gastric cancer cell line, was treated with one of the test agents (0.03 μM) or the vehicle control for 8 h. RNA was isolated from the pretreated cells by using an Isogen™ (Nippongene, Toyama, Japan) kit according to the manu- facturer's specifications. First strand cDNAs were amplified by using oligo(dT) primer and an arbitrary primer. Amplified cDNAs labeled with a radioisotope were electrophoresed on a denaturing polyacrylamide gel and visualized by autoradiography. Up- and downregulated cDNAs were re-amplified, subcloned into the pGEM-T vector (Promega, WI, USA), and sequenced. Nucleotide sequences were analyzed by comparison with the GenBank/EMBL nucleotide database.

4.6. Northern blot

AZ-521 cells were treated with either the vehicle or DB-51630 for 8 h before isolation of total RNA using SV Total RNA Isolation System™ (Promega, WI, USA). For Northern Blot, further purification was carried out using DYNABEADS mRNA Purification Kit™ (Dynal Biotech, Oslo, Norway). Agarose-formaldehyde gel electrophoresis was used for size fractionation of mRNA, and analysis was performed later transferring to Hybond-N+™ membrane (Amersham Biosciences, NJ, USA). p300 RNA was determined by hybridization of a specific digoxigenin (Dig)-labeled p300 RNA probe to the mRNA bound to the membrane. GAPDH RNA was determined by re-hybridization of a specific digoxigenin (Dig)-labeled GAPDH RNA probe to the mRNA bound to the membrane as a control. Alkaline phosphatase-labeled anti-Dig-antibody and CDP-STAR™ (Amersham Biosciences, NJ, USA) were used to detect each RNA probe. Intensity of relevant bands was quantified by densitometry.

4.7. RT-PCR

AZ-521 cells were treated with the test agents for 8 h. An SV Total RNA Isolation System™ (Promega, WI, USA) was used to isolate total RNA. The primers and TaqMan® probes used for TaqMan® PCR are shown in Table 2. An ABI PRISM™ 7700 Sequence Detector (Applied Biosystems, CA, USA) and TaqMan® EZ RT-PCR Kit (Applied Biosystems, CA, USA) were used to acquire and analyze data. The expression level of p300 was determined relatively to the expression of the GAPDH, a house keeping gene. The sequences of the synthesized primers are shown in Table 2. Differential expression of p300 and p21 in agent treated cells was assessed by comparing the p300 and p21/GAPDH ratios in control cells and drug-treated cells.

4.8. In vitro anti-cancer activity evaluation

Cancer cells were plated at a density of 1000 cells/well in 96 well MICROTEST™ plates (BD Biosciences, CA, USA) and cultured overnight. After cell attachment, the test compound was applied at several concentrations and the cells were incubated for additional 72 h. After completion of cell culture, the cells were fixed with 2.5% glutaraldehyde for 15 min and washed 3 times with water. Cells were stained with a 0.05% solution of crystal violet for 15 min and washed three times with water. Crystal violet was extracted with 0.2 mL of 0.05 M NaH_2PO_4–ethanol, and a MTP-32 microplate reader (CORONA, Tokyo, Japan) was used to measure the optical density at a wavelength of 540 nm. The IC_{50} value was defined as the drug concentration needed to produce a 50% reduction of optical density relative to the control, untreated cells.

4.9. In vivo anti-cancer activity against human tumor

Specific pathogen-free male BALB/c nu/nu mice were obtained commercially. Animals were maintained under pathogen free conditions and age-matched 10 weeks of

age at the onset of each experiment. A $2 \times 2 \times 2\ mm^3$ fragment of a human tumor was subcutaneously implanted into BALB/c nude mice. The experimental groups consisted of seven mice each. When the tumor in these animals reached the size of 50–300 mm^3, test agents were administered i.v. on a $qd \times 5 \times 2$ schedule at various doses, beginning on day 1. The body weight was measured twice a week to monitor the toxic potential of the test agent. The mice were sacrificed on day 15 (LOX) or day 28 (AZ-521), and the tumors were removed and weighed. Anti-cancer activity was evaluated as the percentage of tumor weight inhibition (T.W.I.) compared to the mean tumor weight in each test group according to the following formula:

$$\text{T.W.I. } (\%) = (1 - T/C) \times 100$$

T and C represent the mean tumor weight in the test group and the control group, respectively.

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References and notes

1. Ma, D. D.; Rede, T.; Naqvi, N. A.; Cook, P. D. *Biotechnol. Annu. Rev.* **2000**, *5*, 155.
2. Dervan, P. B.; Burli, R. W. *Curr. Opin. Chem. Biol.* **1999**, *3*, 688.
3. Laurance, M. E.; Starr, D. B.; Michelotti, E. F.; Cheung, E.; Gonzalez, C.; Tam, A. W.; Deikman, J.; Edwards, C. A.; Bardwell, A. J. *Nucleic Acids Res.* **2001**, *29*, 652.
4. White, S.; Baird, E. E.; Dervan, P. B. *Chem. Biol.* **1997**, *4*, 569.
5. Simpson, C.; Herr, H.; Courville, K. A. *Clin. J. Oncol. Nurs.* **2004**, *8*, 497.
6. Chang, W.; Gruber, D.; Chari, S.; Kitazawa, H.; Hamazumi, Y.; Hisanaga, S.; Bulinski, J. C. *J. Cell Sci.* **2001**, *114*, 2879.
7. Vo, N.; Goodman, R. H. *J. Biol. Chem.* **2001**, *276*, 13505.
8. Goodman, R. H.; Smolik, S. *Genes Dev.* **2000**, *14*, 1553.
9. Klochendler-Yeivin, A.; Yaniv, M. *Biochim. Biophys. Acta* **2001**, *1551*, M1.
10. Grossman, S. R. *Eur. J. Biochem.* **2001**, *268*, 2773.
11. Ionov, Y.; Matsui, S.; Cowell, J. K. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 1273.
12. Waring, M. J.; Wakelin, L. P. *Nature* **1974**, *252*, 653.
13. Wakelin, S. P.; Waring, M. J. *Biochem. J.* **1976**, *157*, 721.
14. Shafer, R. H.; Waring, M. J. *Biopolymers* **1980**, *19*, 431.
15. Van Dyke, M. M.; Dervan, P. B. *Science* **1984**, *225*, 1122.
16. Wang, A. H.; Ughetto, G.; Quigley, G. J.; Hakoshima, T.; van der Marel, G. A.; van Boom, J. H.; Rich, A. *Science* **1984**, *225*, 1115.
17. Ward, D. C.; Reich, E.; Goldberg, I. H. *Science* **1965**, *149*, 1259.
18. Lee, J. S.; Waring, M. J. *Biochem. J.* **1978**, *173*, 115.
19. Le Pecq, J. B.; Le Bret, M.; Barbet, J.; Roques, B. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 2915.
20. Wakelin, L. P.; Romanos, M.; Chen, T. K.; Glaubiger, D.; Canellakis, E. S.; Waring, M. J. *Biochemistry* **1978**, *17*, 5057.
21. Leng, F.; Priebe, W.; Chaires, J. B. *Biochemistry* **1998**, *37*, 1743.
22. Brana, M. F.; Cacho, M.; Garcia, M. A.; de Pascual-Teresa, B.; Ramos, A.; Dominguez, M. T.; Pozuelo, J. M.; Abradelo, C.; Rey-Stolle, M. F.; Yuste, M.; Banez-Coronel, M.; Lacal, J. C. *J. Med. Chem.* **2004**, *47*, 1391.
23. Liang, P.; Pardee, A. B. *Science* **1992**, *257*, 967.
24. Xiong, Y.; Hannon, G. J.; Zhang, H.; Casso, D.; Kobayashi, R.; Beach, D. *Nature* **1993**, *366*, 701.
25. Gu, Y.; Turck, C. W.; Morgan, D. O. *Nature* **1993**, *366*, 707.
26. Harper, J. W.; Adami, G. R.; Wei, N.; Keyomarsi, K.; Elledge, S. J. *Cell* **1993**, *75*, 805.
27. Zhang, H.; Hannon, G. J.; Beach, D. *Genes Dev.* **1994**, *8*, 1750.
28. Grossman, S. R.; Deato, M. E.; Brignone, C.; Chan, H. M.; Kung, A. L.; Tagami, H.; Nakatani, Y.; Livingston, D. M. *Science* **2003**, *300*, 342.
29. Dornan, D.; Shimizu, H.; Perkins, N. D.; Hupp, T. R. *J. Biol. Chem.* **2003**, *278*, 13431.
30. Agami, R. *Curr. Opin. Chem. Biol.* **2002**, *6*, 829.
31. Kamath, R. S.; Fraser, A. G.; Dong, Y.; Poulin, G.; Durbin, R.; Gotta, M.; Kanapin, A.; Le Bot, N.; Moreno, S.; Sohrmann, M.; Welchman, D. P.; Zipperlen, P.; Ahringer, J. *Nature* **2003**, *421*, 231.
32. Mapp, A. K.; Ansari, A. Z.; Ptashne, M.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3930.
33. Ansari, A. Z.; Mapp, A. K.; Nguyen, D. H.; Dervan, P. B.; Ptashne, M. *Chem. Biol.* **2001**, *8*, 583.
34. Arora, P. S.; Ansari, A. Z.; Best, T. P.; Ptashne, M.; Dervan, P. B. *J. Am. Chem. Soc.* **2002**, *124*, 13067.
35. Chu, E. C.; Chai, J.; Tarnawski, A. S. *Biochem. Biophys. Res. Commun.* **2004**, *320*, 875.
36. Eckner, R.; Ewen, M. E.; Newsome, D.; Gerdes, M.; DeCaprio, J. A.; Lawrence, J. B.; Livingston, D. M. *Genes Dev.* **1994**, *8*, 869.
37. Gayther, S. A.; Batley, S. J.; Linger, L.; Bannister, A.; Thorpe, K.; Chin, S. F.; Daigo, Y.; Russell, P.; Wilson, A.; Sowter, H. M.; Delhanty, J. D.; Ponder, B. A.; Kouzarides, T.; Caldas, C. *Nat. Genet.* **2000**, *24*, 300.
38. Giles, R. H.; Peters, D. J.; Breuning, M. H. *Trends Genet.* **1998**, *14*, 178.
39. Muraoka, M.; Konishi, M.; Kikuchi-Yanoshita, R.; Tanaka, K.; Shitara, N.; Chong, J. M.; Iwama, T.; Miyaki, M. *Oncogene* **1996**, *12*, 1565.
40. Petrij, F.; Giles, R. H.; Dauwerse, H. G.; Saris, J. J.; Hennekam, R. C.; Masuno, M.; Tommerup, N.; van Ommen, G. J.; Goodman, R. H.; Peters, D. J., et al. *Nature* **1995**, *376*, 348.
41. Sobulo, O. M.; Borrow, J.; Tomek, R.; Reshmi, S.; Harden, A.; Schlegelberger, B.; Housman, D.; Doggett, N. A.; Rowley, J. D.; Zeleznik-Le, N. J. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8732.
42. Chan, H. M.; La Thangue, N. B. *J. Cell Sci.* **2001**, *114*, 2363.
43. Bannister, A. J.; Kouzarides, T. *Nature* **1996**, *384*, 641–643.
44. Ogryzko, V. V.; Schiltz, R. L.; Russanova, V.; Howard, B. H.; Nakatani, Y. *Cell* **1996**, *87*, 953.
45. Bayle, J. H.; Crabtree, G. R. *Chem. Biol.* **1997**, *4*, 885–888.
46. Imhof, A.; Yang, X. J.; Ogryzko, V. V.; Nakatani, Y.; Wolffe, A. P.; Ge, H. *Curr. Biol.* **1997**, *7*, 689.
47. Suganuma, T.; Kawabata, M.; Ohshima, T.; Ikeda, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13073.
48. Noguchi, K.; Wakida, M.; Suzuki, K.; Yamada, Y.; Asao, T. PCT Int. Appl. WO 2000,001,672, January 13, 2000.
49. Bhattacharya, S.; Thomas, M. *Biochem. Biophys. Res. Commun.* **2000**, *267*, 139–144.
50. Lee, M. E.; Dyer, D. H.; Klein, O. D.; Bolduc, J. M.; Stoddard, B. L.; Koshland, D. E., Jr. *Biochemistry* **1995**, *34*, 378.