Genome-wide DNA methylation profiling of CpG islands in a morpholino anthracycline derivative-resistant leukemia cell line: p38α as a novel candidate for resistance

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Introduction
Effective leukemia therapy is hampered by drug resistance, which is a serious problem that results from various mechanisms. Pediatric leukemia patients with cancer cells that show resistance to antileukemic agents in vitro have a more dismal prognosis than patients with drug-sensitive leukemia cells (Hollem et al. 2004). The pattern of gene expression in leukemia cells with acquired resistance to standard therapy is probably substantially different compared to leukemia cells prior to the initiation of treatment because drug-resistant subpopulations grow selectively as treatment progresses. Nevertheless, how cells acquire drug resistance is unclear. Many signaling pathways and genes that may affect the response of leukemia cells to therapy have been identified. Because many factors are involved in the mechanism of acquisition of drug resistance, the “one gene: one outcome” hypothesis cannot adequately explain acquired resistance in leukemia (Glasspool et al. 2006). Thus, multiple mechanisms and multiple genes rather than a single pathway or gene likely mediate acquired resistance.
Aberrant methylation, including genome-wide hypomethylation and regional hypermethylation of promoters for genes such as tumor suppressors, is one mechanism of tumorigenesis (Eden et al. 2003). Methylation of eukaryotic DNA occurs at CpG-enriched promoters. Such epigenetic changes are critically involved in acquisition of drug resistance, which is mediated by changes in gene expression that occur following chemotherapy but that are not caused by genetic mutations. Wei et al. (2003) used drug-resistant cell lines and differential methylation hybridization and showed many differences in CpG island methylation and epigenetic regulation after drug treatment. However, this group did not determine which genes were aberrantly methylated in resistant cells. In general, recent studies including ours have investigated only a few CpG island markers in methylation-related drug resistance (Asano et al. 2005).

3′-deamino-3′-morpholino-13-deoxo-10-hydroxycarmi-
nomycin hydrochloride, also known as KRN 8602 (MX2), is a new morpholino anthracycline derivative that acts as a topoisomerase (Topo) IIα inhibitor and is cytotoxic to
tumor cells (Watanabe et al. 1988). MX2 is highly lipophlic and easily passes through the cell membrane in a P-glycoprotein-independent manner (Watanabe et al. 1988). The antitumor effects of MX2 are superior to those of adriamycin (ADR). MX2 is toxic to mouse and human tumor cell lines as well as multidrug-resistant tumor cell lines that express high levels of P-glycoprotein (Watanabe et al. 1991). MX2 may thus be useful for eradicating multidrug-resistant tumors. By continuously exposing cells grown in suspension to increasing amounts of MX2, we previously established the MX2-resistant human myelogenous leukemia cell line K562/MX2, which is derived from the parent cell line K562/P (Asano et al. 2005). K562/MX2 cells show lower levels of Topo IIα mRNA and protein, and the Topo IIα gene in these cells is aberrantly methylated at CpG islands. Thus, drug resistance in K562/MX2 cells may be due to aberrant methylation (Asano et al. 2005). We therefore next investigated the relationship between global gene expression and methylation in drug-resistant cells and identified genes that confer resistance. High-throughput methylation analysis of multiple CpG sites can be performed with the GoldenGate Methylation BeadArray (Illumina Inc. Tokyo, Japan) (Ang et al. 2010). Here, we evaluated the genome-wide methylation status using the methyl array, compared gene expression profiles using microarray, and analyzed the entire profile of altered gene expression with methylation using Gene Ontology (GO) analysis. We found that resistant cells were hypermethylated in whole genes, and that genes involved in gene silencing and the immunological response were most critical for methylation-related altered gene expression. In addition, using key node analysis, p38α mitogen-activated protein kinase (MAPK) was identified as a novel enzyme that may mediate MX2-related resistance. In addition to the K562 cell line, we also established a lymphoblastic leukemia cell line with resistance to MX2 (BALL/MX2). Compared to sensitive cells, p38 kinase activity in both resistant cell lines was increased. Blocking p38 kinase activity and phosphorylated p38α protein with SB203580 or SB20190, which are specific inhibitors of p38 MAPK, or using siRNA to knock down p38α mRNA expression, restored the sensitivity to MX2 in resistant cells, concomitant with decreased expression of p38α mRNA, phosphorylated protein, and kinase activity.

Materials and Methods

Reagents

We used the hydrochloride form of MX2 (Watanabe et al. 1988, 1991). ADR, etoposide, vincristine, and dimethyl sulfoxide, were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phosphate-buffered saline without metal salt solution (PBS (−)) was purchased from Nissui (Tokyo, Japan). RPMI 1640, Hanks’ balanced salt solution without Ca2+ or Mg2+ (HBSS), fetal calf serum, and gentamicin were purchased from Life Technologies, Inc. (Gaithersburg, MD). 5-Aza-
2’-deoxycytidine was purchased from Sigma Aldrich Japan (Tokyo, Japan). SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) and SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole), which are p38 MAPK inhibitors, and SB202474 (4-Ethyl-2-(p-methoxyphenyl)-5-(4’-pyridyl)-1H-imidazole), which is a negative control, were purchased from Calbiochem (Tokyo, Japan). siRNAs were obtained from Ambion (Carlsbad, CA).

Cell lines

Parental cell lines (K562/P, human myelogenous leukemia and BALL-1, human B-cell lymphoblastic leukemia) were purchased from RIKEN (Tsukuba, Japan). BALL-1 (BALL) cell line is established from typical human B-cell leukemia (male) (Miyoshi et al. 1977). K562 cell line is established from pleural effusion with chronic myelogenous leukemia of 53 years old female, which is sensitive to NK cell and can differentiate to erythroid cells (Lozzio and Lozzio 1975). The MX2-resistant cell line was established with limiting dilution using continuous exposure to increasing amounts of MX2 (Asano et al. 2005). MX2-resistant cells were cultured in the absence of MX2 for 2 weeks before use in experiments. The MycoAlert™ mycoplasma detection kit (Lonza Walkersville Inc.,
Tokyo, Japan) was used to confirm the absence of *Mycoplasma* organisms in all cell lines.

**Cytotoxicity assay**

The MTT assay (CellTiter96 AQueous One solution Cell Proliferation Assay, Promega Corp., Madison, WI) or trypan blue exclusion was used to determine cytotoxicity (Asano et al. 2005). Briefly, 1 × 10^5 cells/mL were incubated with various concentrations of MX2, etoposide, ADR, or vincristine for 72 h. Viable cells were counted after performing the MTT assay or trypan blue staining. The combination index method (Zhao et al. 2004) was used to determine the synergistic effect of inhibitors and siRNAs plus MX2.

**5-Aza-2’-deoxyctydine treatment**

Briefly, 1 × 10^5 cells/mL (total 1 × 10^7 cells) were grown for 72 h in a medium that included 10 μmol/L 5-Aza-2’-deoxycytidine. Fresh medium and drug were replaced daily.

**Isolation of genomic DNA and quality assessment**

DNA was extracted from freshly harvested cells with the QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan). Low-percentage (0.5%) agarose gel electrophoresis and low-power voltage were used to assess the quality of the extracted DNA. Genomic DNA was of sufficient quality when a high molecular weight band (>40 kb) was visible and when no strong low molecular weight band (<2.0 kb) was visible on the gel after 3 hours of electrophoresis. Alternatively, an OD260/280 between 1.8 and 2.0 indicated DNA of sufficient quality.

**Bisulphite conversion**

The EZ DNA methylation kit (Zymo Research, Irvine, CA) was used for bisulphite conversion of genomic DNA with modifications for the Illumina Infinium Methylation Assay (Illumina, Tokyo, Japan). Briefly, 1 μg genomic DNA was incubated with 5 μL M-Dilution buffer at 37°C for 15 min. Then, 100 μL CT conversion reagent was prepared as described by the manufacturer and added to the mixture, which was incubated in a thermal cycler for 16 cycles at 95°C for 30 sec and 50°C for 60 sec. Aliquots of bisulphite-converted DNA were added to 96-column plates provided in the kit, and then desulphonated and purified as described by the manufacturer. These samples were used immediately for chip analysis, as described below.

**Illumina inﬁnium human methylation27 beadchip**

The Illumina Infinium Human Methylation27 BeadChip Kit was used for assays with bisulphite-converted genomic DNA. This beadchip includes 27,578 CpG loci in >14,000 human RefSeq genes at single-nucleotide resolution. The reagents included in the kit were used for chip processing and analysis of data. Briefly, 4 μL bisulphite-converted genomic DNA was denatured in 0.014 N sodium hydroxide and neutralized. DNA was then amplified using components included in the kit for 20–24 h at 37°C. After fragmentation, 12 μL of each sample was loaded onto a 12-sample chip, which was inserted into a hybridization chamber as described by the manufacturer. Samples were incubated at 48°C for 16–20 h, chips were washed with wash buffers from the kit, and then chips were incubated in a fluid flow-through station for primer extension and stained using components provided in the kit. The iScan scanner (Illumina) was used for image processing of polymer-coated chips. We used a cutoff level for detection of a P < 0.001, which is the most stringent criterion in the Illumina GoldenGate Methylation array. BeadStudio v3.0 software (Illumina) was used for data extraction. Methylation values for each CpG locus were expressed as a β-value, which is a continuous value from 0 (completely unmethylated) to 1 (completely methylated) and is based on the following equation: 

\[ \beta-value = \frac{\text{signal intensity of methylation-detection probe}}{\text{signal intensity of methylation-detection probe} + \text{signal intensity of non methylation detection probe}} \]

In accordance with the manufacturer's recommendation, results >0.15 as determined by the difference in the β-value between two sets of groups were considered significant.

**RNA isolation, real-time PCR, and microarray analysis**

The Qiagen RNA Mini kit (Qiagen) was used for isolation of total RNA from each sample, and RNA integrity was confirmed following 1% agarose gel electrophoresis. Real-time PCR analysis was performed as described (Yamanishi et al. 2015) using the following primers: p38x sense: 5'-TGCCCGAGCGTTACCAGACC-3' and antisense: 5'-CTGTAAGCTTCTGACATTTC-3'. The Agilent Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies, Tokyo, Japan) was used for in vitro transcription in the presence of Cy3- and Cy5-CTP. Next, 825 ng-labeled complementary RNA from each pair was purified separately, combined, mixed with hybridization buffer prepared using the In Situ Hybridization Plus kit (Agilent Technologies), and added to the microarray. Samples were hybridized to an Agilent 4 × 44k Whole Human Genome microarray (G4112F; Agilent Technologies),
which contains 43,376 coding and noncoding sequences from the human genome, in an Agilent G2545A hybridization oven. Hybridization and washing conditions were as described by the manufacturer in the protocol for oligonucleotide microarray hybridization (Agilent Technologies). Feature Extraction software (version 9.3; Agilent Technologies) and Spotfire software (version 8.0; Spotfire, Cambridge, MA) were used to analyze data from the Agilent G52565BA microarray scanner. Fluorescent spots on the microarrays were considered present or absent. Automatic recognition software (Feature Extraction version 9.3; Agilent Technologies) selected transcripts that were considered present, and quantile normalization was used to normalize data. Spots that failed quality control procedures (those with signal intensity <1.0) were excluded from additional analysis. The possibility of dye-related bias in the microarray results was excluded with an algorithm included in the software that applied normalization factors (linear and lowness normalization). Data were imported into Excel files (Microsoft, Redmond, WA) for subsequent data and statistical analysis (Agilent Technologies provides details of these procedures).

Integration of methyl array data and expression array data

After quantile normalization, we integrated the data from the methyl array and expression array to identify genes that were strongly related to methylation-specific altered expression. We considered increased methylation as a difference in the $\beta$-value of >0.15, decreased methylation as a difference in the $\beta$-value of <0.15, increased expression as >1.5-fold of the expression level, and decreased expression as <1- to 1.5-fold of the expression level.

GO analysis

Functional class scoring analysis based on Biobase Knowledge Library manual curation was used to determine GO classes that were differentially methylated in drug-sensitive compared to drug-resistant cells. A $P$ value for comparison of drug-sensitive and -resistant cells was computed for each gene in a GO class. Multiple testing corrections with the Benjamini and Hochberg false discovery rate (FDR) (Reiner-Benaim 2007) were used to determine the set of $P$ values for a GO class.

Key node search algorithm

The search for signaling molecules (key nodes) in the network vicinity of a gene list can be performed based on only one gene list, or based on a primary gene list with an additional gene list as a secondary set. Genes in the secondary set were incorporated such that the key node algorithm goes through the elements of this gene set. The network path was attracted by the secondary genes, resulting in longer paths that are often cheaper than shorter paths if they include molecules from the secondary set. The algorithm is a feed-forward-based approach that transforms the original weights of the network into new weights. The weights of the resulting network reflect the desired attraction power.

Score: The significance score, used for ranking the key node results, counted the hits that were relative to the respective logarithmized volume $V_i$ that was required to reach every hit.

\[
S = N_0 + \sum_{i=1}^{r} N_i - N_{i-1} \frac{1}{1 + \log(V_i)}
\]

Volume $V_i =$ number of total compounds reachable from the key node within a distance.

Hits $N_i =$ number of targets reached by key node $k$ within distance $i$.

With increasing distance $i$, the volume $V_i$ also increases. The maximum distance is limited by $r$.

FDR

Each individual key node was assigned an FDR value, which represents the probability that the observed rank or higher ranks was occupied by random chance and was estimated on-the-fly by random sampling. The ranking of the key nodes was defined by sorting them according to the score described above in descending order. All key nodes with an observed rank <200 were assigned an FDR value of 1.0 by definition, because their score was considered insufficient. Molecules with no hits were assigned the last rank because the score was 0 in this case.

Z-score

In addition to the FDR, each key node was assigned a Z-score, which measures the deviation of the observed rank of the key node from the expected rank in a random case. The Z-score was divided by the standard deviation.

\[
Z = \frac{X - \mu}{\sigma \mu c^-}
\]

In this formula, the rank distribution was assumed to comply with normal distribution. Key nodes with a Z-score >1.0 were considered significant.

Promoter analysis was performed using the online tool ExPlain 3.1 (http://explain.biobase-international.com/) for detection of overrepresented transcription factor-binding
Table 1. IC50 values for MX2, etoposide, adriamycin, and vincristine with or without 5-Aza-2'-deoxycytidine (SAZ) treatment.

|          | MX2 (nM) | Etoposide (nM) | Adriamycin (nM) | Vincristine (nM) | Carboplatin (µM) |
|----------|----------|----------------|-----------------|-----------------|-----------------|
| K562/P   | 30 ± 4   | 10 ± 4         | 20 ± 3          | 2.0 ± 2.1       | 20 ± 5.1        |
| K562/P with 10 µM 5AZ | 29 ± 6   | 7 ± 4          | 15.0 ± 11.0     | 1.8 ± 2.9       | 22 ± 4.8        |
| K562/MX2 | 200 ± 23*| 94 ± 15*       | 150 ± 20*       | 2.3 ± 1.8       | 18 ± 6.0        |
| K562/MX2 with 10 µM 5AZ | 46 ± 9**| 8 ± 5**        | 130 ± 10.7      | 2.0 ± 1.0       | 20.5 ± 4.8      |

The IC50 values were calculated from the cytotoxicity of various drugs. Data are reported as the mean ± standard deviation from five independent experiments.

*P < 0.05, Cytotoxicity in K562/P versus K562/MX2 cells.

**P < 0.05, Cytotoxicity in K562 cells with versus without 5-Aza-2'-deoxycytidine treatment.

Table 2. Methylation profile in K562/MX2 cells and K562/P cells. List of 30 highly methylated genes in K562/MX2 cells compared to K562/P cells.

| Gene symbol | Difference KMX(→)–P(→) | KMX(→) Beta | P(→) Beta | KMX(→) Beta | Description | GenBank accession |
|-------------|----------------------|-------------|-----------|-------------|-------------|------------------|
| LOC63928    | 0.9727               | 0.9481      | 0.0211    | 0.9471      | Hepatocellular carcinoma antigen gene S20 | NM_022097.1    |
| FLJ36046    | 0.9391               | 0.9696      | 0.0305    | 0.8823      | Hypothetical protein LOC164592 | NM_152612.2    |
| TSC22D3     | 0.9389               | 0.9667      | 0.0278    | 0.9247      | TSC22 domain family; member 3 isoform 2 | NM_004089.3    |
| TMEM22      | 0.9357               | 0.9797      | 0.0439    | 0.9712      | Transmembrane protein 22 | NM_025246.1    |
| HMHA1       | 0.9338               | 0.9662      | 0.0324    | 0.9516      | Minor histocompatibility antigen HA-1 | NM_012292.2    |
| PLXND1      | 0.9304               | 0.9487      | 0.0183    | 0.8712      | Plexin D1 | NM_015103.1    |
| SLC25A22    | 0.9266               | 0.9539      | 0.0274    | 0.8998      | Mitochondrial glutamate carrier 1 | NM_024896.4    |
| FHT         | 0.9205               | 0.9446      | 0.0241    | 0.9417      | Fragile histidine triad gene | NM_020012.1    |
| RGL3        | 0.9184               | 0.9627      | 0.0443    | 0.9448      | Ral guanine nucleotide dissociation stimulator-like 3 isoform 2 | NM_934610.1    |
| RASL10B     | 0.9154               | 0.9406      | 0.0252    | 0.8588      | RAS-like; family 10, member B | NM_033315.2    |
| RHCG        | 0.9143               | 0.9273      | 0.0130    | 0.8476      | Rhesus blood group, C glycoprotein | NM_016321.1    |
| CRIP3       | 0.9143               | 0.9587      | 0.0444    | 0.9346      | Cysteine-rich protein 3 | NM_206922.1    |
| NPB         | 0.9095               | 0.9386      | 0.0291    | 0.8942      | Prepropeptide B | NM_148896.1    |
| H2AFY2      | 0.9072               | 0.9504      | 0.0432    | 0.9387      | Core histone macroH2A2.2 | NM_018649.1    |
| GRIN2D      | 0.9062               | 0.9437      | 0.0375    | 0.8909      | X-methyl-o-aspartate receptor | NM_000883.6    |
| ARHGAP4     | 0.8988               | 0.9757      | 0.0770    | 0.9623      | Rho GTPase-activating protein 4 | NM_001666.2    |
| DRD1P       | 0.8950               | 0.9254      | 0.0304    | 0.8854      | Dopamine receptor D1 interacting protein | NM_015722.2    |
| SYK         | 0.8930               | 0.9655      | 0.0727    | 0.9392      | Spleen tyrosine kinase | NT_008470.18    |
| KIF6        | 0.8921               | 0.9197      | 0.0276    | 0.8709      | Kinesin family member 6 | NM_145027.3    |
| MCL1        | 0.8914               | 0.9758      | 0.0844    | 0.9632      | Melanocortin 1 receptor | NM_002386.2    |
| SLC16A5     | 0.8911               | 0.9846      | 0.0935    | 0.9538      | Solute carrier family 16; member 5 | NM_046952.2    |
| LRRCS6      | 0.8893               | 0.9552      | 0.0658    | 0.9305      | Hypothetical protein LOC115399 | NM_198075.1    |
| SYTL1       | 0.8890               | 0.9215      | 0.0325    | 0.8721      | Synaptotagmin-like 1 | NM_032872.1    |
| EHXY2       | 0.8886               | 0.9217      | 0.0331    | 0.9083      | Epoxide hydrolase 2, cytoplasmic | NM_001979.4    |
| SLC44A2     | 0.8838               | 0.9691      | 0.0854    | 0.9538      | CTL2 protein | NM_020428.2    |
| TMEM58      | 0.8830               | 0.9340      | 0.0510    | 0.9219      | Transmembrane protein 58 | NM_198149.1    |
| AARSD1      | 0.8817               | 0.9436      | 0.0619    | 0.9036      | Hypothetical protein LOC80755 | NM_025267.2    |
| CCND1       | 0.8793               | 0.9226      | 0.0432    | 0.8217      | Cyclin D1 | NM_053056.1    |
| GRB7        | 0.8777               | 0.9499      | 0.0722    | 0.9036      | Growth factor receptor-bound protein 7 | NM_005310.2    |
| CLSTN1      | 0.8743               | 0.9028      | 0.0285    | 0.8493      | Calystemin 1 isoform 2 | NM_014944.3    |

Methylation values for each CpG locus are expressed as a β-value. Difference in KMX(→) – P(→): β-value for K562/MX2 cells minus β-value for K562/P cells without 5-Aza-2'-deoxycytidine treatment.

KMX(→). Beta: β-value for K562/MX2 cells without 5-Aza-2'-deoxycytidine treatment.

P(→). Beta: β-value for K562/P cells without 5-Aza-2'-deoxycytidine treatment.

KMX(+). Beta: β-value for K562/MX2 cells with 5-Aza-2'-deoxycytidine treatment.

P(+). Beta: β-value for K562/P cells with 5-Aza-2'-deoxycytidine treatment.
sites (Stegmaier et al. 2011; Zawacka-Pankau et al. 2011; Takahashi et al. 2015). For the analysis, we selected regions from 1000-bp upstream to 100-bp downstream of the transcription start site of each gene of absolute fold change >4 (Yes set) and <1.12 (No set). The vertebrate_h0.01 set of transcription factors matrix from the TRANSFAC database was used for scanning potential binding sites. We used all promoters of genes with P < 0.01. The high-specific matrices with a Yes/No score >2.1, P < 0.05, and matched promoters P < 0.05 were selected with cutoffs from minSUM. The upstream analysis was performed with distance threshold value of 6 and FDR < 0.05 including expression/transregulation reaction and following curated chains.

**Western blot analysis**

Cells were lysed in RIPA buffer (WAKO Pure Chemical Industries Ltd.) with proteinase (Sigma Aldrich) and phosphatase inhibitors or in 1× Laemmli buffer, and lysates were separated on a SDS-PAGE gel. After transfer to membranes, blots were incubated with primary

| Table 3. Methylation profile in K562/MX2 cells and K562/P cells. List of 30 highly methylated genes in K562/P cells compared to K562/MX2 cells. |
|---|---|---|---|---|---|
| Gene symbol | KMX(−)−P(−) | KMX(−). Beta | KMX(+). Beta | P(+).Beta | Description | GenBank accession |
| FLJ14166 | −0.9012 | 0.0366 | 0.9378 | 0.0288 | Hypothetical protein LOC79616 | NM_024565.4 |
| PAPPA | −0.8892 | 0.0386 | 0.9279 | 0.0414 | Pregnancy-associated plasma protein | NM_002581.3 |
| PLOD2 | −0.8723 | 0.0240 | 0.8963 | 0.0224 | A preproprotein | 5-deoxyxynase 2 isoforrm a precursor |
| KIT | −0.8634 | 0.0489 | 0.9123 | 0.0382 | V-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog precursor | NM_000221.1 |
| EPHB3 | −0.8384 | 0.1107 | 0.9491 | 0.1378 | Ephrin receptor EphB3 precursor | NM_004443.3 |
| TOLLIP | −0.8312 | 0.0787 | 0.9099 | 0.0645 | Toll interacting protein | NM_019009.2 |
| CMKL1 | −0.8311 | 0.0457 | 0.8769 | 0.0488 | Chemokine-like receptor 1 | NM_004072.1 |
| PLK2 | −0.8198 | 0.0780 | 0.8978 | 0.1627 | Polo-like kinase 2 | NM_006622.1 |
| TUBB6 | −0.8149 | 0.1164 | 0.9313 | 0.1080 | Tubulin; beta 6 | NM_032525.1 |
| IMPACT | −0.8127 | 0.0839 | 0.8966 | 0.0642 | Impact homolog | NM_018439.1 |
| HMGB3 | −0.8088 | 0.0302 | 0.8391 | 0.0455 | High-mobility group box 3 | NM_005342.2 |
| GAS1 | −0.7955 | 0.0544 | 0.8500 | 0.0566 | Growth arrest-specific 1 | NM_002048.1 |
| NRP2 | −0.7911 | 0.0671 | 0.8738 | 0.0877 | Neuropilin 2 isoform 2 precursor | NM_003872.2 |
| ONECUT1 | −0.7902 | 0.1143 | 0.9045 | 0.0799 | One cut domain; family member 1 | NM_004498.1 |
| FLJ32130 | −0.7772 | 0.0316 | 0.8088 | 0.0699 | Hypothetical protein LOC146540 | NM_152458.4 |
| PAM | −0.7719 | 0.0897 | 0.8616 | 0.0783 | Peptidylglycine alpha-amidating monooxygenase isoform b; preproprotein | NM_012144.2 |
| PSYD | −0.7697 | 0.1044 | 0.8742 | 0.0925 | Pleckstrin and Sec7 domain containing 4 | NM_012455.2 |
| DNAJC6 | −0.7696 | 0.1047 | 0.8742 | 0.0824 | Dnla (Hsp40) homolog; subfamily C; member 6 | NM_014787.2 |
| C6orf145 | −0.7690 | 0.0152 | 0.7842 | 0.0191 | Hypothetical protein LOC221749 | NM_183373.2 |
| SLRC1A10 | −0.7599 | 0.0236 | 0.7835 | 0.0199 | Salute carrier family 16; member 10 | NM_018959.3 |
| ADCY9 | −0.7540 | 0.0482 | 0.8023 | 0.0681 | Adenylyl cyclase 9 | NM_001116.2 |
| Col111 | −0.7478 | 0.1083 | 0.8561 | 0.0516 | Hypothetical protein LOC284680 | NM_182581.1 |
| BCAS4 | −0.7350 | 0.0243 | 0.7593 | 0.0374 | Toll interacting protein | NM_019009.2 |
| DNA1 | −0.7336 | 0.0525 | 0.7862 | 0.0407 | Dynein; axonemal; intermediate polypeptide 1 | NM_000222.1 |
| SOX3 | −0.7334 | 0.1791 | 0.9125 | 0.1547 | SRY (sex determining region Y)-box 3 | NM_0010974.1 |
| COBL1 | −0.7317 | 0.0314 | 0.7631 | 0.0209 | COBL-like 1 | NM_012144.2 |
| LOC253012 | −0.7124 | 0.0638 | 0.7763 | 0.0467 | Hypothetical protein LOC253012 isoforrm 2 | NM_198151.1 |
| PROKR1 | −0.7018 | 0.0871 | 0.7899 | 0.0719 | G protein-coupled receptor 73 | NM_138964.2 |
| JAKMIP1 | −0.7013 | 0.0787 | 0.7999 | 0.0494 | Multiple coiled-coil GABAB1-binding protein | NM_144720.2 |
| TSPAN18 | −0.6900 | 0.1457 | 0.8357 | 0.1431 | TSPAN18 1 isoform 2 | NM_130782.2 |

Methylation values for each CpG locus are expressed as a β-value. Difference in KMX(−) – P(−): β-value for K562/MX2 cells minus β-value for K562/P cells without 5-Aza-2’-deoxycytidine treatment.
KMX(−). AVG Beta: β-value for K562/MX2 cells without 5-Aza-2’-deoxycytidine treatment.
P(−). AVG Beta: β-value for K562/P cells without 5-Aza-2’-deoxycytidine treatment.
KMX(+). AVG Beta: β-value for K562/MX2 cells with 5-Aza-2’-deoxycytidine treatment.
P(+). AVG Beta: β-value for K562/P cells with 5-Aza-2’-deoxycytidine treatment.
## Table 4: Expression profile in K562/MX2 cells and K562/P cells. List of highly expressed genes in K562/P cells compared to K562/MX2 cells.

| Gene Symbol | Fold change (KMX/C0 / P/C0) | KMX(-) Signal (normalized) | P(C0) Signal (normalized) | KMX(+) Signal (normalized) | P(+) Signal (normalized) | Description |
|-------------|-----------------------------|----------------------------|---------------------------|---------------------------|------------------------|-------------|
| CTAG1A      | 1/804.040                   | 1/18.9461                  | 15233.4490               | 44.4236                   | 98.8608                | Homo sapiens cancer/testis antigen 1A (CTAG1A), mRNA |
| DLK1        | 1/676.138                   | 1/110.0428                 | 74404.1773               | 119.4851                  | 72983.7166             | Homo sapiens delta-like 1 homolog (Drosophila) (DLK1), mRNA |
| VCX3A       | 1/329.081                   | 1/34.8629                  | 11472.7370               | 158.0995                  | 8514.7709              | Homo sapiens variable charge, X-linked 3A (VCX3A), mRNA |
| XAGE1A      | 1/304.000                   | 1/111.6045                 | 33927.7938               | 159.4567                  | 32875.3584             | Homo sapiens X antigen family, member 1A (XAGE1A), mRNA |
| VCX         | 1/280.399                   | 1/27.8574                  | 7811.1865                | 98.0080                   | 6299.8074              | Homo sapiens variable charge, X-linked (VCX), mRNA |
| VCX2        | 1/279.433                   | 1/22.2797                  | 6225.7115                | 101.9774                  | 5343.0137              | Homo sapiens variable charge, X-linked 2 (VCX2), mRNA |
| FHL2        | 1/202.499                   | 1/35.0010                  | 7087.6714                | 28.0545                   | 6380.1560              | Homo sapiens four and a half LIM domains 2 (FHL2), transcript variant 5, mRNA |
| BEX4        | 1/201.518                   | 1/20.8857                  | 4208.8693                | 20.1313                   | 4470.3064              | Homo sapiens brain expressed, X-linked 4 (BEX4), mRNA |
| HCLS1       | 1/170.605                   | 1/62.1245                  | 10598.7536               | 166.5296                  | 9431.3528              | Homo sapiens hematopoietic cell-specific Lyn |
| UCA1        | 1/162.611                   | 1/52.8277                  | 8590.4024                | 45.0836                   | 6804.6158              | Homo sapiens urothelial cancer associated 1 (nonprotein coding) (UCA1), noncoding RNA |
| IFITM2      | 1/150.728                   | 1/69.2778                  | 10442.1126               | 41.0087                   | 9673.5787              | Homo sapiens interferon induced transmembrane protein |
| CYB5A       | 1/136.984                   | 1/53.3125                  | 7302.9771                | 27.7678                   | 5431.9757              | Homo sapiens cytochrome b5 type A (microsomal) (CYB5A), transcript variant 2, mRNA |
| GPR68       | 1/135.231                   | 1/37.0398                  | 5008.9309                | 15.6715                   | 5059.3753              | Homo sapiens G protein-coupled receptor 68 (GPR68), mRNA |
| CD24        | 1/150.728                   | 1/62.1245                  | 8590.4024                | 45.0836                   | 6804.6158              | Homo sapiens CD24 signal transducer mRNA, complete |
| KIAA1324L   | 1/116.956                   | 1/19.5615                  | 2287.8536                | 26.6255                   | 2145.0269              | Homo sapiens KIAA1324-like (KIAA1324L), transcript variant 2, mRNA |
| MAGEC1      | 1/90.8075                   | 1/49.9329                  | 4532.0040                | 81.0197                   | 4937.7946              | Homo sapiens melanoma antigen family C, 1 (MAGEC1), mRNA |
| LAPTM5      | 1/89.9132                   | 1/62.101                   | 1607.0165                | 20.8686                   | 1928.6839              | Homo sapiens lysosomal protein transmembrane |
| PAGE1       | 1/88.9132                   | 1/18.0740                  | 1607.0165                | 20.8686                   | 1928.6839              | Homo sapiens P antigen family, member 1 (PAGE1), mRNA |
| CD33        | 1/85.8386                   | 1/22.5623                  | 1936.6734                | 15.9922                   | 1702.6828              | Homo sapiens CD33 molecule (CD33), transcript variant 2, mRNA |
| APOE        | 1/85.0531                   | 3/20.4242                  | 2849.8391                | 37.1645                   | 3174.8391              | Homo sapiens apolipoprotein E (APOE), mRNA |
| HABOX1      | 1/84.5934                   | 3/77.8706                  | 570.0165                 | 46.4695                   | 650.6957               | Homo sapiens Cys and Cys protein transmembrane |

(Continued)
antibodies against phosphorylated p38α (Cell Signaling Technology, Inc., Danvers, MA) and GAPDH (Wako).

**p38 kinase assay**

The CycLex p38 Kinase Assay kit (CycLex Co., Ltd., Nagano, Japan) was used for the p38 kinase assay.

**Transfection of siRNA**

siRNAs that were used to knock down p38α expression and the corresponding negative control siRNA were purchased from Ambion (Tokyo, Japan). Cells (3 \times 10^3 cells/well) were plated in 96-well plates and incubated for 24 h. Cells were then transfected with 5 pmol control or p38α siRNA using Lipofectamine Plus transfection reagent (Life Technologies, Tokyo, Japan). Twenty-four hours post transfection, the treated cells were incubated with various concentrations of MX2 for 72 h and measured cytotoxicity. For confirming to knock down p38α expression, mRNA were extracted 24 h after transfection, and protein were extracted 96 h after transfection. To establish 100% survival, cells were incubated with vehicle containing Hiperfect alone. Assays were performed in triplicate, and at least three independent experiments were conducted for each condition.

**Methylation-specific polymerase chain reaction analysis for p38α**

For MSP analysis, genomic DNA was obtained and 300 ng of DNA per sample was treated as described before (Yamanishi et al. 2015). Primer pairs for MSP of p38α (Homo sapiens mitogen-activated protein kinase 14 (MAPK14) gene, GenBank: EU332860.1) were designed based on methylated and unmethylated DNA sequences in the promoter region, as follows: p38α1M; pos. 1951-2059: 5'-TATATTGGGTAAAATTTCGGTTTTC-3', 5'-AATACTCCCGTTCCAACTACTACG-3', and p38α1U; 5'-TATATTGGGTAAAATTTTGGTTTTTG-3', 5'-ATACTCCCATTCCAACTACTACACC-3'. p38α2M; pos. 4618-4738: 5'-GTCGGGTGTAGTGGTTTACGT-3', 5'-TTTTAAATAAAAACGAAATTTCACCG-3', and p38α2U; 5'-GGTTGGGTGTAGTGGTTTATGT-3', 5'-TTTTAAAACTAAATCTTACTCTATCACC-3'. p38α3M; pos. 10992-11210: 5'-TTTAGTTTGGAGTGTAGTGGTACGA-3', 5'-AAAAACAAAAAAATCG-3', and p38α3U; 5'-TTTAGTTTGGAGTGTAGTGGTATGA-3', 5'-AAAAAACCAAAAATCACT-3'. p38α4M; pos. 13929-14207: 5'-GTTTAGGTTGGGTGTAGTGGTTTAC-3', 5'-TTTAGTTTGGAGTGTAGTGGTATGT-3', 5'-AAAAACAAAAAAATCG-3'. p38α4U; 5'-GTTTAGGTTGGGTGTAGTGGTTTATGT-3', 5'-AAAAACAAAAAAATCG-3'.
Table 5. Expression profile in K562/MX2 cells and K562/P cells. List of highly expressed genes in K562/MX2 cells compared to K562/P cells.

| Gene symbol | Fold change (KMX(-)/P(-)) | KMX(-) Signal (normalized) | P(-) Signal (normalized) | KMX(+) Signal (normalized) | P(+) Signal (normalized) | Description | Entrez GeneID | Genbank accession |
|-------------|---------------------------|---------------------------|-------------------------|---------------------------|-------------------------|-------------|----------------|------------------|
| HBB         | 258.9561                  | 53109.625                | 205.0914                | 61532.7564                | 234.5827               | Homo sapiens hemoglobin, beta (HBB), mRNA | 3043            | NM_000518        |
| PLAT        | 77.6454                   | 3227.8347                | 41.5715                 | 2953.7541                 | 35.6801                | Homo sapiens plasminogen activator, tissue (PLAT), transcript variant 1, mRNA | 5327            | NM_000930        |
| CLEC2B      | 72.2131                   | 4016.1516                | 55.6153                 | 1469.0889                 | 52.5098                | Homo sapiens C-type lectin domain family 2, member B (CLEC2B), mRNA | BF2213738       | NM_00127         |
| CXCL1       | 66.8582                   | 3750.5358                | 56.0969                 | 1372.3312                 | 58.6276                | Homo sapiens chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (CXCL1), mRNA | 9976            | NM_001151        |
| S100A10     | 46.6281                   | 14773.7249               | 316.8413                | 7658.6691                 | 352.0523               | Homo sapiens S100 calcium binding protein A10 (S100A10), mRNA | 6281            | NM_002966        |
| EMILIN2     | 46.4009                   | 16469.0418               | 354.9296                | 9837.7386                 | 371.1331               | Homo sapiens elastin microfibril interactor 2 (EMILIN2), mRNA | 84034           | NM_032048        |
| PPFIBP1     | 45.6525                   | 1550.9474                | 33.9729                 | 645.4800                  | 26.4176                | Homo sapiens PTPRF interacting protein, binding protein 1 (liprin beta 1) (PPFIBP1), transcript variant 1, mRNA | 8496            | NM_003622        |
| COL6A1      | 43.9149                   | 1951.9319                | 44.4480                 | 1941.1771                 | 37.1725                | Homo sapiens collagen, type VI, alpha 1 (COL6A1), mRNA | 1291            | NM_001848        |
| IL8         | 43.4885                   | 2921.1090                | 67.1697                 | 815.9359                  | 65.7085                | Homo sapiens interleukin 8 (IL8), mRNA | 3576            | NM_000584        |
| BATF3       | 41.7773                   | 5358.6282                | 128.2666                | 2401.9560                 | 140.0571               | Homo sapiens basic leucine zipper transcription factor, ATF-like 3 (BATF3), mRNA | 55509           | NM_018664        |
| PGLYRP4     | 41.3142                   | 1049.9370                | 25.4135                 | 721.4657                  | 19.6105                | Homo sapiens peptidoglycan recognition protein 4 (PGLYRP4), mRNA | 57115           | NM_020393        |
| FN1         | 40.5872                   | 1469.2060                | 36.1987                 | 2108.7846                 | 57.9448                | Homo sapiens fibronectin 1 (FN1), transcript variant 1, mRNA | 2335            | NM_212482        |
| OSBPL6      | 33.8573                   | 1769.1553                | 52.5335                 | 1159.4035                 | 65.3581                | Homo sapiens oxidized low density lipoprotein receptor-like 6 (OSBPL6), mRNA | 114880          | AK_123248        |
| ZDHHC11     | 32.7875                   | 1003.1447                | 30.5954                 | 1332.7551                 | 42.2168                | Homo sapiens zinc finger, DHH-type containing 11 (ZDHHC11), mRNA | 79844           | NM_04786         |
| TSPAN5      | 32.5284                   | 1956.8982                | 60.1596                 | 1961.2544                 | 106.6336               | Homo sapiens tetraspanin 5 (TSPAN5), mRNA | 10098           | NM_005723        |
| TPST1       | 29.7643                   | 1097.9944                | 36.8900                 | 918.5039                  | 49.7456                | Homo sapiens tyrosineprotein sulfotransferase 1 (TPST1), mRNA | 8460            | NM_003596        |
| CTSL2       | 28.0364                   | 3720.2736                | 132.6945                | 2890.063                  | 135.4341               | Homo sapiens cathepsin L2 (CTSL2), mRNA | 1515            | NM_001333        |
| PHLD1A1     | 27.7600                   | 5847.4504                | 210.6430                | 2267.6291                 | 92.4228                | Homo sapiens pleckstrin homology-like domain, family A, member 1 (PHLD1A1), mRNA | 22822           | NM_000518        |
| C20orf56    | 27.7375                   | 2084.2000                | 75.1403                 | 1494.8821                 | 85.9847                | Homo sapiens chromosome 20 open reading frame 56 (C20orf56), noncoding RNA | 140828          | NR_001558        |
| PLOD2       | 26.9148                   | 1185.7967                | 44.0574                 | 1011.9656                 | 30.4028                | Homo sapiens procollagen-lysin, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), transcript variant 1, mRNA | 5352            | NM_182943        |

(Continued)
was performed using the EpiScope MSP kit (Takara Biotechnology Company, Ohtsu, Japan) and the ABI Prism 7500 sequence detection system (Applied Biosystems). Amplification was performed with an initial denaturation at 95°C for 30 sec, 45 cycles of denaturation at 98°C for 5 sec, annealing at 55°C for 30 sec, and extension at 72°C for 60 sec. Subsequently, melting curve analysis was performed on PCR products.

**Apoptosis assay**

Caspase 3/7 activity assays were determined using Caspase-Glo 3/7 assay according to the manufacturer’s instructions in 96-well plates (Promega, Madison, WI). We used 10 μmol/L SB202190, 10 μmol/L SB203580, and 10 μmol/L SB202474 as a pretreatment for 30 min. Then, 2.5 nmol/L of MX2 were added, and the cells were cultured for 1 h and performed caspase 3/7 activity assays.

**Statistical analysis**

The Kruskal–Wallis H test was used for statistical analysis. When a significant difference was detected, we used the Mann–Whitney U test to calculate the significance of differences between each group. Bonferroni–Dunn correction was performed with uncorrected P values by multiplying them by the number of comparisons.

**Results**

**Establishment and features of the MX2-resistant cell line**

MX2-resistant cells were established with constant treatment of MX2-sensitive cells with increasing concentrations of MX2. This subculture of MX2-resistant cells is grown in the continuous presence of 100 nmol/L or 200 nmol/L MX2, but the phenotype remains stable after growth in medium without MX2 for more than 6 months. MX2-resistant K562 cells (K562/MX2 cells) were resistant to both MX2 and ADR and also exhibited cross-resistance to etoposide (Table 1).

**DNA methylation patterns in drug-sensitive and -resistant leukemia cells**

The DNA methylation pattern was clearly different in drug-sensitive and -resistant leukemia cells. After filtering, 19,663 genes were eligible for statistical analysis. Significant results were determined by a difference in the β > 0.15 between the two sets of groups. This analysis identified 4184 genes as differentially expressed between MX2-sensitive and -resistant leukemic cells. Of these,
3229 were hypermethylated, and 955 were hypomethylated in drug-resistant leukemia cells compared with the same genes in the parent cells (Tables 2, 3 Table S1). Lists of all expression profiles including full gene names and gene accession numbers are shown in Table S1. 5-Aza-2’-deoxycytidine treatment produced slight changes in methylation values on hypermethylated genes in K562/MX2 cells (Table 2), but no effect on hypermethylated genes in K562/P cells (Table 3).

**Gene expression patterns in drug-sensitive and -resistant leukemic cells**

After filtering, 22,409 genes were eligible for statistical analysis. The analysis identified 10,515 genes as differentially expressed between MX2-sensitive and -resistant leukemia cells. Of these, 4896 showed higher expression, and 5619 showed lower expression in the drug-resistant leukemia cells compared with those genes in the parent cells (Tables 4, 5). Lists of all expression profiles including full gene names and accession numbers are shown in Table S2. 5-Aza-2’-deoxycytidine treatment induced changes in the expression of a few genes including an increase in low-expression genes in K562/MX2 cells, but expression of most genes was not changed (Table 4, 5).

### Integrated methyl array data and expression array data

Next, we integrated the data from the methyl array and expression array to identify genes with strong methylation-specific changes in expression after quantile normalization. After integration, 9596 genes were eligible for further analysis. These genes were classified into four groups (hypermethylated with lower expression, hypermethylated with higher expression, hypomethylated with higher expression, hypomethylated with lower expression). We identified 326 genes with hypermethylation and

| GO identifier | GO term                                                                 | Ontology                  | #Hits in group | Group size | #Hits expected | P value      |
|---------------|-------------------------------------------------------------------------|---------------------------|----------------|------------|----------------|--------------|
| GO:0050896    | Response to stimulus                                                   | Biological process        | 244            | 5306       | 186            | 1.77776E-06 |
| GO:0070918    | Production of small RNA involved in gene silencing                     | Biological process        | 62             | 876        | 31             | 2.98838E-05 |
| GO:0031047    | Gene silencing by RNA                                                   | Biological process        | 62             | 885        | 31             | 3.07121E-05 |
| GO:0003576    | Extracellular region                                                    | Cellular component        | 88             | 1437       | 51             | 3.52275E-05 |
| GO:0031050    | dsRNA fragmentation                                                     | Biological process        | 62             | 876        | 31             | 3.73548E-05 |
| GO:0048583    | Regulation of response to stimulus                                      | Biological process        | 62             | 871        | 31             | 4.05668E-05 |
| GO:0018479    | Regulation of production of small RNA involved in gene silencing       | Biological process        | 62             | 871        | 31             | 6.08503E-05 |
| GO:0043331    | Response to dsRNA                                                       | Biological process        | 63             | 878        | 31             | 7.05792E-05 |
| GO:0016458    | Gene silencing                                                         | Biological process        | 64             | 962        | 34             | 9.000104926 |
| GO:0016020    | Membrane                                                               | Cellular component        | 222            | 5069       | 178            | 9.000231564 |
| GO:0002376    | Immune system process                                                  | Biological process        | 104            | 1892       | 67             | 9.000282245 |
| GO:0005886    | Plasma membrane                                                        | Cellular component        | 168            | 3486       | 123            | 9.000335645 |
| GO:0070887    | Cellular response to chemical stimulus                                   | Biological process        | 67             | 1070       | 38             | 9.000343859 |
| GO:0006955    | Immune response                                                        | Biological process        | 73             | 1208       | 43             | 9.000359805 |
| GO:0042221    | Response to chemical stimulus                                           | Biological process        | 162            | 3330       | 117            | 9.000378179 |
| GO:0032501    | Multicellular organisational process                                    | Biological process        | 254            | 6016       | 211            | 9.000403796 |
| GO:0006952    | Defense response                                                       | Biological process        | 71             | 1182       | 42             | 9.000572204 |
| GO:0005737    | Cytoplasm                                                              | Cellular component        | 233            | 5640       | 198            | 9.000324465 |
| GO:0048518    | Positive regulation of biological process                              | Biological process        | 157            | 3363       | 118            | 9.000379661 |
| GO:0050776    | Regulation of immune response                                           | Biological process        | 30             | 384        | 14             | 9.000390334 |
| GO:0009986    | Cell surface                                                            | Cellular component        | 23             | 260        | 10             | 9.000402714 |
| GO:0046649    | Lymphocyte activation                                                   | Biological process        | 37             | 525        | 19             | 9.000417652 |
| GO:0042110    | T-cell activation                                                      | Biological process        | 29             | 372        | 14             | 9.000430733 |
| GO:0009611    | Response to wounding                                                    | Biological process        | 62             | 1078       | 38             | 9.00060105 |
| GO:0046651    | Lymphocyte proliferation                                                | Biological process        | 19             | 202        | 8              | 9.00060727 |
| GO:0010033    | Response to organic substance                                           | Biological process        | 94             | 1836       | 65             | 9.000627684 |
| GO:0044421    | Extracellular region part                                               | Cellular component        | 55             | 925        | 33             | 9.000629094 |
| GO:0070661    | Leukocyte proliferation                                                 | Biological process        | 19             | 204        | 8              | 9.000639204 |
| GO:0002697    | Regulation of immune effector process                                   | Biological process        | 18             | 185        | 7              | 9.000653073 |
| GO:0032943    | Mononuclear cell proliferation                                          | Biological process        | 19             | 204        | 8              | 9.00066772 |

**Table 6.** Analysis of 30 significant GO terms.

GO, Gene Ontology.
lower expression in K562/MX2 cells (Table S3A), 173 genes with hypermethylation and higher expression in K562/MX2 cells (Table S3B), 71 genes with hypomethylation and higher expression in K562/MX2 cells (Table S3C), and 61 genes with hypomethylation and lower expression in K562/MX2 cells (Table S3D).

**GO analysis in drug-sensitive and -resistant leukemic cells**

GO analysis was performed using the dataset for 631 genes with either significantly higher or lower expression combined with higher or lower methylation in drug-resistant leukemia cells (K562/MX2) compared with drug-sensitive leukemia cells (K562/P) (Table S4A). In K562/MX2 cells that responded poorly to MX2, etoposide, and doxorubicin, selective enrichment of genes with significantly altered expression and methylation was found in ontology categories related to the response to stimuli, gene silencing, the extracellular region, and the immune response (Table 6, Table S4A, B). The methylation and expression status of each gene indicated that half of the genes were highly methylated with lower expression in resistant cells (Table S4B).

**Key node search**

Using the key node search, we found that p38α was an important factor in methylation-related MX2 resistance.

**Increased phosphorylated p38α protein in MX2-resistant leukemia cells and decreased phosphorylated p38α protein after pretreatment with p38α MAPK inhibitors**

We examined the expression levels of phosphorylated p38α protein in MX2-resistant leukemia cells compared with sensitive parent cells (Fig. 1). Phosphorylated p38α protein was increased in MX2-resistant cells compared to parent cells. The specific inhibitors of p38 MAPK, SB203580 and SB202190, effectively decreased the levels of phosphorylated p38α protein in MX2-resistant leukemia cells (K562/MX2, BALL/MX2) (Fig. 1).

**Increased p38 kinase activity in MX2-resistant leukemia cells and decreased p38 kinase activity after pretreatment with p38 MAPK inhibitors**

We next examined the p38 kinase activity in MX2-resistant leukemia cells compared with sensitive parent cells (Fig. 2A and B). The p38 kinase activity was increased in MX2-resistant cells compared to parent cells. Using specific inhibitors of p38 MAPK, SB203580 and SB202190, effectively decreased the p38 kinase activity in MX2-resistant leukemia cells (K562/MX2, BALL/MX2), but SB202474 (negative control) did not decrease the activity (Fig. 2A and B).

**Increased cytotoxicity with MX2 in MX2-resistant leukemia cells following pretreatment with p38 MAPK inhibitors**

First, we determined the optimal concentration of SB202190, SB203580, and SB202474 in K562 and BALL cells. Leukemia cells were incubated with various concentrations of these drugs for 72 hours, and the viability was...
measured. The IC50 values in BALL/P cells for SB202190, SB203580, and SB202474 were 400 ± 123 μmol/L, 200 ± 6 μmol/L, and 380 ± 88 μmol/L, and in BALL/MX2 cells were 1200 ± 320 μmol/L, 190 ± 67 μmol/L, and 700 ± 267 μmol/L, respectively. The IC50 values in K562/P cells were 560 ± 160 μmol/L, 240 ± 62 μmol/L, and 980 ± 80 μmol/L, and in K562/MX2 cells were 250 ± 57 μmol/L, 220 ± 35 μmol/L, and 1200 ± 378 μmol/L, respectively. Based on these values and a previous report (Planchard et al. 2012), we used 10 μmol/L SB202190, 10 μmol/L SB203580, and 10 μmol/L SB202474 as a pretreatment for 30 min. Then, various concentrations of MX2 were added, and the cells were cultured for 72 h. SB202190 or SB203580 pretreatment significantly increased the cytotoxicity of MX2 in BALL/MX2 cells and K562/MX2 cells, but not in parent cells (Fig. 3A and B). The combination index values of MX2 plus SB202190, SB203580, or SB202474 were 0.99, 1.08, and 1.08 in BALL/P cells, 0.06, 0.10, and 1.01 in BALL/MX2 cells, 1.18, 0.99, and 0.92 in K562/P cells, and 0.18, 0.18, and 1.51 in K562/MX2 cells, respectively. These results strongly suggested that MX2 synergistically acted with SB202190 or SB203580 in MX2-resistant cells, but MX2 did not act synergistically with SB202474 (negative control).

Increased p38α mRNA expression in MX2-resistant leukemia cells and decreased p38α mRNA expression after pretreatment with siRNAs to knock down p38α MAPK expression

We next examined the p38α mRNA expression in MX2-resistant leukemia cells compared with sensitive parent...
cells (Fig. 4). The p38α mRNA expression was increased in MX2-resistant cells compared to parent cells. Using siRNAs (siRNA1, 2, 3) to knock down p38α effectively decreased the p38α mRNA and protein expression in MX2-resistant leukemia cells (K562/MX2, BALL/MX2) (Fig. 4A–D).

**Increased cytotoxicity with MX2 in MX2-resistant leukemia cells following pretreatment with siRNAs for p38α MAPK**

Pretreatment with siRNAs to knock down p38α mRNA expression significantly increased the cytotoxicity of MX2 in BALL/MX2 cells and K562/MX2 cells, but not in parent cells (Fig. 5). The combination index values of MX2 plus siRNA for knock down of p38α and negative control siRNA were 0.98, 0.96, and 0.99 for siRNA1, 2, 3, and 0.97 for negative control siRNA in BALL/P cells, 0.05, 0.04, and 0.08 for siRNA1, 2, 3, and 1.02 for negative control siRNA in BALL/MX2 cells, 0.98, 0.95, and 0.93 for siRNA1, 2, 3, and 0.95 for negative control siRNA in K562/P cells, and 0.15, 0.13, and 0.18 for siRNA1, 2, 3, and 0.97 for negative control siRNA in K562/MX2 cells, respectively. These results strongly suggested that MX2 synergistically acted with siRNAs in MX2-resistant cells.

**CpG islands in p38α might contribute to changes in expression in MX2-resistant leukemia cell lines**

We measured the methylation status in the p38α gene by MSP analysis in BALL/P, BALL/MX2, K562/P, and K562/MX2 cells. MX2-resistant cell lines showed more methylation in p38α gene (position 1951, 10992 in BALL and K562, 4618 in BALL, 13929 in K562) we examined, but some of CpG islands (position 4618 in K562, and 13929 in BALL) showed more methylation in parent cells (Fig. 6).

**Caspase activity with SB202190, SB203580, and MX2 increased in MX2-resistance leukemia cells**

Caspase 3 (and caspase 7) showed decreased proteolytic activity in MX2-resistant cells and slightly increased proteolytic activity at 30-min exposure with SB202190 and SB203580. And, marked enhanced proteolytic activity was shown at 1 h exposure with 2.5 nmol/L of MX2 in MX2-resistant cell lines treated with SB202190 and SB203580. (Fig. 7A and B).

**Discussion**

Childhood leukemia is the most common childhood cancer. The majority of children can be cured with current therapies, although around 20% of children relapse, and their outcome remains dismal. Although reinduction
regimens with higher doses of antileukemic drugs with or without stem cell transplantation are used to treat relapsed leukemia, the remission rate has not improved. Therefore, further dose intensification is not a viable option for improving outcomes, and other novel therapeutic options are necessary. Understanding the

Figure 4. Increased p38α mRNA expression in MX2-resistant leukemia cells and decreased p38α mRNA and protein expression after pretreatment with siRNAs to knock down p38α MAPK. (A and B) p38α mRNA expression was measured in MX2-resistant leukemia cells and sensitive parent cells. p38α mRNA expression was increased in MX2-resistant cells compared to parent cells. siRNAs (siRNA1, 2, 3) to knock down p38α effectively decreased p38α mRNA expression in MX2-resistant leukemia cells (BALL/MX2(A), K562/MX2(B)). The data shown are from six independent experiments. P < 0.05: BALL/P versus BALL/MX2. P < 0.02: BALL/MX2(−) versus BALL/MX2 + siRNA. P < 0.03: BALL/MX2(−) versus BALL/MX2 + siRNA2. P < 0.01: BALL/MX2(−) versus K562/MX2 + siRNA. P < 0.03: K562/MX2(−) versus K562/MX2 + siRNA2. P < 0.01: K562/MX2(−) versus K562/MX2 + siRNA3. (C) and (D) p38α protein was investigated in MX2-resistant leukemia cells and sensitive parent cells. siRNAs (siRNA1, 2, 3) to knock down p38α effectively decreased p38α protein expression in MX2-resistant leukemia cells (BALL/MX2(C), K562/MX2(D)). The data shown are representative data from two independent experiments. MAPK, mitogen-activated protein kinase.
The mechanism of drug resistance is essential prior to exploring new strategies against relapse. We previously reported that aberrant methylation of genes for key enzymes involved in drug metabolism is a novel mechanism of drug resistance (Asano et al. 2005). Exploitation of the methodology for analyzing the methylation status throughout the entire genome and the development of statistical methods for large datasets have enabled exploration and new insight into epigenetics and drug resistance (Hogan et al. 2011). Here, we found novel mechanisms involving p38α in resistance to MX2 using high-throughput methylation analysis of multiple CpG sites and GO and key node analyses.

Inhibition of p38 MAPK activation by pharmacological inhibitors increased the cytotoxicity of MX2 in MX2-resistant leukemia cell lines, but not in MX2-sensitive cell lines. Our results suggest that adding p38 MAPK inhibitors will decrease the resistance to MX2 in MX2-resistant leukemia cell lines by partially decreasing p38 activity.

The p38 MAPK pathway, which was initially identified as playing a role in stress and the inflammatory response, has a tumor suppressor function as well. The p38 MAPK pathway suppresses tumorigenesis by controlling the cell cycle, cell differentiation, cell proliferation, oncogene-induced and replicative senescence, contact inhibition, the...
DNA damage response, and induction of apoptosis. p38 MAP kinase also inhibits apoptosis in several types of cells, including multiple myeloma cells (Navas et al. 2006; Wen et al. 2008). p38 activation mediates tamoxifen resistance in estrogen receptor-positive breast tumors (Gutiérrez et al. 2005). p38 inhibition enhances the sensitivity of multiple myeloma cells to arsenic trioxide and bortezomib (Wen et al. 2010). However, the details of the mechanism of enhanced drug sensitivity remain unclear. Our current study is the first to show that cytotoxicity due to increased inhibition of p38 MAPK is related to aberrant methylation in drug-resistant leukemia cells.

The gold standard of current treatment against relapsed or refractory leukemia is allogeneic stem cell transplantation. This treatment strategy is effective for patients in complete remission, but not in patients who have not achieved complete remission. Although increasing the dose of cytotoxic drugs increases the cure rate and the rate of complete remission in relapsed patients, the cure rate has recently reached a plateau. A novel strategy to achieve complete remission in relapsed patients is needed. We believe that epigenetics is a major mechanism of drug resistance (Asano et al. 2005; Yamanishi et al. 2015). We analyzed the genome-wide methylation status in MX2-resistant leukemia cells and found that p38α was a key enzyme in MX2-related drug resistance. This strategy for finding the key enzyme from the viewpoint of epigenetic changes may be a powerful concept for exploring new drugs to combat drug resistance. A p38 inhibitor may be a novel candidate for leukemia treatment.

In conclusion, our study showed that the p38α signaling pathway is involved in MX2-induced drug resistance. Inhibition of p38 MAPK restored the sensitivity to MX2 in MX2-resistant leukemia cell lines. Thus, p38 inhibitors may provide new chemotherapeutic options for overcoming drug resistance in the treatment of cancer. Further studies on the mechanisms of p38 inhibitors in drug resistance and the development of effective p38-specific antagonists with low toxicity are expected to improve the clinical effects of chemotherapy.

Author Contribution

Participated in research design: Takeshi Asano. Conducted experiments: Takeshi Asano, Hidehiko Narazaki, and Atsushi Fujita. Contributed new reagents or analytic tools: Takeshi Asano. Performed data analysis: Takeshi Asano. Wrote or contributed to the writing of the manuscript: Takeshi Asano.

Disclosures

None declared.
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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Methylation profile of K562/P and K562/MX2 cells.

Table S2. Gene expression profile of K562/P and K562/MX2 cells.
Table S3. (A) List of genes with hypermethylation and lower expression in K562/MX2 cells. (B) List of genes with hypermethylation and higher expression in K562/MX2 cells. (C) List of genes with hypermethylation and higher expression in K562/MX2 cells. (D) List of genes with hypomethylation and lower expression in K562/MX2 cells.

Table S4. List of genes for Gene Ontology analysis. (B) Gene Ontology results and gene list.