Expression of Aryl Hydrocarbon Receptor Nuclear Translocator Enhances Cisplatin Resistance by Upregulating MDR1 Expression in Cancer Cells

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Received May 7, 2013; accepted July 31, 2013

ABSTRACT

The identification of molecular pathways in cancer cells is important for understanding the cells’ underlying biology and for designing effective cancer therapies. We demonstrate that the expression of aryl hydrocarbon receptor nuclear translocator (ARNT) is critical during the development of cisplatin resistance. The reduced expression of ARNT was correlated with cisplatin-induced cell death in drug-sensitive cells. In addition, suppression of ARNT reversed the characteristics of cisplatin-resistant cells, making these cells cisplatin-sensitive, and significantly enhanced caspase-3 activation, DNA fragmentation, and apoptosis. The inhibition of colony formation, regulated by cisplatin, was more significant in ARNT-knockdown cells than in parental cells. In a xenograft analysis of severe combined immunodeficiency mice, cisplatin also efficiently inhibited ARNT-deficient c4 tumors but not ARNT-containing vT2 tumor formation. Furthermore, the downregulation of multidrug resistance 1 (MDR1) expression and retention of drugs in cells caused by suppression of ARNT, resulting in the resensitization of drug-resistant cells to cisplatin, was observed. When overexpressed, ARNT interacted with Sp1 to enhance the expression of MDR1 through Sp1-binding sites on the MDR1 promoter, resulting in a reversal of the effect of cisplatin on cell death. In addition, ARNT-induced MDR1 expression was inhibited in Sp1-knockdown cells. These results reveal previously unrecognized, multifaceted functions of ARNT in establishing the drug-resistant properties of cancer cells by the upregulation of MDR1, highlighting ARNT’s potential as a therapeutic target in an important subset of cancers.

Introduction

The aryl hydrocarbon receptor (AhR) nuclear translocator (ARNT), also known as hypoxia-inducible factor (HIF)-1α, is a member of the basic helix-loop-helix–period, ARNT, single-minded (bHLH-PAS) family of transcription factors. It serves as a dimerization partner for a number of other transcription factors, such as the AhR, HIF-1α, single-minded, and c-Jun proteins (Kewley et al., 2004; Chang et al., 2009). Previous studies suggested that ARNT is a new regulatory factor that interacts with c-Jun and bridges the binding of c-Jun with Sp1 to regulate epidermal growth factor (EGF)-induced cyclooxygenase-2 (COX-2), 12(15)-lipoxygenase, and p21WAF1/CIP1 gene expression under normoxic conditions, thus contributing to tumorigenesis (Chang et al., 2009; Huang et al., 2010). In addition, ARNT is essential for normal embryonic development (Kozak et al., 1997). Loss of ARNT results in reduced tumor growth, decreased angiogenesis, and an increased response to radiotherapy (Carmeliet et al., 1998). Inactivation of ARNT suppresses the development of liver hemangiomas, polycthemia, and HIF-induced gene expression (Rankin et al., 2005). In contrast to ARNT, however, HIF-1α is insufficient to suppress the development of von Hippel-Lindau (VHL)–associated polycythemia (Rankin et al., 2005). This observation indicates that the development of VHL-associated vascular tumors in the liver depends on a functional ARNT, but not in an HIF-1α/hypoxia-dependent manner. Although
ARNT is ubiquitously and constitutively expressed in cells, and the translocator's functions under hypoxic conditions and in the presence of stimulating xenobiotics have been largely discussed, little is known about ARNT's role in response to cancer-therapeutic drugs, such as cisplatin, in normoxia.

Cisplatin revolutionized the treatment of solid tumors of the testes and ovaries and is the first-line drug for treating human cervical cancer, the second most common cancer among women in the world. In chemotherapy, the drug remains a cornerstone for treating various malignant tumors (Kelland, 2007). Cisplatin induces cytotoxicity through the formation of cisplatin-DNA adducts (Takahara et al., 1995), which lead to irreparable DNA damage and cell death. Understanding the molecular basis of such cisplatin-mediated apoptosis could lead to strategies for therapeutic benefits. Despite its success in treating certain cancers, the major challenge of cisplatin chemotherapy is drug resistance. Possible mechanisms of acquired resistance to cisplatin include the inability of cisplatin to reach its target DNA due to diminished cisplatin uptake, increased efflux, or increased cellular glutathione and metallothioneins, and the inhibition of apoptosis induction after formation of cisplatin-DNA adducts due to increased DNA repair, spoiled apoptosis, or altered oncogene expression (Dempke et al., 2000; Siddik, 2003; Wang and Lippard, 2005; Stewart, 2007). Although these mechanisms have been identified, it is still a challenge to overcome drug resistance. Therefore, accurate elucidation of the mechanisms involved in regulating drug resistance is an important issue in cancer therapy.

The resistance of cancer cells to anticancer drugs, such as paclitaxel, vinblastine, etoposide, doxorubicin, and cisplatin, which comprehensively differ in their mechanisms of action and molecular structures, often occurs due to the enhanced expression of ATP-binding cassette (ABC) transporters. These transporters can use the energy of ATP hydrolysis to transport a wide range of substances across cell membranes (Leonard et al., 2003; Takara et al., 2006; Chattopadhyay et al., 2008). The multidrug resistance (MDR) efflux pumps ABC subfamily B member 1 (P-glycoprotein), ABCB1 (MRP1), ABCB2 (MRP2), and ABCG2 (BCRP and MXR) actively expel numerous types of drugs from cancer cells, thereby conferring resistance to those agents (Leslie et al., 2005). Among the ABC transporters, P-glycoprotein/MDR1 mediates resistance to a wide variety of anticancer agents, and the regulation of MDR1 expression might contribute to the development of appropriate agents to combat MDR and improve chemotherapeutic efficacies (Liu et al., 2007). Because tumorigenesis usually involves normoxic conditions, and given that ARNT is involved in response to cancer-therapeutic drugs, such as cisplatin, in normoxia.

Western Blotting. An analytical 10% SDS-PAGE was performed, and 30 μg of protein was analyzed, unless stated otherwise. For immunoblotting, proteins in the SDS gels were transferred onto a polyvinylidene difluoride membrane by an electroblot apparatus. Antibodies against human caspase 3 (Cell Signaling Technology, Danvers, MA), MDR1 (Gene Tex, Irvine, CA), ARNT and β-actin (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as the primary antibodies. Mouse or rabbit IgG antibodies coupled to hors eradish peroxidase were used as secondary antibodies. An enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL) was used for detection.

DNA Fragmentation Assay. Cells treated with or without cisplatin were collected; washed in phosphate-buffered saline; lysed in a solution containing 10 mM Tris HCl, pH 8.0, 10 mM EDTA, and 0.5% Triton X-100; digested with 0.1 mg/ml RNase A at 37°C for 1 hour; and then centrifuged at 12,000g for 25 minutes to pellet chromosomal DNA. The supernatant was digested with 1 mg/ml proteinase K at 50°C for 2 hours in the presence of 1% sodium dodecyl sulfate, extracted with phenol and chloroform, precipitated in cold ethanol, and subjected to electrophoresis on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. DNA fragments were visualized by ultraviolet light transillumination. Photographs were taken with the aid of a computer-assisted image processor.

Materials and Methods

Cell Culture. The cell lines of A375, A431, and human cervical cancer HeLa and SiHa were grown at 37°C under 5% CO₂ in 10-cm plastic dishes containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. In this series of experiments, cells were treated with 50 ng/ml EGF (Pepro Technology, Rocky Hill, NJ) in culture medium supplemented with 10% fetal bovine serum, unless stated otherwise. The cell lines of c4 and vT2 were grown in same manner but with minimal essential media and 0.1 mg/ml G418 for vT2 cells. C4 and vT2 cells originate from the same cell type, whereas c4, a 3,4-benzopyrene-resistant mutant clone (c4) of the mouse hepatoma Hepa-1c1c7 cell line, was examined for a single point mutation, leading to replacement of Gly326 with Asp between two internal repeats in the highly conserved PAS domain that causes the defective function of ARNT; the vT2 cell line was derived from c4 cell line, possesses a complete transfected ARNT cDNA, and expresses the ARNT gene (Numayama-Tsuruta et al., 1997). Drug-resistance schedules were used to develop the sublines resistant to cisplatin. HeLa cells were exposed to cisplatin for a 9-month period. The resistance subline obtained by this procedure is denoted as HeLa R.

MDR1 and ARNT primers (sense, 5’-TGGTTCAAACTTCTGCTCCTGA-3’ and 5’-CATTCAGGCGCAGAGACCACTT-3’; antisense, 5’-ATTGACGGTGT-3’ and 5’-CCATCACCATCTTCCAGGAG-3’), Cu²⁺-transporting ATPase subunit β1 (ATP1B1) primers (sense, 5’-GCCAGGATTAACACAGATTCCT-3’ and 5’-TGGGTCCAGCCATTGCCTCT-3’; antisense, 5’-CCATCACCATCTTCCAGGAG-3’), Cu²⁺-transporting ATPase β polypeptide (ATP7B) primers (sense, 5’-CTCTGGTCTGCTATTAGCAGGT-3’ and 5’-CCATCACCATCTTCCAGGAG-3’), MDR1 primers (sense, 5’-GCCACCATATGGTCAAGGCG-3’ and 5’-GTTCAAACCTCTCTTCTGTA-3’), and glyceraldehyde-3-phosphate dehydrogenase primers (sense, 5’-CCACTCACCCTTCGAGAG-3’ and 5’-CTGCTTACACACTCCCTTTG-3’) were used. The PCR products were separated by 1% agarose–gel electrophoresis and visualized with ethidium bromide staining.
**Plasmid Construction.** A 1500 bp fragment from the human MDR1 promoter region was PCR-amplified from human genomic DNA and subcloned into luciferase plasmid PGL2 at the Nhe I and Hind III sites as the pMDR1 plasmid. The forward and reverse primers were 5’-CTAGCTAGGAAAGTGGAAATCCATCTCAGAC-3’ and 5’-CCCAAGC TTGTCTCCAGCTACATCTCCACGG-3’. The mutants at Sp1 site (pMDR1–Sp1 mutation [Sp1m]) were constructed by the site-directed mutagenesis method. Synthetic primers are shown as follows: pMDR1-Sp1-1m mutant primer 5’-GGAGAAGGGCTTCTGCTATGATTGTY TGGATAGTGGAAATCCATCTCAGAC-3’, and pMDR1–Sp1-2m mutant primer 5’-GGAGAAGGGCTTCTGCTATGATTGTY TGGATAGTGGAAATCCATCTCAGAC-3’. Mutated positions in the DNA sequences are underlined. The vector sequences were confirmed by DNA sequencing.

**Transfection of Cells with ARNT siRNA Oligonucleotides.** Transient transfection of cells with 30 nM ARNT siRNA oligonucleotides was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions but with slight modifications. For use in transfection, 2 μl of Lipofectamine 2000 was incubated with ARNT siRNA or scrambled siRNA (Invitrogen) in 1 ml of Opti-MEM medium for 30 minutes at room temperature. After a change from Opti-MEM medium to 2 ml of fresh culture medium, cells were incubated for an additional 24 hours, unless stated otherwise.

**MTT Assay.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as a cytotoxicity assay for the HeLa, HeLa R, c4, and vT2 cells. Briefly, cells (2 × 104 cells/ml) were seeded into 96-well plates and treated with varying concentrations of cisplatin for 36 hours. The MTT assay was performed using a commercially available MTT assay kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. The remaining viable cells with MTT dye uptake were determined by measuring the optical density at 490 nm in an enzyme-linked immunosorbent assay reader. Values represent mean ± S.E. of six determinations.

**Apoptosis Analysis by Flow Cytometry.** Quantification of apoptosis induced by cisplatin was performed with Annexin V and propidium iodide staining according to the manufacturer’s instructions (BioVision, San Francisco, CA). Briefly, 1 × 106 cells were resuspended in Annexin V binding buffer and stained with Annexin V–phycoerythrin and propidium iodide (1 μg/ml). After incubation at room temperature, apoptotic cells were quantified by flow cytometry using the Cell Lab Quanta SC (Beckman Coulter, Brea, CA).

**Transfection of Cells with Plasmids and Luciferase Assay.** Luciferase vectors bearing wild-type (pMDR1) or Sp1 mutants (pMDR1-Sp1m) of the MDR1 gene promoter were used. Transient transfection of cells with plasmids was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions but with slight modification. The luciferase activity in cell lysate was determined as described previously (Chang et al., 2009).

**DNA Affinity Precipitation Assay.** Quantitation of the change in ARNT and Sp1 binding to the MDR1 promoter element was achieved by DNA affinity precipitation assay according to the method reported previously (Chang et al., 2009). In brief, 5’-biotinylated oligonucleotides corresponding to the sense (–56 to –46 bp and +258 to +269 bp) and antisense strands of the MDR1 promoter element were annealed. The DNA affinity precipitation assay was performed by incubating 2 μg of biotinylated DNA probe with 200 μg of nuclear extract and 20 μl of streptavidin–agarose beads in saline on ice at room temperature for 1 hour with rotation. Beads were collected and washed three times with cold phosphate-buffered saline. The binding proteins were eluted by loading buffer and separated by SDS-PAGE, followed by Western blot analysis probed with specific antibodies.

**Cell-Counting Assay.** Quantitation of the viability of cisplatin-treated cells was achieved by a Cell Counting Kit–8 according to the manufacturer’s instructions (258992; Sigma–Aldrich, St. Louis, MO). Briefly, 5000 cells were dispensed to each well of 96-well plate. After cells were treated with cisplatin for 24 hours, 10 μl of the Cell Counting Kit–8 solution was added into each well. After 4 hours, the absorbance at 450 nm was measured using a microplate reader.

**Colonic Assay.** To determine the long-term effects of ARNT on cisplatin-inhibited colony formation, cells were treated with cisplatin for 8 hours. After being rinsed with fresh medium, cells were allowed to grow for 6 days to form colonies, which were then stained with crystal violet (0.4 g/l; Sigma–Aldrich).

**Xenograft Analysis.** Female severe combined immunodeficiency (SCID) mice (at around 6–7 weeks of age) were randomly injected with c4 or vT2 cells. In total, 1 million cells in 200 μl phosphate-buffered saline were subcutaneously injected into both flanks of each mouse. The mice were monitored for the appearance of tumors and were sacrificed at the indicated times. The volume of the tumors was measured. Tumor size was measured by external caliper, and tumor volume was calculated using a standard formula as follows: V = height × width × depth. All mice were obtained from the National Cheng Kung University Laboratory Animal Center (Tainan, Taiwan) and the National Laboratory Animal Center (Tainan, Taiwan). All animal experiments in this study were approved by the Laboratory Animal Committee of National Cheng Kung University.

**Statistical Analysis.** In all experiments, statistical significance was analyzed by Student’s t test. P < 0.05 was considered significant.

## Results

**Expression of ARNT Regulates Cisplatin-Induced Cancer Cell Death.** To test whether ARNT has any effects on cancer therapy, we first characterized cancer cell survival in response to cisplatin treatment in sensitive HeLa cells and HeLa cells with acquired resistance (HeLa R). As shown in Fig. 1A, cisplatin significantly reduced the cell viability of HeLa but not HeLa R cells. Interestingly, the reduction in ARNT in cisplatin-treated cells was correlated with cell death (Fig. 1C). To confirm whether the expression of ARNT was accompanied by cisplatin resistance, the hepatoma c4 cell line, in which ARNT is degraded by replacing Gly326 with asparagine, and vT2 cells, which were derived from c4 cells possessing complete transected ARNT cDNA (Numayama-Tsuruta et al., 1997) (Fig. 1C), were used in a cell survival

### Fig. 1. Effect of cisplatin on cell survival and ARNT expression. (A and B) HeLa, HeLa R, c4, and vT2 cells were treated with 30 μM cisplatin alone or not treated (control). After 36 hours, an MTT assay was performed as described in Materials and Methods. Error bars denote mean ± S.E.M. (n = 3). (C) Cells were treated with various concentrations of cisplatin for 36 hours. Lysates of cells were prepared and subjected to SDS-PAGE and analyzed by Western blotting with antibodies against ARNT and actin.
Fig. 2. ARNT siRNA enhances cisplatin (CDDP)-induced apoptosis in resistant cells. Cells were transfected with 30 nM ARNT siRNA oligonucleotides and scrambled oligonucleotides (SC) by lipofection. (A) After treatment with 30 μM cisplatin for 36 hours, cell lysates were prepared and subjected to SDS-PAGE and analyzed by Western blotting with antibodies against ARNT and actin. (B and C) Apoptotic fractions were determined by flow cytometry.
analysis. As shown in Fig. 1B, cisplatin inhibited cell survival in c4 but not vT2 cells. These results imply that ARNT expression is correlated with cisplatin-induced cell death.

**Knockdown of ARNT Reduces Cisplatin Resistance in Cancer Cells.** Based on the findings that ARNT expression may confer resistance to cisplatin, we assessed the role of ARNT in cisplatin-induced cell death by knocking down ARNT in resistant cells. In addition to the human cervical cancer cell line HeLa, SiHa, which is another type of cervical cancer, was used. The results showed no reduction in ARNT in the cisplatin-treated condition (Fig. 2A). The knockdown efficiency of ARNT in SiHa cells was confirmed by a Western blot analysis (Fig. 2A). A flow cytometry–based cell-cycle analysis revealed that SiHa cells were indeed more resistant to cisplatin than were HeLa cells (Fig. 2B). However, the resistance of SiHa cells to cisplatin was eliminated by knockdown of ARNT (Fig. 2B). To further confirm that ARNT expression is required for cancer cells to be resistant to cisplatin, c4 and vT2 cells were separately treated with cisplatin. The absence and presence of ARNT in c4 and vT2 cells, respectively, were confirmed, as shown in Fig. 1C. As shown in Fig. 2C, apoptosis of c4 but not vT2 cells dramatically increased during cisplatin treatment.

To further study whether the sensitization of resistant cells to cisplatin is regulated by ARNT, we first studied cisplatin-induced apoptosis by examining the activation of caspase-3 and DNA fragmentation in ARNT siRNA-treated resistant cells. As shown in Fig. 3A, lane 3, and B, lane 2, activation of caspase-3 was observed when sensitive HeLa and c4 cells were treated with cisplatin. In contrast, resistant vT2, HeLa R, and SiHa cells did not show any apoptotic index in the cisplatin-treated condition (Fig. 3A, lane 1 and B, lane 1), whereas ARNT siRNA-sensitized SiHa and HeLa R cells exhibited apoptosis with cisplatin treatment (Fig. 3A, lane 8, and B, lane 9). Conversely, cisplatin-induced activation of caspase-3 was inhibited by overexpression of ARNT in sensitive HeLa cells (Fig. 3C). Upon analyzing the fragmented DNA, which is a hallmark of apoptosis, we observed fragmentation in cisplatin-treated HeLa and c4 cells (Supplemental Fig. 1A, lanes 3 and 12). In addition, when ARNT was knocked down in drug-resistant cells, DNA fragmentation dramatically increased with cisplatin treatment (Supplemental Fig. 1A, lane 8, and B, lane 8). The results indicated that ARNT siRNA caused...
drug-resistant cells to become sensitized to chemotherapeutic treatment by activating the apoptosis pathway.

To study whether ARNT’s participation in the regulation of cisplatin-induced cell death is universal, stable cell lines with ARNT knockdown were generated by stably transfecting melanoma A375 cells with an expression vector encoding a short hairpin RNA against ARNT (Fig. 4A). The effect of cisplatin on these short hairpin ARNT (shARNT) cells was examined using cell viability and flow cytometric analyses. As shown in Fig. 4, B and C, stable shARNT cell lines were more sensitive to cisplatin-induced cell death in a dose-dependent manner. These results reveal that the effects of ARNT in cisplatin treatment occur in various types of cancer cells.

ARNT Regulates Cancer Cell Tumorigenicity and Therapeutic Efficacy in SCID Mice Treated with Cisplatin. We further examined the effect of ARNT inhibition on the therapeutic efficacy of cisplatin in vitro and in vivo using colony formation and xenograft analyses, respectively. As shown in Fig. 5A, although cisplatin induced a partial inhibition of colony formation in control cells, dramatic inhibition was observed in shARNT cell lines when cells were treated with the same concentration of cisplatin. In addition, our results indicated that vT2 but not c4 cells expressed the ARNT protein (Fig. 1C). We injected c4 or vT2 cells subcutaneously into the flanks of SCID mice to study the effect of ARNT on cisplatin-inhibited tumor formation. As shown in Fig. 5, B and C, the growth rate of c4 cells was slower than that of the vT2 groups during the early stages of tumor formation. This result was consistent with the previous finding that ARNT is required during the early stages of growth of the tumors but is less necessary in later stages (Shi et al., 2010). Cisplatin treatment showed no inhibition of SCID mouse tumor growth in the vT2 group, which had a high level of ARNT expression (Fig. 5D). However, cisplatin treatment resulted in a significant reduction in tumor volume in the c4 group (Fig. 5C). These results indicated that cisplatin resistance is caused, at least in part, by ARNT overexpression.

ARNT Contributes to Drug Efflux by Upregulating ABC Transporters. Based on the observation that ARNT

![Fig. 4. Stable ARNT-knockdown cells are more sensitive to cisplatin treatment. (A) The ARNT-deficient cell lines were selected by infecting A375 cells with lentivirus containing an expression vector encoding a short hairpin RNA (shRNA) against ARNT. Expressions of ARNT and α-tubulin were analyzed in shARNT #1 and -#2 cells or negative control shLacZ-Z cells by Western blot (WB) using anti-ARNT and anti-tubulin antibodies. P, parental cells; Z, shLacZ cells. (B) ARNT deficient (shARNT) A375 cells were treated with cisplatin (CDDP) for 24 hours. Cell viability was analyzed using the Cell Counting Kit–8 assay. Statistical significance (**P < 0.01) between Z and shARNT was analyzed by Student’s t test. P, parental cells; Z, shLacZ cells. (C) ARNT deficient (shARNT) A375 cells were treated with cisplatin for 24 hours. The apoptotic cells were examined using Annexin V and propidium iodide (PI) staining in flow cytometric analysis. The apoptosis ratio was calculated. *late apoptosis; #early apoptosis.](image-url)
expression altered the sensitivity of cancer cells to cisplatin, we were curious about the mechanisms of ARNT-related cisplatin resistance. In general, ABC transporters play functional roles in regulating drug resistance by exporting drugs out of cells. To assess whether drug efflux is involved in cell resistance to cisplatin-induced apoptosis, drugs expelled from resistant cells were examined using conditioned medium collected from cisplatin-treated cells, and the activation of caspase-3 was detected. As shown in Fig. 6, A and B, conditioned media harvested from cisplatin-treated HeLa R and vT2 cells, respectively, induced caspase-3 activation in HeLa and c4 cells. Conversely, no significant activation of caspase-3 was observed in HeLa R cells treated with conditioned media (Fig. 6A). However, the conditioned medium harvested from cisplatin-treated HeLa R cells dramatically enhanced the activation of caspase-3 and cell death in ARNT-knockdown HeLa R cells (Fig. 6, A and C). These results suggest that HeLa R and vT2 cells resisted cisplatin by pumping the drug into the medium. To clarify whether ARNT indeed regulates drug efflux, the efflux activity of MDR was studied in ARNT-knockdown cells. MDR1 inhibition was examined by measuring calcein acetoxymethyl ester retention in HeLa and HeLa R cells using an MDR assay as shown in Fig. 6D, cisplatin enhanced the retention of calcein AM in sensitive HeLa cells, indicating inhibitory effects on MDR1 activity. Conversely, calcein AM was significantly expelled from HeLa R cells, even with cisplatin treatment. However, HeLa R cells lost the ability to exclude calcein AM from cells in ARNT-knockdown condition. The results indicated that ARNT mediated cisplatin resistance by controlling the efflux of drugs from cancer cells.

To clarify whether ARNT mediates drug efflux by regulating ABC or other transporters, the expression of the ABC transporter, MDR1, and cation transporters such as Na+/K+-transporting ATPase subunit β and Cu2+-transporting ATPase β polypeptide were detected in cisplatin-resistant cells. As shown in Supplemental Fig. 2, no change in ATP1B1 gene expression was observed in cisplatin-treated, ARNT siRNA-treated, or combination-treated HeLa R cells. In addition, ATP7B gene expression was partially inhibited in the ARNT-knockdown condition (Supplemental Fig. 2). Furthermore, cisplatin significantly induced ATP1B1 but not ATP7B expression in HeLa cells. This finding shows that the response of sensitive cells to cisplatin-induced cell damage might not be due to the downregulation of cation transporters. We next clarified whether the expression of MDR1 is regulated by ARNT. As shown in Fig. 7A, MDR1 expression was inhibited in cisplatin-treated HeLa and c4 cells but not in HeLa R or vT2 cells. MDR1 expression was higher in resistant vT2 and HeLa R cells than in sensitive c4 and HeLa cells (Fig. 7A). Importantly, ARNT siRNA significantly inhibited the expression of MDR1 in cisplatin-resistant SiHa and HeLa R cells (Fig. 7B). To examine whether regulation of the expression of MDR1 by ARNT is a common phenomenon, the contribution of ARNT to the upregulation of MDR1 expression was also confirmed in various shARNT cell lines. As shown in Fig. 7C, MDR1 protein levels were significantly
reduced in ARNT-deficient melanoma A375 and cervical cancer A431 cell lines. These results suggest that ARNT contributes to cisplatin resistance by the regulation of MDR1 expression.

**ARNT Regulates MDR1 Expression through the Binding of the ARNT/Sp1 Complex to the Gene Promoter.** To confirm that ARNT regulates MDR1 expression through transcriptional activation of the gene, a vector containing the human MDR1 gene promoter was used in a reporter assay. As shown in Fig. 8A, MDR1 promoter activity was significantly reduced in ARNT-deficient cell lines. To complement the loss-of-function studies, cells were transfected with 30 nM ARNT short hairpin RNA nontargeting form of ARNT (pARNT-KDR). The expression of ARNT derived from pARNT-KDR was verified in shARNT cell lines (Supplemental Fig. 3A). The transcriptional functions of pARNT-KDR and wild-type pARNT were also verified by inducing hypoxia response element promoter activity under hypoxic conditions (Supplemental Fig. 3B). Indeed, the overexpression of ARNT derived from pARNT and pARNT-KDR enhanced MDR1 promoter activity in parental cells and restored the promoter activity in ARNT-knockdown cells, respectively (Fig. 8, B and C). These results suggest that ARNT contributes to transcriptional activation of the MDR1 promoter. The literature states that Sp1-binding sites on the gene promoter, such as 12(S)-lipoxygenase and p21WAF1/CIP1, were directly targeted by ARNT, resulting in the induction of gene expression (Huang et al., 2010). To determine whether the binding of Sp1 and ARNT to the gene promoter is responsible for ARNT induction, we performed DNA affinity immunoprecipitation assays. As shown in Fig. 9A, Sp1 and ARNT bound to Sp1-binding sites but bound much less to a mutated Sp1 sequence on the MDR1 promoter. In addition, binding of ARNT to the MDR1 promoter was inhibited in Sp1-knockdown cells (Fig. 9B). To determine whether two Sp1-binding sites (−56 to −46 bp and +258 to +269 bp) located in the promoter region were embedded in transcriptionally active sequences, DNA constructs with mutations at Sp1-binding sites were transfected.
into cells. Comparing pMDR1 with the wild-type Sp1 binding sequence, a significant decrease in ARNT-stimulated response was observed in pMDR1 Sp1-1m and pMDR1 Sp1-2m, and complete elimination of the ARNT response was detected in pMDR1 Sp1-1/2m in cells (Fig. 9C; Supplemental Fig. 4A), indicating that Sp1-binding sites are essential for ARNT-mediated MDR1 gene expression. In addition, ARNT-induced MDR1 promoter activity was also inhibited in Sp1-knockdown cells (Fig. 9D; Supplemental Fig. 4B). These results revealed that the binding of the ARNT/Sp1 complex to Sp1-binding sites is essential for the activation of the MDR1 gene promoter.

Discussion

The development of an MDR phenotype is a major cause of treatment failure in cancer therapy. Importantly, the upregulation of drug efflux ABC transporters, such as MDR1 and ABCG2, was identified and characterized as transporting and conferring resistance to virtually the entire spectrum of cancer drugs. Consequently, the upregulation of these genes can cause MDR in cancers (Gottesman et al., 2002; Szakács et al., 2004). However, the strategies to overcome MDR are still limited. Herein, we report, for the first time, that activation of the ARNT pathway is critical in the development of drug-resistant properties by the induction of MDR1 in cancer cells. In studying mechanisms for drug resistance, genetic alterations within a tumor cell population are selected during drug treatment (Sharma et al., 2010). However, other studies identified nonmutational mechanisms of drug resistance. For example, drug efflux is associated with epigenetic changes in the promoter region of MDR1 and chromatin alterations. Hypermethylation of CpG dinucleotides within the MDR1 promoter region is closely associated with the low expression of MDR1 in various cancer cell lines and tissues (El-Osta et al., 2002; Baker et al., 2005). In this study, we found that upregulation of MDR1 by ARNT improved cisplatin efflux and initiated the resistant properties of cancer cells. These results indicated that the transcriptional regulation of gene expression by transcription factors, such as ARNT, is an alternative pathway that regulates MDR1's functions, especially at the level of drug efflux.

ARNT is essential to maintain the normal functions of HIF-1α, HIF-2α, and the AhR, which are required for cellular responses to hypoxia (HIF proteins) and environmental toxins (AhR) (Kewley et al., 2004). ARNT-containing dimers were reported to regulate the expression of various genes in response to differential stimuli. These promoters have multiple potential ARNT-binding sites. Under hypoxic conditions, HIF-1α/ARNT dimers activate the transcription of numerous target genes whose the promoters contain a binding motif termed the hypoxia-response element (Semenza, 2001). Our previous studies indicated that ARNT regulates the transcriptional activation of genes that do not contain ARNT-binding sites, such as COX-2, 12(S)-lipoygenase, and p21WAF1/CIP1 (Chang et al., 2009; Huang et al., 2010). Herein, we provided evidence to show that ARNT indeed regulates gene expression in a hypoxia response element–independent manner. MDR1 expression can be regulated by ARNT through Sp1-binding sites. These results indicate that ARNT either directly or indirectly binds to DNA by forming bHLH-PAS heterodimers and interacting with other transcription factors to regulate gene expression. By the alternative mechanism of extending the effect of ARNT on gene expression in different environments, this feature broadens the role that ARNT plays in physiologic and pathophysiologic functions.
In addition to ARNT, several transcription factors, including FoxM1, HIF, HOXC6, and pCAF, have been reported to be involved in regulating MDR1 expression and drug resistance under various cellular conditions. Consistent with what was observed in ARNT-regulated MDR1 expression and cisplatin resistance, the knockdown of FoxM1 in cells that are resistant to cisplatin can induce apoptosis by reducing breast cancer (BRCA)-associated gene 2 expression (Kwok et al., 2010). In addition, HOXC6 increases resistance to chemotherapeutic drugs by inducing MDR1 in oral cancer cells (Kim et al., 2013). These results indicated that transcription factors mediate cisplatin resistance in different manners, including by controlling drug efflux and DNA repair pathways. Regarding the regulation of cisplatin resistance, several studies have also shown that the activation of signaling pathways determines the activation of these factors and drug resistance. The activation of polo-like kinase 1 by growth factor–activated signaling pathways such as phosphoinositide 3-kinase/Akt and Ras/Raf/mitogen-activated protein kinase results in the phosphorylation and activation of FoxM1 (Le et al., 2005; Fu et al., 2008), leading to cisplatin resistance (Spankuch et al., 2006). Although the mechanism involved in the activation of ARNT is still unclear, we found that EGF enhanced the nuclear accumulation and phosphorylation of ARNT in cells (Chang et al., 2009), and the effect was inhibited by an MEKI/2 inhibitor (data not shown). This finding suggests that the EGF-activated extracellular signal-regulated kinase signaling pathways may be involved in the activation of ARNT.

The cofactor associated with ARNT’s regulation of MDR1 expression is not well characterized under normoxic conditions. However, we found that the binding of Sp1/ARNT to Sp1-binding sites on the MDR1 promoter was essential for ARNT-induced MDR1 promoter activity. Kim et al. also showed that the downregulation of HDAC1 by RNA silencing induced an increase in the interaction of pCAF with Sp1 and the binding of C/EBPβ and pCAF to an Sp1-binding site on the MDR1 promoter, resulting in increased MDR1 expression in cancer cells (Kim et al., 2009). Among these factors, Sp1 also provides a docking site for ARNT to enhance p21WAF1/CIP1 and 12(S)-lipoxygenase gene expression (Huang et al., 2010). This finding indicates that possible interactions between ARNT, Sp1, C/EBPβ, and pCAF may control the expression of MDR1 in a specific cell type or condition. Although MDR1 expression is also induced in hypoxia (Comerford et al., 2004), a functional HIF heterodimer (HIF-1α/ARNT) is a prerequisite for the observed upregulation of MDR1 expression under hypoxic conditions (Wartenberg et al., 2003). In this study, we found that ARNT induced the expression of MDR1 in...
normoxia. This finding reveals that regardless of the oxygen concentration in cancer, ARNT, but not HIF-1α, plays a pivotal role in controlling MDR1 expression.

Interestingly, we found that ARNT levels were reduced in cisplatin-treated sensitive cells. However, very few studies have investigated how ARNT is controlled and how this translocator responds to stimulation. Recently, tumor necrosis factor-α–activated nuclear factor-κB was shown to induce expression of ARNT (van Uden et al., 2011). Our previous studies also revealed that EGF enhances nuclear accumulation of ARNT (Chang et al., 2009; Huang et al., 2010). The stabilization of p53 induced by severe hypoxia results in the activation of caspases and, in turn, cleaves ARNT and turns off HIF-induced gene expression. The activation of caspase-3 and caspase-9 cleaves ARNT at the amino acid 151 Asp and leads to the loss of the bHLH domain (Suzuki et al., 2001). In addition, the other clue that molecules involved in cisplatin-induced cell death may be correlated with ARNT degradation is BRCA1, which is known to regulate cell cycle progression, DNA repair, apoptosis, and transcription (Rosen et al., 2003). The methylation of BRCA1 accounts for not only the genomic instability but also the cisplatin sensitivity of a large fraction of ovarian cancers (Taniguchi et al., 2003). BRCA1 also interacts with ARNT and stabilizes the ARNT protein under xenobiotic (2,3,7,8-tetrachlorodibenzo[b,e][1,4]-dioxin [TCDD]) stress (Kang et al., 2006). Thus, we proposed that reduced levels of BRCA1 may cause cisplatin-induced ARNT instability in sensitive cells. Identification of the BRCA1/ARNT complex in cisplatin-induced cell death will be an important issue in our future studies.

In correlating ARNT with tumorigenesis, several points should be recognized. First, the development of VHL-associated vascular tumors in the liver depends on functional ARNT (Rankin et al., 2005). Although HIF-1α is overexpressed in most tumors, certain tumors do not stain positive for this factor (Zhong et al., 1999). This issue makes analysis of ARNT important for determining the role of HIF in vascular tumors. Second, ARNT-deficient embryos show reduced levels of vascular endothelial growth factor protein and an increased rate of apoptotic hematopoietic cells (Ramirez-Bergeron et al., 2006). Third, genes that control cell proliferation and metastasis in normoxia, such as EGF-induced COX-2, 12(S)-lipoxigenase, and p21[WRAP53]-dependent expression of p21[WRAP53]/CIP1 are regulated by ARNT (Chang et al., 2009; Huang et al., 2010). In particular, stably ARNT-expressing vT2 cell–injected mice,
not c4 cell—injected mice, were resistant to cisplatin. This finding suggests that ARNT contributes to tumorigenesis and cisplatin resistance. In addition, cisplatin-induced cytotoxicity in cancer is critically dependent on the phosphorylation and subsequent degradation of the EGFR receptor (EGFR) (Ahsan et al., 2010). Because ARNT plays roles in regulating EGFR-induced gene expression, as mentioned above, the degradation of the EGFR induced by cisplatin in sensitive cells may also cause inactivation of ARNT in cell nuclei, resulting in reduced cell survival. Therefore, ARNT not only is a valid target in cisplatin-resistant tumors but can also mediate EGFR inhibitor resistance. Taken together, these findings yield the first identification of the bHLH-PAS family of transcription factors, which regulates expression of an ABC drug transporter through Spt1-binding sites on the promoter, reducing the retention of chemotherapeutic drugs in cells and protecting cells from drug-induced DNA damage. These results suggest a new target for the circumvention of tumor multidrug resistance.

Authorship Contributions

Participated in research design: W.-Cha. Chang, W.-Chi. Chang, Chen.

Conducted experiments: Chan, Kalpana, Chen.

Contributed new reagents or analytic tools: W.-Cha. Chang, Chen.

Performed data analysis: Chan, Kalpana, Chen.

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References


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