Chemical suppression of an oncogenic splicing variant of AIMP2 induces tumour regression

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AIMP2 (aminoacyl-tRNA synthetase-interacting multifunctional protein 2) is a potent tumour suppressor that induces apoptosis in response to various oncogenic signals. AIMP2-DX2, an exon2-deleted splicing variant of AIMP2, is up-regulated in lung cancer and competitively suppresses the pro-apoptotic activity of AIMP2, resulting in tumorigenesis. In the present study we report that BC-DXI01, a synthetic compound, specifically reduces the cellular levels of AIMP2-DX2 through selective degradation of the AIMP2-DX2 mRNA transcript. We found that BC-DXI01-mediated cell death positively correlates with AIMP2-DX2 expression in the lung cancer cell lines tested. Administration of BC-DXI01 in a AIMP2-DX2-driven tumour xenograft model led to reduced tumour sizes and volumes of up to 60% in comparison with vehicle-treated mice group, consistent with decreases in AIMP2-DX2 transcript and protein levels. Taken together, our findings suggest that tumorigenic activity of AIMP2-DX2 can be controlled by the small chemical BC-DXI01, which can selectively suppress the AIMP2-DX2 mRNA transcript.

Key words: aminoacyl-tRNA synthetase-interacting multifunctional protein 2 (AIMP2)-DX2, chemical suppression, lung cancer, mRNA, splicing variant.

INTRODUCTION

ARSs (aminoacyl-tRNA synthetases) are essential enzymes for protein synthesis, conjugating amino acids to their cognate tRNAs. In higher eukaryotes, nine different ARSs form a MSC (multi-tRNA synthetase complex) comprising non-enzymatic factors and AIMPs (ARS-interacting multifunctional proteins) [1]. The three AIMPs, AIMP1/p43, AIMP2/p38 and AIMP3/p18, function as scaffolding proteins for the assembly and integrity of the MSC. However, they are capable of dissociating from the MSC whereupon they participate in various regulatory pathways [1].

Among the AIMPs, AIMP2, which consists of four exons, is a potent tumour suppressor, which triggers growth-arrest signalling of TGF-β (transforming growth factor-β). This growth-arrest signalling occurs by its enhancement of ubiquitin-mediated degradation of FBP (FUSE-binding protein), which is a transcriptional activator of the proto-oncogene Myc [2]. In addition, upon TNF-α (tumour necrosis factor-α) stimulation, AIMP2 induces cell apoptosis by facilitating ubiquitination of TRAF2 (TNF-receptor-associated factor 2) [3]. AIMP2 also reinforces pro-apoptotic signalling by protecting the tumour suppressor p53 from MDM2 (murine double minute 2)-mediated degradation under DNA damage stress [4]. The anti-proliferative and pro-apoptotic roles of AIMP2 have been supported by an in vivo mouse model, whereby Aimp2-heterozygous mice, as compared with wild-type mice, were shown to be more vulnerable to tumorigenesis in lung, colon and skin carcinogenesis xenograft models [5].

Interestingly, AIMP2-DX2, an isoform of AIMP2 in which exon2 is deleted by alternative splicing, has been detected in cancer cell lines and tissues [6]. Like AIMP2, AIMP2-DX2 binds to FBP, TRAF2 and p53; however, it competes with AIMP2 for binding to these target proteins, consequently inhibiting the tumour-suppressive roles of AIMP2 [6,7]. The expression ratio of AIMP2-DX2 to AIMP2 positively correlates with the malignancy of lung cancers in patients, as well as chemoresistance of ovarian cancer [6,7]. These data suggest that AIMP2-DX2 might be an attractive target for developing cancer therapeutics.

Lung cancer is one of the most frequently occurring cancer types in humans, and is the leading cause of cancer-related death worldwide [8]. The need for an effective anti-lung cancer drug is obvious; however, current lung cancer treatment is principally dependent on cytotoxic drugs. Only a few targeted drugs are available to treat lung cancer due to the poor understanding of the mechanism of lung carcinogenesis, and, as a result, effective targets are scarce [9]. Since the expression of AIMP2-DX2 adversely affects lung cancer progression and patient survival [6], inhibitors of AIMP2-DX2 expression may prove effective therapeutic agents for lung cancer. Indeed, knockdown of AIMP2-DX2 in a lung cancer mouse model by RNAi has been shown to significantly suppress tumorigenesis in the lung [6]. However, an effective small-molecule inhibitor of AIMP2-DX2 has not yet been reported. In the present paper we report that a small molecule

Abbreviations used: ARS, aminoacyl-tRNA synthetase; AIMP, ARS-interacting multifunctional protein; AIMP2-F, full-length AIMP2; FBP, FUSE-binding protein; IHC, immunohistochemistry; MDM2, murine double minute 2; MSC, multi-tRNA synthetase complex; NSCLC, non-small-cell lung cancer; RT, reverse transcription; TNF, tumour necrosis factor; TRAF2, TNF-receptor-associated factor 2.

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termed BC-DXI01 specifically inhibits the expression of AIMP2-DX2 through down-regulation of its mRNA transcript, and we show the molecule has anti-tumorigenic activity in an in vivo mouse model.

EXPERIMENTAL

An expanded Experimental section is available in the Supplementary Online Information (at http://www.biochemj.org/bj/454/bj4540411add.htm).

Compounds

A total of 2231 small compounds were sorted from ChemDiv’s synthetic chemical library to maximize the coverage of structural diversity. To prepare BC-DXI01, ethyl 3-chloro-3-oxopropanoate (13.4 g, 0.089 mol) and triethylamine (1.25 ml, 0.089 mol) were added to a mixture of 4-aminobenzoic acid (10.0 g, 0.073 mol) in acetonitrile (200 ml). The reaction mixture was stirred at room temperature (25°C) for 4 h, and all volatiles were removed under reduced pressure. The residue was dissolved in water and acidified to pH 3 with concentrated HCl, the resulting precipitates were filtered and collected as an off-white solid (13.0 g, 75% yield): 1H-NMR (DMSO-d6, 300 MHz): δ 12.72 (s, 1 H), 10.46 (s, 1 H), 7.88 (d, 1 H, J 8.7 Hz), 7.65 (d, 1 H, J 8.7 Hz), 4.10 (q, 2 H, J 7.2 Hz), 3.47 (s, 2 H), 1.17 (t, 3 H, J 8.1 Hz); MS (ES +) m/z calculated for C28H41N5O8S (M+) as a white solid: 607.2, found 607.0.

Animal experiments complied with the University Animal Care and Use Committee guidelines at Seoul National University. The tumour mass was obtained by subcutaneous injection of H460 cell lines in 5-week-old BALB/c Slc-nu nude male mice. Tumour mass was extracted and dissected to 3 mm3 fragments and subcutaneously transplanted into healthy nude mice using a trocar and allowed to grow. When the tumour mass volume reached 80–117 mm3, mice were divided into control and treatment groups (n = 8 per group), and vehicle or BC-DXI01 (50 mg/kg) was introduced via intraperitoneal (4 days) and intrasubcutaneous (24 days) injections. The vehicle was prepared by mixing 10% DMSO and 90% mixture (TWEEN 80/PEG 400/distilled water, 16:4:25, by vol.). Mice were monitored every day and tumour volumes and body weights were checked two times a week. After killing the mice, tumour weights were measured and subjected to IHC (immunohistochemistry).

RESULTS

Identification of compounds which reduce the cellular AIMP2-DX2 level

Since AIMP2-DX2 was previously suggested as a novel therapeutic target against NSCLC (non-small-cell lung cancer) [6], we set up the firefly luciferase assay system to screen a chemical library (2231 chemicals) for small molecules that inhibit the cellular level of AIMP2-DX2 (see Supplementary Figure S1 at http://www.biochemj.org/bj/454/bj4540411add.htm). In the screening system, H460 cells were transfected with pGL2-AIMP2-DX2 to express luciferase-tagged AIMP2-DX2 and four compounds were identified to reduce showed an over 40% decrease in the luminescence of AIMP2-DX2 compared with the DMSO control were chosen. Selected compounds were further tested to monitor the cytotoxicity based on the MTT assay using WI-26 normal lung cells.

Small compound screening based on the luciferase assay

The AIMP2-DX2 gene was cloned into the pGL2 control vector by HindIII digestion to generate luciferase-tagged protein. pGL2-AIMP2-DX2 was transfected into cell lines using FuGene HD (Roche), according to the manufacturer’s instructions. After a 12 h incubation, cells were counted and seeded into 96-well plates (104 cells/well) and incubated for 12 h. A total of 2231 small compounds (ChemDiv) dissolved in DMSO were added to the pGL2-AIMP2-DX2-transfected H460 cell lines at a 10 μM concentration in serum-free medium. After a 4-h incubation, cells were lysed and analysed using a firefly luciferase assay system (Promega) to detect luminescence. The compounds which
cellular levels of AIMP2-DX2 by over 40% compared with control (Figure 1A). Those included compound 101, 4-[[3-ethoxy-1,3-dioxopropl]amino]-benzoic acid; compound 259, phenol, 4-[[bis(2-hydroxyethyl)amino]methyl]-2,6-bis(1,1-dimethylthyl); compound 266, 2,6-pyridinedicarboxylic acid, 2,6-bis[1-methyl-2-(1-methylethoxy)-2-oxoethyl] ester; and compound 1341, 1H-indene-1,3(2H)-dione, 2-(1,3-dihydro-3-oxo-2H-indol-2-ylidene). A further round of screening was performed using the MTT assay to select chemicals without cytotoxic activity against the normal lung cell line (Figure 1B). From this assay, compound 101 {4-[[3-ethoxy-1,3-dioxopropl]amino]-benzoic acid, CAS number 130217-49-1} (designated as BC-DXI01) was selected as the primary hit for further investigation (Figure 1C).

Specific suppression of the AIMP2-DX2 transcript by BC-DXI01

To examine the concentration-dependent inhibitory effect of BC-DXI01 on the AIMP2-DX2 level, 0.04, 0.4 and 4 μM BC-DXI01 or DMSO only were added to H460 cells transfected with pGL2-AIMP2-DX2. BC-DXI01 effectively reduced AIMP2-DX2 expression on the basis of luminescence (Figure 2A). The effect of BC-DXI01 on endogenous AIMP2-DX2 protein was also analysed by immunoblotting, and the same result was observed (see Supplementary Figure S2A at http://www.biochemj.org/bj/454/bj4540411add.htm). Since 0.4 μM BC-DXI01 provided a robust suppressive effect, we used 0.4 μM for additional experiments. Next, we monitored the duration of efficacy by treating H460 cells with BC-DXI01 for the indicated times and analysed the levels of AIMP2-DX2 by immunoblotting (Figure 2B, upper panel). The AIMP2-DX2 level decreased significantly within 2 h, and decreased progressively to an almost undetectable level within 12 h after treatment. BC-DXI01 did not affect the cellular level of AIMP2-F, indicating that BC-DXI01 specifically suppresses AIMP2-DX2.

To see whether BC-DXI01 influences the level of the AIMP2-DX2 mRNA transcript, total RNA was extracted from the H460 cells, and the AIMP2-F and AIMP2-DX2 mRNA levels were analysed using RT–PCR with primer pairs which could simultaneously detect both AIMP2-F and AIMP2-DX2 transcripts. Interestingly, levels of the AIMP2-DX2 transcript were decreased from 2 h after BC-DXI01 treatment and continuously reduced as shown for AIMP2-DX2 protein levels. However, levels of the AIMP2-F transcript were unchanged by BC-DXI01 treatment (Figure 2B, lower panel, top gel). PCR cycles were reduced to detect AIMP2-F only without saturation and the same result was observed (Figure 2B, lower panel, upper middle gel). The effect of BC-DXI01 on the AIMP2-DX2 transcript was confirmed using the AIMP2-DX2-specific primers and the result showed that, unlike AIMP2-F, AIMP2-DX2 expression was specifically reduced by BC-DXI01 treatment (Figure 2B, lower panel, lower middle gel). The reduction in the AIMP2-DX2 transcript level was observed from 30 min after chemical treatment, and steadily declined thereafter (see
Supplementary Figure S2B). When transcription was blocked by actinomycin D treatment, the degradation of AIMP2-DX2 mRNA was accelerated by BC-DXI01, showing a comparatively rapid reduction in AIMP2-DX2 mRNA compared with that of the actinomycin D-untreated cells (see Supplementary Figure S2C).

Since AIMP2-DX2 is an alternative splicing variant of AIMP2, we examined whether BC-DXI01 modulates the splicing of AIMP2-DX2. As a control, we transfected H460 cells with the pGINT plasmid, which comprises a splicing cassette capable of reporting alternative splicing by the expression of GFP [10]. We also transfected the pGINT-exon2 and pGINT-exon2-A152G plasmids [6], which are used for the monitoring of AIMP2-DX2-specific splicing. The fluorescence of GFP observed by the transfection of pGINT, pGINT-exon2 and pGINT-exon2-A152G was not affected by the treatment of BC-DXI01, suggesting that it is the mature mRNA transcript of AIMP2-DX2, but not the splicing process, that is the target of BC-DXI01 (see Supplementary Figure S2D).

To test whether BC-DXI01 modulates the cellular level of mature AIMP2-DX2 mRNA, we designed a DNA hybridization probe which specifically recognizes the exon1–3 junction sequence of the mature AIMP2-DX2 transcript, and examined AIMP2-DX2 mRNA levels by Northern blotting. We observed reduced levels of mature AIMP2-DX2 transcript after BC-DXI01 treatment (Figure 2C). Taken together, these results suggest that the decrease in AIMP2-DX2 protein by BC-DXI01 is due to BC-DXI01-mediated degradation of the mature AIMP2-DX2 transcript.

To understand the mechanism of BC-DXI01-mediated degradation of the mature AIMP2-DX2 transcript, we synthesized RNA fragments of AIMP2 or the AIMP-DX2 transcript via in vitro transcription (Figure 2D) and examined the binding affinity of BC-DXI01 to each RNA fragment using a filter-binding assay. BC-DXI01 showed apparent association with the fragments of AIMP2-DX2 transcripts which contain the exon1–3 junction sequence. However, this association was not observed in DX2-f85 and AIMP2-f573, which lacked the exon1–3 junction sequence (Figure 2E). We confirmed interaction between BC-DXI01 and the RNA fragments with the exon1–3 junction sequence by pulling down the compound-RNA complex and performing RT–PCR (Figure 2F). Since BC-DXI01 specifically bound to AIMP2-DX2 RNA fragments with the exon1–3 junction sequence, we suggest that BC-DXI01-mediated degradation of the mature AIMP2-DX2 transcript may be induced by the direct binding of BC-DXI01 to the region spanning the exon1–3 junction of the AIMP2-DX2 transcript.

**Effect of BC-DXI01 on lung cancer cells**

To examine the effect of AIMP2-DX2 inhibition on the viability of lung cancer cells, H460 cells were treated with BC-DXI01 and harvested at 24, 48 and 72 h. Using the MTT assay, we found that cell death was proportional to the dosage and duration of BC-DXI01 treatment, with 40% of treated cells viable at the maximal dosage of 4 μM and duration of 72 h of treatment while cell viability was maintained at nearly 100% in the control group (Figure 3A). BC-DXI01-induced cell apoptosis was also confirmed by flow cytometry-based cell cycle analysis, which showed a dose-dependent increase in the sub-G1 fraction (Figure 3B). In addition, we found that, in BC-DXI01-treated H460 cells, active caspase 3, a marker of apoptosis, was present in levels proportional to the concentration of BC-DXI01 treatments (Figure 3C). We also found that active caspase 3 levels were inversely correlated with expression of PARP-1 [poly(ADP-ribose) polymerase 1], a cell proliferation marker, and AIMP2-DX2 (see Supplementary Figure S3 at http://www.biochemj.org/bj/454/bj4540411add.htm). Furthermore, a thymidine incorporation assay showed BC-DXI01-mediated inhibition of H460 cell proliferation (Figure 3D). Taken together, these data suggest that BC-DXI01 inhibits the anti-apoptotic and pro-proliferative effects of AIMP2-DX2.

**Positive correlation between AIMP2-DX2 levels and the pro-apoptotic activity of BC-DXI01**

To determine the effects of BC-DXI01 in various NSCLC cell lines, and also to see whether its effect is affected by AIMP2-DX2, we analysed the cellular levels of AIMP2-DX2 in ten NSCLC cell lines, and compared them with that of the normal lung cell line WI-26. First, we found that, in BC-DXI01-untreated cell lines, H1972 and H1171 cells expressed similar levels of AIMP2-DX2 protein as the control, whereas the other eight lung cancer cell lines expressed higher than normal levels of AIMP2-DX2 protein (Figure 4A, middle gels). We then tested the effects of BC-DXI01 treatment, and found an obvious reduction in AIMP2-DX2 levels in all of the cancer cell lines, except for H1972 and H1171 cells in which AIMP2-DX2 levels were intrinsically low. Whereas AIMP2-DX2 levels varied to some small degree between the tested cell lines, AIMP2-F levels were not affected by BC-DXI01 treatment (Figure 4A, middle gels). A cell viability analysis using the MTT assay revealed that BC-DXI01-mediated cell death was apparent in almost all of the cell lines tested, except for WI-26, H1975, H1972 and H1171 cells (Figure 4B). The susceptibility to BC-DXI01-mediated cell death generally showed a positive correlation with the cellular levels of AIMP2-DX2, apart from some outliers (Figure 4C), suggesting that the apoptotic activity of BC-DXI01 mainly resulted from the suppression of AIMP2-DX2.
Chemical suppression of an oncogenic splicing variant of AIMP2

415

Figure 4 The effect of BC-DXI01 on various NSCLC cell lines

(A) WI-26 normal lung cells and other NSCLC cell lines (A549, H460, H1299, H1975, HCC44, HCC15, HCC2108, H1972, H358 and H1171) were treated with BC-DXI01 for 4 h. The effects of BC-DXI01 on the AIMP2-DX2 and AIMP2-F levels in these cell lines were examined using immunoblotting. (B) Cell death effects were analysed using the MTT assay after 48 h treatment with BC-DXI01. Results are means ± S.D. and the experiment was repeated three times. (C) The level of AIMP2-DX2 protein in each lung cell line was quantified using densitometry. A scatter diagram was plotted to analyse the correlation between BC-DXI01-induced cell death and AIMP2-DX2 levels. The dotted line shows the trend line, which was obtained by ruling out HCC44, H358, H1975 and H1972 cell lines.

Inhibitory effect of BC-DXI01 on tumorigenesis in vivo

To test the antitumour activity of BC-DXI01, we established a xenograft mouse model using H460 cells. Since BC-DXI01 did not show good solubility in water, we synthesized a sodium salt form of BC-DXI01 to improve its solubility. The salt form of BC-DXI01 induced degradation of the AIMP2-DX2 transcript with similar efficacy to BC-DXI01 (see Supplementary Figure S4A at http://www.biochemj.org/bj/454/bj4540411add.htm). After once-daily administration of the salt form of BC-DXI01 for 28 days, mice were killed and tumour masses were analysed. Compared with the control group, tumour growth in the BC-DXI01-treated group was apparently inhibited (Figure 5A). Tumour volumes and weights were reduced by up to 60% following BC-DXI01 treatment (Figures 5B and 5C). However, BC-DXI01 did not affect the body weights of treated mice (see Supplementary Figure S4B). To see whether BC-DXI01 induced cell death in the tumour region, we monitored the activation of caspase 3 in the tumour region by IHC and found a significant increase in apoptotic cells, validating the apoptotic activity of BC-DXI01 in vivo (Figure 5D). A decrease in the cellular levels of AIMP2-DX2 and its mRNA transcript was also observed in the BC-DXI01-treated group (see Supplementary Figure S4C). These results suggest that BC-DXI01-mediated AIMP2-DX2 suppression can retard tumour growth in vivo.

DISCUSSION

AIMP2 regulates the stability of well-known oncogenes and tumour suppressors via context-dependent protein–protein interactions. AIMP2-DX2 shows increased expression in cancers, and competes with AIMP2 in various signalling pathways to compromise the anti-tumorigenic activity of AIMP2. Thus targeting AIMP2-DX2 could provide an effective therapeutic strategy to treat cancer. In the present study, we performed a screening with 2231 chemical libraries to identify small chemicals that can reduce the levels of AIMP2-DX2 and identified
BC-DXI01, which has a novel mechanism to reduce the AIMP2-DX2 mRNA transcript, resulting in anti-tumorigenic activity in vivo.

By analysing AIMP2-DX2 levels in various NSCLC cell lines, we found that most tested NSCLC cells appear to require AIMP2-DX2 for their survival, except for a few exceptional cases. For instance, the viability of H1975 cells was not significantly affected, although BC-DXI01 treatment successfully suppressed the AIMP2-DX2 level. Perhaps AIMP2-DX2 levels can be also regulated via unknown mechanisms in some cancer cells. The impact of this oncogenic splicing variant on cell survival would need more investigation for better understanding of the AIMP2-DX2 roles in cancer biology.

BC-DXI01 showed specific inhibitory activity to AIMP2-DX2, but not to AIMP2. Interestingly, BC-DXI01 targets the mature mRNA transcript of AIMP2-DX2 and consequently functions as an RNA-interfering chemical molecule. It may induce specific degradation of the AIMP2-DX2 transcript through direct binding to the exon1–3 junction region, although other possibilities can also be considered that could explain the activity of BC-DXI01. The exact mode of action for BC-DXI01 awaits further investigation in molecular detail. Nonetheless, the present study suggests the possibility of chemical-mediated RNA regulation for the development of specific RNA-targeting therapy.

Splicing variants have been recognized as attractive cancer diagnostic markers and drug targets because the controlled switching between splicing variants is closely related to tumour progression [11]. CD44, Wilms’ tumour 1, BRCA1/2 (breast cancer early-onset 1/2), PSA (prostate-specific antigen), the kallikrein gene, osteopontin are the representative genes where splicing regulation between splicing variants is closely related to tumour progression [11,12]. RNA and ribonucleoprotein complexes have received attention as new therapeutic targets, because disruption in their activities has been identified as the cause of numerous diseases [13].

To date, the major obstacle for the development of RNA-regulating therapeutics lies in the difficulty of delivering antisense oligonucleotides or siRNAs in a tissue-specific manner [13,14]. Chemical-based therapeutics might help overcome this drawback, but, so far, only a few compounds which modulate the spliceosmal proteins or translational machinery have been developed to control alternative splicing of HIV-1 [13,15]. Development of chemicals which can regulate the mature transcript of human mRNA may provide an opportunity for the control of human mRNA without delivery concerns. Optimizing the effects of BC-DXI01 and determining its working mechanism at the molecular level would contribute to the progress in developing pharmaceutical compounds to satisfy the unmet needs in target-based therapy.

AUTHOR CONTRIBUTION

Hee Sook Lee performed the experiments, analysed the data and wrote the paper. Dae Gyu Kim and Young Sun Oh carried out experiments and analysed the data. Nam Hoon Kwon designed and performed the experiments, analysed the data and wrote the paper. Jin Young Lee performed the experiments. Doyeon Kim and Song-Hwa Park performed in vivo experiments. Jong-Hwan Song, Sunkyung Lee and Jongeook Lee provided materials. Jung Min Han helped to perform the experiments and analyse the data. Bum-Joon Park helped to design the experiments. Sunghoon Kim designed the experiments, analysed the data and wrote the paper.
**SUPPLEMENTARY ONLINE DATA**

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**EXPERIMENTAL**

**Cell culture**

WI-26, a human embryonic lung fibroblast cell line, was obtained from the Korea Cell Line Bank. The other NSCLC cell lines, A549, H460, H1299, H1975, HCC44, HCC15, HCC2108, H1972, H358 and H1171, were purchased from A.T.C.C. (Manassas, VA, U.S.A). Cell lines were maintained in DMEM (Dulbecco’s modified Eagle’s medium) (WI-26 cells) or RPMI 1640 (other NSCLC cell lines) medium complemented with 10% FBS and 1% penicillin/streptomycin and kept in a 5% CO2 incubator at 37°C. For chemical treatment, cells were incubated with compounds in serum-free or 0.5% serum-containing medium. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock solution (5 mg/ml) was 10-fold diluted and 10 μl of diluted solution was added to each well containing 200 μl of medium and incubated for 30 min. The precipitated crystal was dissolved in 100 μl of DMSO (Sigma). Absorbance was measured at 420 nm using a microplate reader (Sunrise, TECAN).

**Flow cytometry**

H460 cells (1 × 10⁴ cells) were seeded in 96-well plates and treated with compounds for the indicated times in 0.5% serum-containing medium. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed with the reagent according to the manufacturer’s guidelines.

**MTT assay**

H460 cells were treated with BC-DXI01 (0.04, 0.4 and 4 μM) in 0.5% serum-containing medium for 48 h. The treated cells were fixed with 70% ethanol for 1 h at 4°C, washed twice with ice-cold PBS, and stained with propidium iodide (50 μg/ml) containing 0.1% sodium citrate, 0.3% Nonidet P40 and 50 μg/ml RNase A for 40 min. The cells were subjected to flow cytometry (FACSCalibur, Becton-Dickinson) to evaluate the apoptotic cells by counting the sub-G1 cells. For each sample, 20000 cells were analysed using Cell Quest Pro software.

**Thymidine incorporation assay**

H460 cells were treated with BC-DXI01 (0.04, 0.4 and 4 μM) in 0.5% serum-containing medium for 48 h. [3H]Thymidine at 1 μCi/ml was added to the culture medium and was incubated for 4 h. The incorporated thymidine was measured using a liquid scintillation counter (Wallac).

**IHC**

Dissected tumours were fixed and stored in 10% neutral-buffered formalin. Tissues were processed, paraffin-embedded,
and sectioned at $6 \mu m$ thickness. After deparaffinization, antigen retrieval was performed by boiling sections for 5 min in sodium citrate solution (0.01 M, pH 6). Sections were incubated at 4°C overnight with rabbit anti-(active caspase 3) antibody (1:200 dilution, Cell Signaling Technology) in PBST (0.3% Triton X-100 in PBS) containing 5% goat serum. After washing, sections were incubated with biotin-conjugated anti-(rabbit IgG) (1:100 dilution, Sigma) antibody and ExtrAvidin-peroxidase (1:50 dilution, Sigma) at room temperature for 1 h each. Sections were developed with DAB (3, 3-diaminobenzidine) substrate for 30 min, counterstained with haematoxylin and imaged under the microscope.

**In vitro transcription**

To generate RNA fragments of AIMP2 and AIMP2-DX2 transcripts, PCR was performed with specific primers. A common forward primer with a T7 promoter sequence, 5′-TAATA- CGA CTCA TATA AATGC CAGT GACG AGT AAG-3′, was used for every reaction in combination with each reverse primer. Specific reverse primers, 5′-GGGGAGGCCGGTAC- ATGCAGTTGG-3′, 5′-GGGGAGGCCGGGGTTTGGCTGATTCA-3′, 5′-GTTTCAGGACGCTTGTGACCGAG-3′, 5′-GTTTGAGCAGCAGTATGGCTC-3′, 5′-TCACCTAA GGAGCTGTTGACAGCTTA TGCTTCT-3′, and 5′-ATTCTTCAAATTA-AAGTGAATCCC-3′, were used for the amplification of DX2-f85, DX2-f185, DX2-f285, DX2-f485, DX2-full and AIMP2-f573 respectively. Amplified PCR products were purified and RNA was synthesized by in vitro transcription using each PCR template and T7 RNA polymerase (Promega). For the filter-binding assay, RNA was radioactively labelled using $[^{32}P]$UTP.

**Filter-binding assay (dot blot assay)**

Biotinylated BC-DXI01 and radioactively labelled RNA (10000 c.p.m.) were incubated in the binding buffer (20 mM Tris/HCl, pH 7.4, 75 mM KCl, 10 mM MgCl$_2$ and 5% glycerol) at room temperature for 30 min. The reaction mixtures were filtered through a streptavidin-coated matrix biotin-capture membrane (Promega) using a 96-well Minifold filtration apparatus. Dried membranes were exposed, and signals were detected by autoradiography.

**Pull-down assay for BC-DXI01 and RNA complex**

Synthesized RNA fragments (2 μg) were added to the biotinylated BC-DXI01 in the RNA-binding buffer (200 mM KCl, 20 mM NaCl, 0.05% Nonidet P40, 10% glycerol, 2 mM DTT, 2 mM MgCl$_2$ and 20 mM Hepes, pH 7.8), and incubated for 1 h at 30°C. After cross-linking by the addition of 1% formaldehyde at room temperature for 10 min, the RNA–BC-DXI01 complex was pulled down using streptavidin–Sepharose beads (1 h incubation with rotation). After washing and incubation with DNase I (Fermentas) to remove contaminating DNA, RNA was extracted with phenol/chloroform and precipitated by ethanol. Each RNA fragment was detected via RT–PCR using a common forward primer, 5′-ATGC CGGATGTACAGGTAAG-3′, and the specific reverse primer described in the In vitro transcription section above.

**Figure S1** Schematic diagram for the chemical screening based on luciferase assay

AIMP2-DX2 was cloned into the pGL2 control vector, fused to the 5′ end of firefly luciferase and expressed in H460 cells. The protein level of AIMP2-DX2 was monitored by luminescence (left-hand panel). When chemicals inhibit the expression of AIMP2-DX2 at the mRNA or protein level, luciferase activity is decreased and the efficacy of compounds can be calculated (right-hand panel). P, promoter.

**Figure S2** BC-DXI01 induces degradation of the AIMP2-DX2 transcript without affecting the splicing process

(A) BC-DXI01 was treated to H460 cells for 4 h and the amounts of AIMP2-DX2 and AIMP2-F were detected by immunoblotting. (B) The effect of BC-DXI01 (0.4 μM) on the mRNA transcript levels was monitored at early time points via RT–PCR. (C) H460 cells were pre-incubated with actinomycin D (Act.D, 2 μM) for 4 h and then treated with BC-DXI01 for the indicated time periods. The mRNA level of AIMP2-DX2 was examined via RT–PCR (upper panel). The band intensity of AIMP2-DX2 was measured and the relative percentage of AIMP2-DX2 mRNA is presented (lower panel). (D) pGINT, pGINT-exon2 wild-type (WT) or pGINT-exon2 A152G mutant were transduced into H460 cells and incubated with DMSO (control) or BC-DXI01. Expression of GFP was monitored using fluorescence microscopy.

**Figure S3** BC-DXI01 induces apoptosis of H460 lung cancer cells

H460 cells were treated with the indicated concentrations of BC-DXI01 for 48 h. Expression of AIMP2-DX2 and PARP-1 was analysed via immunoblotting.
Figure S4  Antitumour activity of BC-DXI01 in a mouse xenograft model

(A) H460 cells were treated with the sodium salt form of BC-DXI01 and the mRNA level of AIMP2-DX2 was analysed using RT–PCR. Original BC-DXI01 was used as a positive control. (B) Body weights of mice administered with the BC-DXI01 salt form or vehicle were measured twice a week. Results are means±S.D. (n = 8 per group). (C) Protein and mRNA levels of AIMP2-DX2 were analysed from the tumours and the results are presented. IB, immunoblot; RT, RT–PCR.