Intracellular Cl⁻ level

Fig. (2). Formation of intracellular cisplatin.

The equilibrium of cisplatin is affected by physiological conditions of intracellular pH and chloride concentration. The charged species under low Cl⁻ and/or acidic conditions (the H_2O-Cl, H_2O-OH and H_2O-H_2O species) are the most active forms.

been linked to the MAPK signaling cascade [22]. Another kinase, the c-Abl tyrosine kinase, is also activated by cisplatin. This kinase phosphorylates p73 and induces apoptosis [23]. The c-Abl pathway is also associated with the JNK signaling pathway, which is a member of the MAPK family [24]. The evidence therefore suggests that DNA damage signals might undergo crosstalk with each other.

Protein phosphatase is also involved in the cisplatin-induced signaling pathway through regulating the cellular phosphorylation state. Nuclear Src homology 2 domain-containing tyrosine phosphatase (SHP-2) was constitutively associated with c-abl and its phosphatase activity was significantly enhanced in response to DNA damage. It was reported that cells lacking SHP-2 showed markedly decreased apoptosis in response to DNA-damaging agents, such as cisplatin and γ-irradiation [25]. Furthermore, cisplatin has been shown to interact with the tumor-suppressor phosphatase and tensin homolog (PTEN), which plays an important role in cell growth and apoptosis [26]. The enzymatic activity of protein tyrosine phosphatases (PTPs) containing PTEN is often regulated by a redox system, including thioredoxin-1 [27, 28]. Apoptosis signal-regulating kinase 1 (ASK1) is a MAPK kinase kinase that activates p38 and JNK cascades, and is activated in response to oxidative stress [29]. Protein phosphatase 5 interacts with ASK1 and inhibits its activity [30]. These results provide

evidence that protein phosphatase is an important modulator of apoptosis through cisplatin-induced DNA damage and oxidative stress, and that it contributes to drug sensitivity.

Reactive oxygen species (ROS) are produced upon various stress stimulations—including ultraviolet (UV) irradiation and cytotoxic agents such as cisplatin—and are closely involved in stress-induced apoptosis. ROS production can enhance sensitivity to cisplatin through activation of the JNK or p38 pathways [31], or through Fas aggregation [32]. Furthermore, it has been recently reported that cisplatin could induce apoptosis in the absence of DNA damage, through ER stress [4]. Cisplatin induced the activation of the calcium-dependent protease calpain, which activated caspase-3 and ER-specific caspase-12 in cytoplasts [4]. These data suggest that the ER might be the non-nuclear target of cisplatin.

Cisplatin-induced apoptotic pathways are complicated, as cisplatin might cause different stresses, such as DNA damaging, oxidative and ER stresses. Various cisplatin-induced stress signals can activate each pathway through specific transcription factors that act as the ultimate drug targets. Cell death or survival in response to cisplatin might be dependent on the relative intensity of, and the crosstalk between, these signal pathways. Fig. (3) shows a schematic summary of cisplatin-induced cellular signaling involved in cell death and survival. Further studies will lead to a better
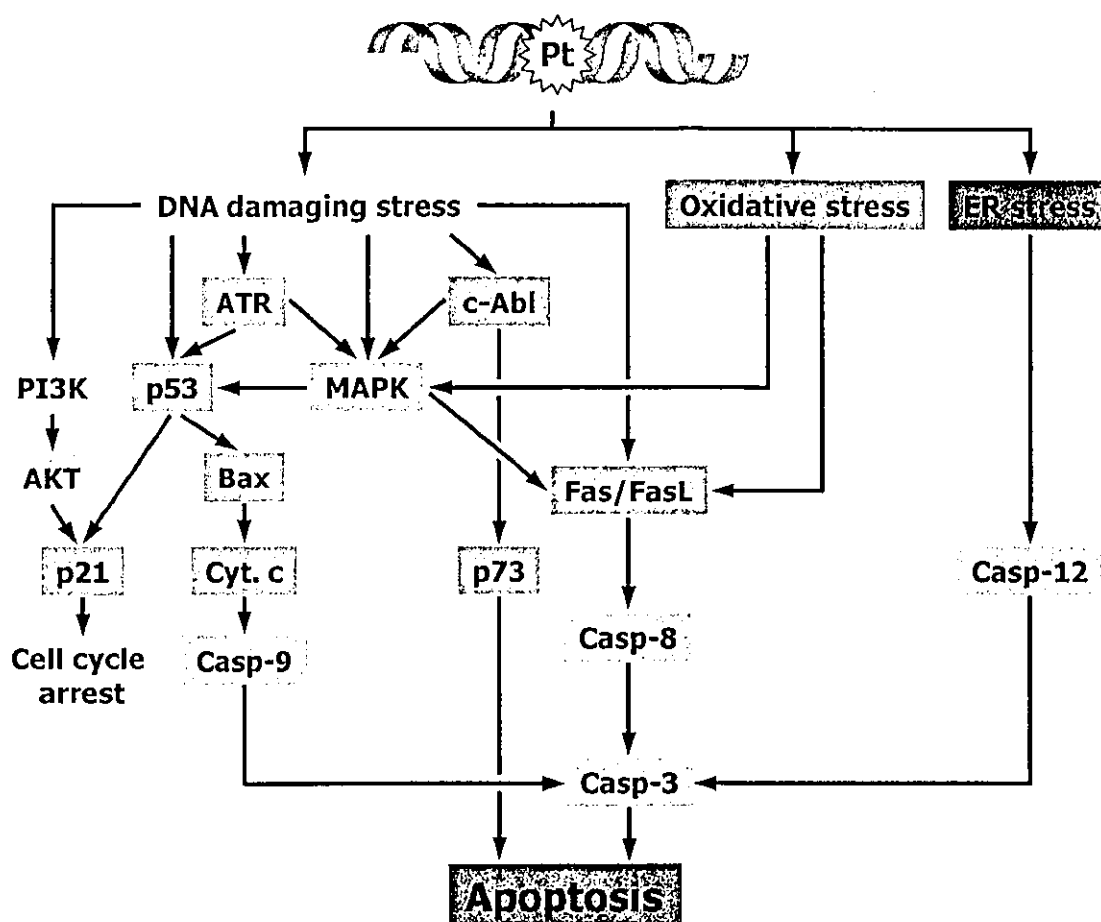
Cisplatin-induced apoptosis

Fig. (3). Schematic summary of cisplatin-induced damaging signals.

Cisplatin induces different damaging signals, such as DNA damaging, oxidative and ER damaging stresses. These stresses can activate each pathway through specific phosphorylation cascades, which include transcription factors as the ultimate targets. The fate of cancer cells in cisplatin treatment is determined by the relative intensity of, and the crosstalk between, these signaling pathways.

understanding of the mechanisms involved in cisplatin-induced apoptosis.

MECHANISMS OF CISPLATIN RESISTANCE

The development of cisplatin resistance by tumor cells is a major clinical limitation in cancer chemotherapy. This resistance might arise due to changes in the biochemical pharmacology of cisplatin. Cisplatin resistance is induced through various mechanisms, including the reduction of cisplatin accumulation inside cancer cells [5]. One of the several possible efflux pumps for cisplatin is the multidrug resistance-associated protein 2 (MRP2; also designated cMOAT). MRP2 is a member of the MRP gene family and these ABC membrane proteins have been connected with the efflux of various drugs [33]. A recent study has shown that expression of MRP2 coincides with resistance to cisplatin [34] and Koike *et al.* have demonstrated that cisplatin sensitivity is increased by antisense MRP2 constructs [35]. These data give an insight into the relationship between MRP2 expression and drug resistance. Moreover, the copper

transporters ATP7A and ATP7B have been shown to be involved in cisplatin efflux [36], and have potential as clinical markers in ovarian cancer specimens [37, 38]. However, the P-glycoprotein, which is a membrane channel encoded by the multidrug resistance 1 (*MDR1*) gene, has been reported not to participate in cisplatin resistance [39].

In another mechanism of resistance, increased activity of intracellular pathways of thiol production—including glutathione (GSH), metallothionein and thioredoxin—can contribute to the detoxification of cisplatin [5]. A small fraction of the intracellular cisplatin can bind to genomic DNA. However, a major fraction, about 60% of the intracellular cisplatin, is conjugated with GSH [40]. GSH is one of the most abundant SH-containing molecules, which can interact with cisplatin through the catalytic action of glutathione *S*-transferase π (GSTπ). GS-platinum complexes, which show inactivated cytotoxicity, are discharged from cancer cells via the glutathione conjugate export pump (GS-X pump) [1, 2]. GSTπ and γ-glutamylcysteine synthetase (γ-GCS), which is the enzyme involved in GSH biosynthesis,

were also shown to be associated with cisplatin resistance [41, 42]. Metallothionein is rich in thiol-containing cysteine and is presumed to function in the detoxification of heavy metals such as cadmium. Overexpression of metallothionein has been observed in cisplatin-resistant cell lines [43]. Thioredoxin, which is another intracellular thiol, is a redox-active protein induced by various stresses and secreted from cells. Cellular levels of thioredoxin, thioredoxin reductase and glutaredoxin are associated with cisplatin resistance, as are GSH and metallothionein [5, 44]. The glutathione adducts, which are GS-platinum complexes, inhibit the thioredoxin and glutaredoxin systems; thus, these results are consistent with the correlation between increased thioredoxin and cisplatin resistance [45]. Moreover, it has been recently reported that thioredoxin, acting as a downstream effector of Smad7; inhibitor of transforming growth factor (TGF)-β1 signaling, could suppress cisplatin-induced apoptosis in pancreatic cancer [46]. Reduced thioredoxin is also an inhibitor of ASK1 [29], and peroxiredoxin that is dependent upon thioredoxin activity might protect cancer cells from apoptosis caused by cisplatin-induced oxidative stress [47].

The cytotoxicity of cisplatin is ascribed to the formation of cisplatin-DNA adducts, and to the induction of DNA damage signals and apoptosis. The following damage-recognition proteins have been identified: HMG domain proteins (high-mobility group 1 and 2 (HMG1/2), mtTFA and hUBF); transcription factors lacking an HMG domain (TATA-binding protein (TBP), YB-1 and ZNF143); nucleotide excision repair (NER) proteins (XPE, XPA); and mismatch repair (MMR) proteins (hMutSα and hMSH2) (Table (1) [48-57]). These proteins can recruit repair complexes to damaged regions or shield them by inhibiting DNA damage signals.

The NER system is a vital pathway in the removal of cisplatin-DNA adducts and in the repair of DNA damage. First, damage-recognition proteins, such as XPA, detect cisplatin-DNA adducts. Then, XPG and ERCC1/XPF complexes make 3' and 5' incisions, respectively. Cisplatin-induced DNA damage regions are excised and these gaps are repaired in a proliferating cell nuclear antigen (PCNA)-dependent manner [2]. Cellular defects in the NER system have resulted in hypersensitivity to cisplatin [1, 2, 5] and NER related proteins, such as ERCC1 and XPA, have been overexpressed in cisplatin-resistant ovarian cancers [58, 59]. Recently, it has been shown that transcription-coupled NER is more closely related to cisplatin resistance than global genomic NER [60]. ERCC1 was shown to physically interact with a MMR protein, MSH2; these proteins might act

cooperatively in cisplatin resistance [61]. The MMR system consists of various proteins, including MSH2, MSH3, MSH6 and MLH1. A defective MMR pathway in cisplatin resistance is associated with microsatellite instability [62] and these repair proteins were also demonstrated to contribute to drug resistance [63]. These data represent NER and MMR pathways as critical mechanisms in cisplatin resistance. The Fanconi anemia-BRCA1 pathway also regulates cisplatin sensitivity [64, 65]. BRCA1 colocalizes at DNA damage lesions and interacts with various DNA repair proteins residing within a large DNA repair protein complex known as the BRCA1-associated genome-surveillance complex; this indicates that BRCA1 is a critical component of multiple repair pathways [66]. However, there is no evidence that BRCA1 directly binds to cisplatin-modified DNA. A recent report from Wang and Lippard has demonstrated that cisplatin treatment could induce the phosphorylation of histone H3 at serine 10, mediated by the p38 signaling pathway and acetylation of histone H4 [67]. These chromatin modifications are thought to be involved in drug resistance, because they increase the accessibility of DNA for transcription factors and DNA repair proteins.

In general, tumor cells upregulate glycolysis and can grow in a severe microenvironment with hypoxia and/or acidosis; therefore, pH regulators are upregulated in highly proliferative cancer cells to avoid intracellular acidification [68, 69]. We have previously shown that subunit genes of one of the pH regulators, vacuolar H^+-ATPase (V-ATPase), are induced by cisplatin treatment and are overexpressed in cisplatin-resistant cell lines [70]. Intracellular pH was markedly higher in cisplatin-resistant cell lines than in sensitive parental cell lines. Furthermore, DNA-binding activity of cisplatin was markedly increased in acidic conditions [13, 70]. The DNA topoisomerase II inhibitor TAS-103, which can induce intracellular acidosis [71], also enhanced expression of the V-ATPase subunit genes [72]. In addition, we found that combining the V-ATPase inhibitor bafilomycin A1 with cisplatin or TAS-103 could enhance drug-induced apoptosis in cancer cells [70, 72]. Thus, elevated expression of pH regulators, such as V-ATPase subunit genes, contributes to the avoidance of apoptosis induced by intracellular acidosis and/or the drug cytotoxicity of cisplatin and TAS-103.

TRANSCRIPTION FACTORS INVOLVED IN CISPLATIN RESISTANCE

Resistance to cisplatin is orchestrated via several mechanisms (as described above). These might be regulated

Table 1.   Cisplatin-Induced Damage-Recognition Proteins

	Protein	References
Transcription factors possessing an HMG domain	mtTFA*, UBF	49, 50
Transcription factors lacking an HMG domain	TBP, YB-1*, ZNF143*	51-53
HMG domain proteins	HMG1/2, SRY, SSRP1	48, 142, 143
Repair proteins	XPE, XPA, MutSα, MSH2	54-57
Chromatin protein	Histone H1	144

* indicates transcription factors focused on in this article.

by various transcription factors, which are often activated in response to cisplatin treatment. We now realize that molecular mechanisms of DNA damage signaling and cisplatin resistance are much more complex than was previously predicted. Transcription factors participate not only in gene expression, but also in DNA repair and apoptosis at the end of all signal transduction and stress-induced pathways. Various classified molecules mutually interact and function in nuclei; these interaction profiles might be altered by DNA damage, indicating that analysis of protein-interaction profiles is critical for future post-genomic research in cancer treatment. Fig. (4) illustrates DNA damage signaling and the ways in which this pathway might be associated with drug resistance, DNA repair and apoptosis. Cisplatin-induced transcription factors are closely involved in cisplatin resistance, and investigation of their mechanisms of action might allow us to overcome drug resistance. Furthermore, these transcription factors might be promising potential targets for clinical cancer treatment. Table (2) shows a summary of transcription factors and interacting molecules involved in cisplatin resistance.

p53/p73

The p53 tumor-suppressor gene family proteins p53 and p73 are central to the cellular response to DNA damage. These proteins accumulate in nuclei after DNA damage and control cell proliferation [73, 74]. Cisplatin treatment can stabilize p53 through ATR- and MAPK-induced phosphorylation of p53 at serine 15; this treatment also induces p53 downstream genes [21, 75]. Several genes transcriptionally controlled by p53 have been identified, including the CDK inhibitor *p21/Waf1/Cip1* gene, the growth arrest and DNA damage-inducible *GADD45* gene and the pro-apoptotic *bax* gene [73]. Another significant role of p53 is its possible involvement in DNA repair. p53 preferentially associates
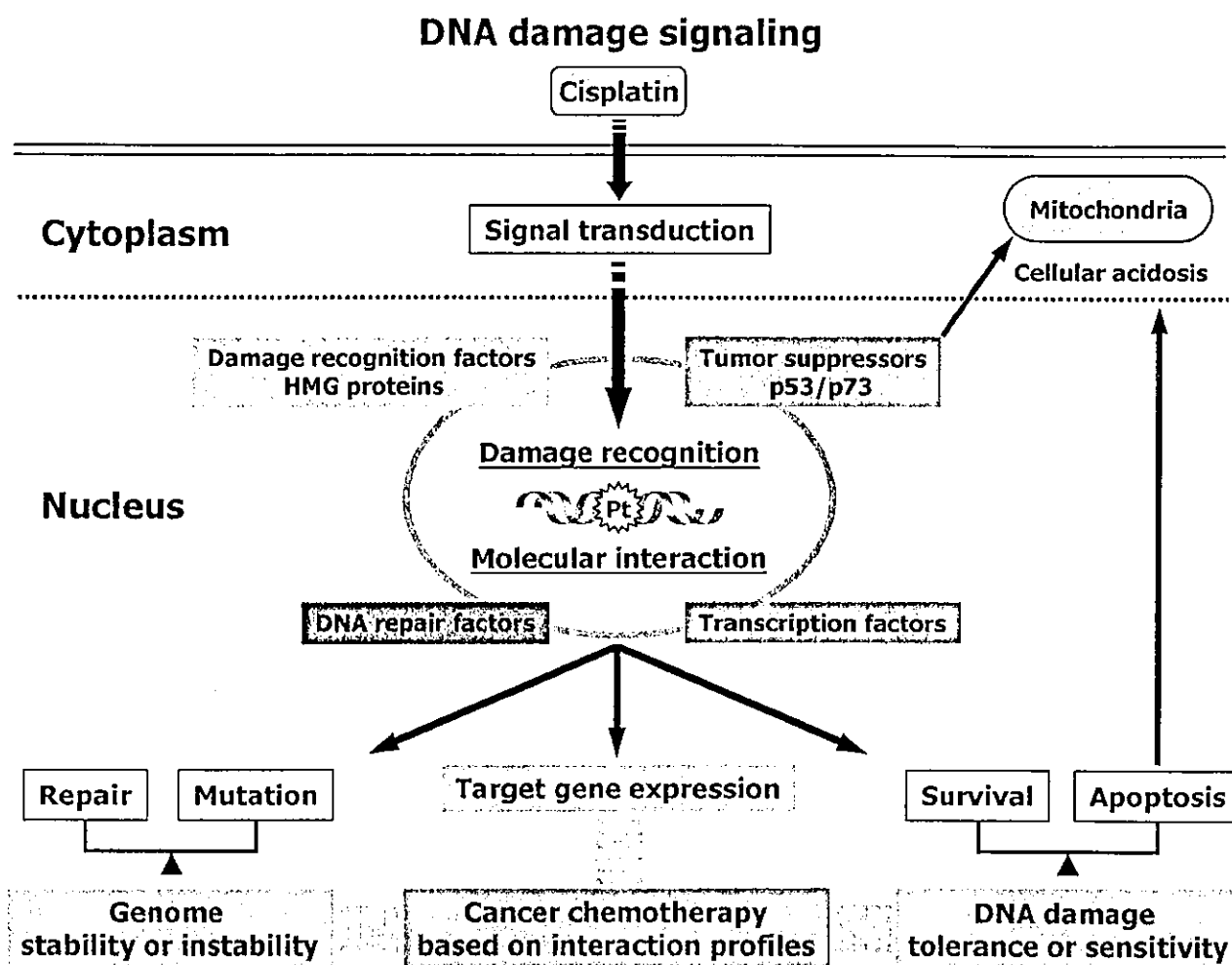

Fig. (4). Flow diagram illustrating the cellular effects of cisplatin.

Cisplatin treatment can activate various classified molecules, including DNA damage-recognition factors, DNA repair factors and transcription factors involved in cisplatin resistance via cisplatin-induced signal transductions. These factors mutually interact and function in nuclei, and interaction profiles might be altered by DNA damage. Thus, these molecular interactions are closely involved in genome stability, DNA damage tolerance and damage-induced apoptosis. The analysis of protein-interaction profiles is critical for future post-genomic research in cancer chemotherapy.

Cisplatin Resistance and Transcription Factors

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Table 2. Transcription Factors Involved in Cisplatin Sensitivity

Transcription factor	Target gene	Interacting protein involved in drug resistance
p53/p73	p21, GADD45, Bax	c-Myc [83], YB-1 [85], CTF2 [104], TBP [73], mtTFA [127], HMG1 [105], XPB [76], XPD [76]
Myc/Max	Myc/Max, YB-1	p53/p73 [83]
YB-1*	MDR1, cyclin A/B1, TopoIIα, Fas, PTP1B, GPX1	p53 [85], PCNA [52], MSH2 [92], Ku80 [92], DNA polymerase δ [92], WRN protein [92]
CTF2	HMG1	p53/p73 [104]
ATF4	ZNF143, CHOP	Nrf1/2 [110, 111]
ZNF143*	mtTFA, MRP S11	unknown
mtTFA*		p53 [127]
Oct1	ATP6L (V-ATPase c subunit), TrxR	Sp1 [145], HMG2 [136]
Sp1	ATP6L (V-ATPase c subunit), DNA-PKcs, VEGF, TrxR	p53 [137], Oct1 [145], c-Jun [137], NF-κB [146], TBP [146], p38 [147], BRCA1 [137]

* indicates specific recognition of cisplatin-modified DNA
Bracketed numbers indicate references.

with damaged DNA and interacts with DNA repair proteins, such as XPB and XPD [76]. Additionally, wild-type p53, but not mutant p53, has been demonstrated to exert intrinsic 3'→5' exonuclease activity [77]. Cancer cells carrying loss-of-function mutants of p53 are also less sensitive to anti-cancer agents [12].

p73 possesses structural and functional similarities to p53, and is able to activate p53-responsive genes and induce apoptosis. p73 was initially cloned from neuroblastoma cell lines and is involved in the development of the central nervous system [74]. The c-Abl tyrosine kinase can activate p73 by phosphorylating the p73 protein and induce apoptosis in response to DNA damage [23]. However, it has recently been reported that p73α overexpression is associated with resistance to DNA damaging agents [10]. These findings indicate that the major roles of p73 remain unclear and might be different from those of p53 in tumor cells. Further investigation is necessary to understand the functional differences between p53 and p73 in drug resistance.

c-Myc

The oncoprotein c-Myc is a transcription factor that binds to E-box and transactivates various genes. c-Myc has been shown to function in many cellular processes, including cell proliferation, differentiation and transformation [78]. However, c-Myc is also able to induce apoptosis under certain conditions, such as deprivation of survival factors or treatment with anti-cancer agents [79, 80]. The mechanisms of c-Myc-induced cell growth and apoptosis remain unclear.

A recent report has shown that c-Myc downregulation is involved in cisplatin sensitivity in human melanoma cells [81]. Low expression of c-Myc or c-Myc downregulation by antisense oligonucleotides resulted in increased susceptibility to cisplatin, via glutathione depletion [82]. We have previously demonstrated that p73 interacts with c-Myc to regulate gene expression via E-box binding [83]. Furthermore, p73 stimulated the interaction of Max; the dimerization partner of the Myc oncoprotein with c-Myc and promoted binding of the c-Myc/Max complex to its target

DNA. Our findings might help explain the complicated functions of c-Myc in drug resistance of cancer cells.

Y-Box Binding Protein-1 (YB-1)

YB-1 is the most highly evolutionarily conserved nucleic-acid-binding protein and is a member of the cold-shock domain (CSD) protein superfamily. It functions in various biological processes, including transcriptional regulation, translational regulation, DNA repair, drug resistance and cell proliferation [84, 85]. YB-1 is a transcription factor, which was first identified by its ability to bind to the inverted CCAAT box (Y-box) of the MHC class II promoter. YB-1 has also been shown to regulate the expression of various genes through a Y-box in promoter regions, including *MDR1, cyclin A/B1, DNA topoisomerase IIα, Fas* and Protein tyrosine phosphatases 1B (*PTP1B*) [85-87].

YB-1 comprises three domains: a variable N-terminal domain; a highly conserved nucleic-acid-binding domain (the CSD); and a C-terminal basic and acidic amino-acid cluster domain (called a B/A repeat) [84, 85]. The N-terminal domain is thought to be a *trans*-activation domain and the CSD has an affinity for double-stranded DNA *in vitro*. The C-terminal region functions as either a nucleic acid-binding domain or a protein-protein interaction domain; the C-terminal domain also has a strong affinity for single-stranded DNA/RNA *in vitro* and is involved in dimerization [88]. YB-1 has pleiotropic functions, which are conferred through molecular interactions with a diverse range of proteins. It regulates human gene expression via interactions with transcription factors, including p53, p65, AP2, CTCF and Smad3 [85, 89]. YB-1 also interacts with the RNA-binding proteins IRP2 and hnRNPK to regulate mRNA translation and splicing, respectively [90, 91]. An examination of its physical partners might help to elucidate the integrated functions of YB-1.

YB-1 might be one of the components necessary for DNA repair. We previously reported that YB-1 preferentially binds to cisplatin-modified DNA, similarly to HMG domain

proteins [52]. This is the first evidence that a sequence-specific transcription factor can recognize cisplatin-modified DNA. YB-1 interacts with PCNA, which is necessary for nucleotide-excision repairs [52], in addition to DNA repair proteins such as MSH2, DNA polymerase δ, Ku80 and WRN protein [92]. YB-1 also possesses 3′→5′ exonuclease and endonucleolytic activities [88, 92], and is thus thought to be involved in base-excision repair. Furthermore, YB-1 preferentially binds to RNA containing 8-oxoguanine, which suggests that it might be able to detect damaged RNA molecules [93].

YB-1 is mainly localized in the cytoplasm. When cells are challenged with anti-cancer agents, hyperthermia or UV irradiation, YB-1 is immediately translocated from the cytoplasm to the nucleus [85, 94]. We have demonstrated that promoter activity of the *MDR1* gene increases in response to various environmental stresses in a Y-box-dependent manner [95]. We have also shown that YB-1 is overexpressed in human cancer cell lines, which are resistant to cisplatin, and that cisplatin sensitivity is increased by antisense YB-1 constructs [96]. Moreover, the disruption of one allele of the *YB-1* gene increased sensitivity to cisplatin and mitomycin C in mouse embryonic stem cells [97]. Additionally, increased YB-1 expression in clinical specimens has been reported to be significantly correlated with tumor progression and poor prognosis in lung cancer, ovarian cancer, prostate cancer and synovial sarcoma [85, 98, 99]. Interestingly, it has been shown that YB-1 can recognize the selenocysteine insertion-sequence element in glutathione peroxidase (*GPX1*) gene transcripts, suggesting that increased YB-1 might enable the effective translation of selenoproteins under cisplatin-induced oxidative stress [100]. These data indicate that YB-1 might have the capacity to protect the genome from DNA damaging agents in cancer cells, might play an important role in drug resistance and, thus, might be a new molecular target in cancer treatment.

CCAAT-Binding Transcription Factor 2 (CTF2)

The CCAAT-binding transcription factor/nuclear factor I (CTF/NF-I) family of ubiquitous transcription factors was initially discovered as part of an adenovirus-DNA replication complex. CTF/NF-I group proteins recognize the sequence TTGGC(N$_5$)GCCAA and are involved in the transcriptional regulation of various genes [101, 102]. CTF2 is one of four different splice variants of the CTF/NF-I protein. We have previously determined that CTF2 is overexpressed in cisplatin-resistant cells, and that overexpression of this transcription factor might be responsible for the trans-activation of the *HMG1* gene [103]. p53 and p73 physically interact with CTF2 and reciprocally regulate *HMG1* gene expression; p73α upregulates the activity of the *HMG1* gene promoter and enhances the DNA binding activity of CTF2, although p53 does not [104].

HMG1 (also designated HMGB1) and HMG2—the nonhistone chromosomal proteins—are ubiquitously expressed in higher eukaryotes, function as class II transcription factors and preferentially bind to cisplatin-modified DNA [48]. Furthermore, physical interaction of HMG1 with p53 enhances binding of cisplatin-modified DNA [105]. HMG1 and HMG2 have been implicated in cisplatin

resistance [106, 107]. CTF2 might thus be a potential target in overcoming cisplatin resistance, because it could regulate *HMG1* gene expression in cancer cells. However, Wei *et al.* demonstrated recently that *HMG1* knockout of mouse embryonic fibroblasts has no effect on cisplatin sensitivity [108]. Further studies are needed to establish the mechanisms involving CTF2 and HMG1/2 in cisplatin resistance.

Activating Transcription Factor 4 (ATF4)

ATF4 is a member of the ATF/cyclic AMP-responsive element-binding (CREB) family of transcription factors, and is widely expressed in a variety of tissues and tumor cell lines. It has been reported previously that ATF4 forms a homodimer *in vitro* and binds to the consensus ATF/CRE site TGACGTCA [109]. Various stress-inducible genes, including DNA repair genes, contain an ATF/CRE site in their promoter regions. ATF4 interacts with nuclear-factor erythroid 1 (Nrf1)- and Nrf2-related factors, which are recruited to the antioxidant-response element and regulate the expression of genes encoding enzymes with antioxidant or detoxification functions [110, 111]. Moreover, ATF4-null cells show impaired expression of genes involved in glutathione biosynthesis and resistance to oxidative stress [112]. Additionally, ATF4 participates in ER stress-induced gene expression and transactivates ER stress response-related protein; CHOP (a CCAAT/enhancer-binding protein (C/EBP) family protein) in response to amino-acid starvation [113]. Thus, these data might provide insights into the relation between ATF4 expression and cisplatin resistance. Transcription profiling by cDNA arrays that are inducible by genetic-suppressor elements has demonstrated that ATF4 is upregulated in drug-resistant cells [114]. ATF2, which is another member of the ATF/cAMP-responsive element binding family, is phosphorylated via JNK activation following cisplatin treatment. Phosphorylated ATF2 plays a critical role in drug resistance by promoting p53-independent DNA repair [115].

We have previously shown that ATF4 is a cisplatin-induced gene and is overexpressed in cisplatin-resistant cell lines [116]. ATF4 expression in human lung cancer cell lines correlated significantly with cisplatin sensitivity, and two stable transfectant ATF4-overexpressing derivatives of human lung cancer A549 cells were less sensitive to cisplatin than the parental cells. This is the first demonstration that ATF4 closely correlates with resistance to cisplatin. Cellular levels of ATF4 expression might aid the prediction of cisplatin efficacy; however, further study of the expression of ATF4 target genes is necessary to clarify the relationship between ATF4 expression and cisplatin resistance.

Zinc-Finger Factor 143 (ZNF143)

ZNF143 is a zinc-finger transcription factor and is the human homologue of selenocysteine tRNA gene-transcription activating factor (Staf), which was identified originally in the frog. Staf is involved in transcriptional regulation of snRNA type and mRNA promoters transcribed either by RNA polymerase II or III [117]. Two human Staf homologues, ZNF143 and ZNF76, were recently isolated; these are 84 and 64% identical to *Xenopus* Staf, respectively [118]. ZNF143 is required for transcriptional activation of

selenocysteine transfer RNA, which mediates the incorporation of selenocysteine into selenoproteins, such as glutathione peroxidase and thioredoxin reductase [119].

We recently characterized one cisplatin-inducible gene, the mitochondrial ribosomal protein S11 (*MRP S11*) gene [53], which is a component of the ribosomal small subunit and binds to 12S rRNA [120]. Although there is no evidence that MRP S11 is directly related to DNA damage signals, it has been shown that the apoptosis-related protein DAP3 is a component of the small subunit of the mitochondrial ribosome (mitoribosome) [121]. This suggests that the mitoribosome, with MRP S11 as one of the constituents, might be involved in the apoptotic pathway. The Staf binding site is located in the promoter region of the *MRP S11* gene. Functional analysis of the MRP S11 promoter showed that ZNF143 regulates both basal and cisplatin-inducible promoter activities. Expression of the *ZNF143* gene was upregulated, while *ZNF76*, which is another human homologue of Staf, was downregulated by cisplatin treatment in cancer cells. We also found that γ-irradiation and anticancer agents, such as etoposide and adriamycin, induced the expression of *ZNF143*. Furthermore, ZNF143 preferentially recognizes cisplatin-modified DNA, as is YB-1 [53]. It is important to investigate whether elevated expression of ZNF143 induces cisplatin resistance and to identify the molecules interacting with ZNF143.

Mitochondrial Transcription Factor A (mtTFA)

Mitochondria are the major site of ROS production in eukaryotic cells. The accumulation of ROS, which causes oxidative damage to the mitochondrial DNA (mtDNA), has been implicated in aging, cancer and various degenerative diseases [122]. mtDNA is more susceptible to oxidative damage than genomic DNA because of its lack of nucleosome structures, and mainly contains an oxidized form of the guanine base 8-oxo-7, 8-dihydroguanine (8-oxo-dG) [123]. Moreover, mtDNA might easily form cisplatin-adducts similar to 8-oxo-dG induced oxidative stress.

mtTFA is a member of the HMG-box protein family [124] that stimulates the transcription of mitochondrial genes by binding to the mitochondrial D-loop region. Nuclear HMG-box proteins, including HMG1/HMG2, are ubiquitous

in higher eukaryotic cells and bind preferentially to cisplatin-damaged DNA [48, 49]. mtTFA is essential not only for mitochondrial gene expression but also for mtDNA maintenance and repair [125]. Additionally, increased apoptosis has been observed in *mtTFA*-knockout animals, suggesting that mtTFA plays an important role in apoptosis [125]. We have previously demonstrated that mtTFA binds preferentially to oxidatively damaged DNA containing 8-oxoguanine in addition to cisplatin modified DNA [126], whereas HMG1/HMG2 does not bind. Furthermore, the binding affinity of mtTFA for oxidized DNA is higher than that of mtMYH, which has DNA glycosylase activity and protects mtDNA from the mutagenic effects of oxidized DNA.

We examined the number of cisplatin-targeted sequences in mtDNA from databases (NCBI; National Center for Biotechnology Information, and UCSC Genome Browser) and found that cisplatin-targeted sequences, such as G-stretch sequences, are more numerous in humans and gorillas than in rodents, frogs and flies. Interestingly, G-stretch sequences appear much more frequently in mtDNA than in nuclear DNA in humans (Table (3)). These observations suggest that mitochondria might be the main targets of cisplatin in human cancer cells. We previously showed that p53 physically interacts with mtTFA in mitochondria [127]. Binding of mtTFA to cisplatin-modified DNA was significantly enhanced by p53, similar to HMG1, whereas binding to oxidized DNA was inhibited. Thus, the interaction of p53 with mtTFA might contribute to cisplatin-induced apoptosis. The amount of mtTFA protein was also increased after cisplatin treatment [127]. Moreover, there is a Staf-binding site in the promoter region of the *mtTFA* gene, indicating that mtTFA is upregulated by the transcription factor ZNF143 during cisplatin treatment (data not shown). These findings suggest that mtTFA might act as a pivotal decision center in mechanisms of protection from cisplatin-induced apoptosis, and could become a potential target for cancer chemotherapy.

Other Transcription Factors

Activator protein-1 (AP-1) is a transcription factor that induces various genes involved in cell proliferation,

Table 3.   Number of Cisplatin-Targeted DNA Sequences in Mitochondrial DNA

	GG	AG	GGG	GGGG	GGGGG	Total number of mtDNA	Accession number
Human (Expectation) (Nuclear DNA)	2,202 2,071 1,919±319	2,233 2,071 2,292±101	703 518 501±77	250 129 118±25	85 32 35±14	16,565	AY255136
Gorilla	2,137	2,185	667	232	77	16,364	NC_001645
Rat	1,696	2,041	440	113	36	16,300	NC_001665
Mouse	1,501	2,015	346	83	22	16,300	AJ512208
Xenopus	1,536	2,174	331	72	9	17,553	M10217
Drosophila	615	1,332	98	26	9	16,019	NC_001322

Expectation indicates the probable number of each DNA sequence in human mitochondrial DNA.
Nuclear DNA indicates the average of numbers of cisplatin-targeted DNA sequence in human nuclear DNA, which encodes each gene such as *Sp1* (NM_138473), *YB-1* (NM_004559), *ZNF143* (NM_003442) and *collagen type-1 α2* (NM_000089) by way of example.

differentiation, tumor invasion and apoptosis [128]. A recent study has shown that an adenovirus expressing a dominant-negative form of AP-1, lacking DNA binding capability, is able to selectively inhibit cisplatin resistance [129]. Furthermore, downregulation of c-Jun and c-fos, which are components of the AP-1 transcription complex, by antisense oligonucleotides increases cisplatin sensitivity [130, 131]. c-Jun expression is also closely linked with cellular GSH content [130].

Nuclear factor-κB (NF-κB) is a transcription factor involved in the inflammatory and immune responses, which also functions as an anti-apoptotic molecule in TNF- and cancer therapy-induced apoptosis [132]. Moreover, it has been reported recently that NF-κB activation via inhibition of the ERK signaling pathway increases resistance to cisplatin in human cervical carcinoma cells [133].

Oct1, which is a member of the POU (Pit-Oct-Unc) homeodomain family, is ubiquitously expressed and plays a role in activating the transcription of various genes [134]. Recently, Oct1 was shown to be induced after cells are treated with UV irradiation and anti-cancer agents, including cisplatin, etoposide, camptothecine and TAS-103 [135, 72]. The V-ATPase c subunit gene, pH regulator, is overexpressed in cisplatin-resistant cell lines [70] and is induced by treatment with anti-cancer agents in an Oct1-dependent manner [72]. We previously showed that intracellular pH is significantly higher in cisplatin-resistant cell lines than in sensitive parental cell lines, and that *in vitro* cisplatin-DNA cross-link formation is markedly enhanced in low pH conditions [70]. Therefore, intracellular pH is involved in drug sensitivity and Oct1 might play a critical role in intracellular pH regulation. Furthermore, HMG2, which recognizes cisplatin-modified DNA, functionally interacts with the octamer transcription factors Oct1, Oct2 and Oct6 to enhance transcriptional activity [136]. Although there is no direct evidence to implicate Oct1 expression with resistance to cisplatin, these data indicate that Oct1 is potentially involved in drug resistance.

Sp1 is a member of the C2-H2 zinc-finger family and was one of the first transcription factors to be identified in mammalian cells. Sp1 binds to GC boxes within promoter regions and regulates various genes, including housekeeping genes and genes involved in cell growth [137]. It has been previously shown that Sp1 regulates nitric oxide (NO)-induced expression of the DNA-dependent protein-kinase catalytic subunit (DNA-PKcs), which is one of the key enzymes involved in the repair of double-stranded DNA breaks, to protect cells from DNA damaging agents such as X-ray radiation, adriamycin, bleomycin and cisplatin [138]. Interestingly, Sp1 and Oct1 also bind to the promoter of the human thioredoxin reductase 1 gene, which has antioxidant and redox regulatory functions that are involved in resistance to cisplatin [139].

Hypoxia, acidosis and a transient decrease in intracellular pH are common characteristics of solid tumors [68]. Tumor cells can survive under severely energy-deficient and acidic conditions, having developed several mechanisms to escape from cellular acidosis such as pH regulators [68]. One of the cellular pH regulators is the proton pump V-ATPase; its expression is thought to be regulated by Sp1 in addition to

Oct1 [69, 72]. We have also shown that the DNA binding activity of Sp1 and its interaction with TBP are increased in low pH conditions [140]. Therefore, it is favorable for Sp1 to function well at low pH, enabling the cell to grow rapidly. A recent report from Wang *et al.* has shown that strong Sp1 expression is detectable in human gastric cancers and might be a significant predictor of survival [141]. Sp1 might thus be involved in cisplatin resistance and be a potentially promising molecular target for cancer chemotherapy.

SUMMARY

This review has focused on the transcription factors involved in the sensitivity of solid tumors to cisplatin. Transcription factors are DNA binding proteins, so it is possible that they are able to recognize DNA damage and participate in DNA repair action. Several transcription factors are activated by cisplatin treatment. We have previously reported various transcription factors—including YB-1, CTF2, ATF4, ZNF143 and mtTFA—that act as inducing genes involved in drug resistance and DNA repair. YB-1 and ZNF143 might directly participate in DNA repair reactions, because both transcription factors can preferentially recognize DNA damage. Furthermore, transcription factors with upregulated expression in cisplatin-resistant cells, such as YB-1, ATF4, ZNF143, Oct1 and Sp1, might also control the expression of the proteins that are involved in redox processes induced by cisplatin. We believe that transcription factors might represent a potential target for cancer chemotherapy. However, only nuclear hormone receptors are widely recognized as good targets for chemotherapy against hormone-dependent tumors. Molecular interactions with transcription factors induced by DNA damage are thought to be important for either survival or apoptosis, suggesting that these interactions might be also promising targets for cancer chemotherapy. Further studies to identify DNA damage-induced transcription factors and characterize functions of these factors might allow us to understand cisplatin sensitivity and to develop novel molecular-targeted drugs in future.

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ABBREVIATIONS

ASK1	=	Apoptosis signal-regulating kinase 1
ATF4	=	Activating transcription factor 4
ATR	=	Ataxia telangiectasia mutated and Rad3-related protein
CTF/NF-I	=	CTF/nuclear factor I
CTF2	=	CCAAT-binding transcription factor 2
ER	=	Endoplasmic reticulum
ERK	=	Extracellular signal-regulated kinase
GPX	=	Glutathione peroxidase
GSH	=	Glutathione
GST	=	Glutathione S-transferase

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HMG	=	High-mobility group
JNK	=	c-Jun N-terminal kinase
MAPK	=	Mitogen-activated protein kinase
MDR1	=	Multidrug resistance 1
MMR	=	Mismatch repair
MRP2	=	Multidrug resistance-associated protein 2
mtDNA	=	Mitochondrial DNA
mtTFA	=	Mitochondrial transcription factor A
NER	=	Nucleotide-excision repair
NF-κB	=	Nuclear factor-κB
Nrf1	=	Nuclear-factor erythroid 1
ROS	=	Reactive oxygen species
Staf	=	Selenocysteine tRNA gene-transcription activating factor
TBP	=	TATA-binding protein
TrxR	=	Thioredoxin reductase
UV	=	Ultraviolet
V-ATPase	=	Vacuolar H⁺-ATPase
YB-1	=	Y-box binding protein-1
ZNF143	=	Zinc-finger factor 143

REFERENCES

[1]   Cohen, S. M.; Lippard, S. J. *Prog. Nucleic Acid. Res. Mol. Biol.*, 2001, *67*, 93.
[2]   Jamieson, E. R.; Lippard, S. J. *Chem. Rev.*, 1999, *99*, 2467.
[3]   Wang, X.; Martindale, J. L.; Holbrook, N. J. *J. Biol. Chem.*, 2000, *275*, 39435.
[4]   Mandic, A.; Hansson, J.; Linder, S.; Shoshan, M. C. *J. Biol. Chem.*, 2003, *278*, 9100.
[5]   Siddik, Z. H. *Oncogene*, 2003, *22*, 7265.
[6]   Johnsson, A.; Zeelenberg, I.; Min, Y.; Hilinski, J.; Berry, C.; Howell, S. B.; Los, G. *Br. J. Cancer*, 2000, *83*, 1047.
[7]   El-Deiry, W. S. *Oncogene*, 2003, *22*, 7486.
[8]   Melino, G.; De Laurenzi, V.; Vousden, K. H. *Nat. Rev. Cancer*, 2002, *2*, 605.
[9]   Niedner, H.; Christen, R.; Lin, X.; Kondo, A.; Howell, S. B. *Mol. Pharmacol.*, 2001, *60*, 1153.
[10]   Vikhanskaya, F.; Marchini, S.; Marabese, M.; Galliera, E.; Broggini, M. *Cancer Res.*, 2001, *61*, 935.
[11]   Dumont, P.; Leu, J. I.; Della Pietra, A. C. 3rd; George, D. L.; Murphy, M. *Nat. Genet.*, 2003, *33*, 357.
[12]   Lowe, S. W.; Bodis, S.; McClatchey, A.; Remington, L.; Ruley, H. E.; Fisher, D. E.; Housman, D. E.; Jacks, T. *Science*, 1994, *266*, 807.
[13]   Chau, Q.; Stewart, D. J. *Cancer Chemother. Pharmacol.*, 1999, *44*, 193.
[14]   Sato, S.; Kigawa, J.; Minagawa, Y.; Okada, M.; Shimada, M.; Takahashi, M.; Kamazawa, S.; Terakawa, N. *Cancer*, 1999, *86*, 1307.
[15]   Mansouri, A.; Ridgway, L. D.; Korapati, A. L.; Zhang, Q.; Tian, L.; Wang, Y.; Siddik, Z. H.; Mills, G. B.; Claret, F. X. *J. Biol. Chem.*, 2003, *278*, 19245.
[16]   Wada, T.; Penninger, J. M. *Oncogene*, 2004, *23*, 2838.
[17]   Persons, D. L.; Yazlovitskaya, E. M.; Cui, W.; Pelling, J. C. *Clin. Cancer Res.*, 1999, *5*, 1007.
[18]   Hayakawa, J.; Ohmichi, M.; Kurachi, H.; Ikegami, H.; Kimura, A.; Matsuoka, T.; Jikihara, H.; Mercola, D.; Murata, Y. *J. Biol. Chem.*, 1999, *274*, 31648.
[19]   Mitsuuchi, Y.; Johnson, S. W.; Selvakumaran, M.; Williams, S. J.; Hamilton, T. C.; Testa, J. R. *Cancer Res.*, 2000, *60*, 5390.
[20]   Dan, H. C.; Sun, M.; Kaneko, S.; Feldman, R. I.; Nicosia, S. V.; Wang, H. G.; Tsang, B. K.; Cheng, J. Q. *J. Biol. Chem.*, 2004, *279*, 5405.
[21]   Damia, G.; Filiberti, L.; Vikhanskaya, F.; Carrassa, L.; Taya, Y.; D'incalci, M.; Broggini, M. *Neoplasia*, 2001, *3*, 10.
[22]   Zhang, Y.; Ma, W. Y.; Kaji, A.; Bode, A. M.; Dong, Z. *J. Biol. Chem.*, 2002, *277*, 3124.
[23]   Gong, J. G.; Costanzo, A.; Yang, H. Q.; Melino, G.; Kaelin, W. G. Jr.; Levrero, M.; Wang, J. Y. *Nature*, 1999, *399*, 806.
[24]   Kharbanda, S.; Pandey, P.; Yamauchi, T.; Kumar, S.; Kaneki, M.; Kumar, V.; Bharti, A.; Yuan, Z. M.; Ghanem, L.; Rana, A.; Weichselbaum, R.; Johnson, G.; Kufe, D. *Mol. Cell Biol.*, 2000, *20*, 4979.
[25]   Yuan, L.; Yu, W. M.; Yuan, Z.; Haudenschild, C. C.; Qu, C. K. *J. Biol. Chem.*, 2003, *278*, 15208.
[26]   Schondorf, T.; Becker, M.; Gohring, U. J.; Wappenschmidt, B.; Kolhagen, H.; Kurbacher, C. M. *Anticancer Drugs*, 2001, *12*, 797.
[27]   Cho, S. H.; Lee, C. H.; Ahn, Y.; Kim, H.; Kim, H.; Ahn, C. Y.; Yang, K. S.; Lee, S. R. *FEBS Lett.*, 2004, *560*, 7.
[28]   Meuillet, E. J.; Mahadevan, D.; Berggren, M.; Coon, A.; Powis, G. *Arch. Biochem. Biophys.*, 2004, *429*, 123.
[29]   Takeda, K.; Matsuzawa, A.; Nishitoh, H.; Ichijo, H. *Cell Struct. Funct.*, 2003, *28*, 23.
[30]   Morita, K.; Saitoh, M.; Tobiume, K.; Matsuura, H.; Enomoto, S.; Nishitoh, H.; Ichijo, H. *EMBO J.*, 2001, *20*, 6028.
[31]   Benhar, M.; Dalyot, I.; Engelberg, D.; Levitzki, A. *Mol. Cell Biol.*, 2001, *21*, 6913.
[32]   Huang, H. L.; Fang, L. W.; Lu, S. P.; Chou, C. K.; Luh, T. Y.; Lai, M. Z. *Oncogene*, 2003, *22*, 8168.
[33]   Borst, P.; Evers, R.; Kool, M.; Wijnholds, J. *J. Natl. Cancer Inst.*, 2000, *92*, 1295.
[34]   Taniguchi, K.; Wada, M.; Kohno, K.; Nakamura, T.; Kawabe, T.; Kawakami, M.; Kagotani, K.; Okumura, K.; Akiyama, S.; Kuwano, M. *Cancer Res.*, 1996, *56*, 4124.
[35]   Koike, K.; Kawabe, T.; Tanaka, T.; Toh, S.; Uchiumi, T.; Wada, M.; Akiyama, S.; Ono, M.; Kuwano, M. *Cancer Res.*, 1997, *57*, 5475.
[36]   Katano, K.; Kondo, A.; Safaei, R.; Holzer, A.; Samimi, G.; Mishima, M.; Kuo, Y. M.; Rochdi, M.; Howell, S. B. *Cancer Res.*, 2002, *62*, 6559.
[37]   Samimi, G.; Varki, N. M.; Wilczynski, S.; Safaei, R.; Alberts, D. S.; Howell, S. B. *Clin. Cancer Res.*, 2003, *9*, 5853.
[38]   Nakayama, K.; Kanzaki, A.; Ogawa, K.; Miyazaki, K.; Neamati, N.; Takebayashi, Y. *Int. J. Cancer*, 2002, *101*, 488.
[39]   Wada, H.; Saikawa, Y.; Niida, Y.; Nishimura, R.; Noguchi, T.; Matsukawa, H.; Ichihara, T.; Koizumi, S. *Exp. Hematol.*, 1999, *27*, 99.
[40]   Ishikawa, T.; Ali-Osman. F. *J. Biol. Chem.*, 1993, *268*, 20116.
[41]   Cullen, K. J.; Newkirk, K. A.; Schumaker, L. M.; Aldosari, N.; Rone, J. D.; Haddad, B. R. *Cancer Res.*, 2003, *63*, 8097.
[42]   Iida, T.; Mori, E.; Mori, K.; Goto, S;. Urata, Y.; Oka, M.; Kohno, S.; Kondo, T. *Int. J. Cancer*, 1999, *82*, 405.
[43]   Kasahara, K.; Fujiwara, Y.; Nishio, K.; Ohmori, T.; Sugimoto, Y.; Komiya, K.; Matsuda, T.; Saijo, N. *Cancer Res.*, 1991, *51*, 3237.
[44]   Becker, K.; Gromer, S.; Schirmer, R. H.; Muller, S. *Eur. J. Biochem.*, 2000, *267*, 6118.
[45]   Arner, E. S.; Nakamura, H.; Sasada, T.; Yodoi, J.; Holmgren, A.; Spyrou, G. *Free Radic. Biol. Med.*, 2001, *31*, 1170.
[46]   Arnold, N. B.; Ketterer, K.; Kleeff, J.; Friess, H.; Buchler, M. W.; Korc, M. *Cancer Res.*, 2004, *64*, 3599.
[47]   Chung, Y. M.; Yoo, Y. D.; Park, J. K.; Kim, Y. T.; Kim, H. J. *Anticancer Res.*, 2001, *21*, 1129.
[48]   Hughes, E. N.; Engelsberg, B. N.; Billings, P. C. *J. Biol. Chem.*, 1992, *267*, 13520.
[49]   Chow, C. S.; Whitehead, J. P.; Lippard, S. J. *Biochemistry*, 1994, *33*, 15124.
[50]   Treiber, D. K.; Zhai, X.; Jantzen, H. M.; Essigmann, J. M. *Proc. Natl. Acad. Sci. USA*, 1994, *91*, 5672.
[51]   Jung, Y.; Mikata, Y.; Lippard, S. J. *J. Biol. Chem.*, 2001, *276*, 43589.
[52]   Ise, T.; Nagatani, G.; Imamura, T.; Kato, K.; Takano, H.; Nomoto, M.; Izumi, H.; Ohmori, H.; Okamoto, T.; Ohga, T.; Uchiumi, T.; Kuwano, M.; Kohno, K. *Cancer Res.*, 1999, *59*, 342.
[53]   Ishiguchi, H.; Izumi, H.; Torigoe, T.; Yoshida, Y.; Kubota, H.; Tsuji, S.; Kohno, K. *Int. J. Cancer*, 2004, *111*, 900.
[54]   Chu, G.; Chang, E. *Proc. Natl. Acad. Sci. USA*, 1990, *87*, 3324.

[55]   Jones, C. J.; Wood, R. D. *Biochemistry,* 1993, *32,* 12096.

[56]   Duckett, D. R.; Drummond, J. T.; Murchie, A. I.; Reardon, J. T.; Sancar, A.; Lilley, D. M.; Modrich, P. *Proc. Natl. Acad. Sci. USA,* 1996, *93,* 6443.

[57]   Mello, J. A.; Acharya, S.; Fishel, R.; Essigmann, J. M. *Chem. Biol.,* 1996, *3,* 579.

[58]   Dabholkar, M.; Bostick-Bruton, F.; Weber, C.; Bohr, V. A.; Egwuagu, C.; Reed, E. *J. Natl. Cancer Inst.,* 1992, *84,* 1512.

[59]   States, J. C.; Reed, E. *Cancer Lett.,* 1996, *108,* 233.

[60]   Furuta, T.; Ueda, T.; Aune, G.; Sarasin, A.; Kraemer, K. H.; Pommier, Y. *Cancer Res.,* 2002, *62,* 4899.

[61]   Lan, L.; Hayashi, T.; Rabeya, R. M.; Nakajima, S.; Kanno, S.; Takao, M.; Matsunaga, T., Yoshino, M.; Ichikawa, M.; Riele, H.; Tsuchiya, S.; Tanaka, K.; Yasui, A. *DNA Repair (Amst),* 2004, *3,* 135.

[62]   Mayer, F.; Gillis, A. J.; Dinjens, W.; Oosterhuis, J. W.; Bokemeyer, C.; Looijenga, L. H. *Cancer Res.,* 2002, *62,* 2758.

[63]   Vaisman, A.; Varchenko, M.; Umar, A.; Kunkel, T. A.; Risinger, J. I.; Barrett, J. C.; Hamilton, T. C.; Chaney, S. G. *Cancer Res.,* 1998, *58,* 3579.

[64]   Taniguchi, T.; Tischkowitz, M.; Ameziane, N.; Hodgson, S. V.; Mathew, C. G.; Joenje, H.; Mok, S. C.; D'Andrea, A. D. *Nat. Med.,* 2003, *9,* 568.

[65]   Quinn, J. E.; Kennedy, R. D.; Mullan, P. B.; Gilmore, P. M.; Carty, M.; Johnston, P. G.; Harkin, D. P. *Cancer Res.,* 2003, *63,* 6221.

[66]   Wang, Y.; Cortez, D.; Yazdi, P.; Neff, N.; Elledge, S. J.; Qin, J. *Genes Dev.,* 2000, *14,* 927.

[67]   Wang, D.; Lippard, S. J. *J. Biol. Chem.,* 2004, *279,* 20622.

[68]   Torigoe, T.; Izumi, H.; Ise, T.; Murakami, T.; Uramoto, H.; Ishiguchi, H.; Yoshida, Y.; Tanabe, M.; Nomoto, M.; Kohno, K. *Anticancer Drugs,* 2002, *13,* 237.

[69]   Izumi, H.; Torigoe, T.; Ishiguchi, H.; Uramoto, H.; Yoshida, Y.; Tanabe, M.; Ise, T.; Murakami, T.; Yoshida, Y.; Nomoto, M.; Kohno, K. *Cancer Treat. Rev.,* 2003, *29,* 541.

[70]   Murakami, T.; Shibuya, I.; Ise, T.; Chen, Z. S.; Akiyama, S.; Nakagawa, M.; Izumi, H.; Nakamura, T.; Matsuo, K.; Yamada, Y.; Kohno, K. *Int. J. Cancer,* 2001, *93,* 869.

[71]   Kluza, J.; Lansiaux, A.; Wattez, N.; Mahieu, C.; Osheroff, N.; Bailly, C. *Cancer Res.,* 2000, *60,* 4077.

[72]   Torigoe, T.; Izumi, H.; Ishiguchi, H.; Uramoto, H.; Murakami, T.; Ise, T.; Yoshida, Y.; Tanabe, M.; Nomoto, M.; Itoh, H.; Kohno, K. *J. Biol. Chem.,* 2002, *277,* 36534.

[73]   May, P.; May, E. *Oncogene,* 1999, *18,* 7621.

[74]   Yang, A.; McKeon, F. *Nat. Rev. Mol. Cell Biol.,* 2000, *1,* 199.

[75]   Persons, D. L.; Yazlovitskaya, E. M.; Pelling, J. C. *J. Biol. Chem.,* 2000, *275,* 35778.

[76]   Wang, X. W.; Yeh, H.; Schaeffer, L.; Roy, R.; Moncollin, V.; Egly, J. M.; Wang, Z.; Freidberg, E. C.; Evans, M. K.; Taffe, B. G.; Bohr, V. A.; Weeda, G.; Hoeijmakers, J. H. J.; Forrester, K.; Harris, C. C. *Nat. Genet.,* 1995, *10,* 188.

[77]   Mummenbrauer, T.; Janus, F.; Muller, B.; Wiesmuller, L.; Deppert, W.; Grosse, F. *Cell,* 1996, *85,* 1089.

[78]   Marcu, K. B.; Bossone, S. A.; Patel, A. J. *Annu. Rev. Biochem.,* 1992, *61,* 809.

[79]   Askew, D. S.; Ashmun, R. A.; Simmons, B. C.; Cleveland, J. L. *Oncogene,* 1991, *6,* 1915.

[80]   Dong, J.; Naito, M.; Tsuruo, T. *Oncogene,* 1997, *15,* 639.

[81]   Citro, G.; D'Agnano, I.; Leonetti, C.; Perini, R.; Bucci, B.; Zon, G.; Calabretta, B.; Zupi, G. *Cancer Res.,* 1998, *58,* 283.

[82]   Biroccio, A.; Benassi, B.; Fiorentino, F.; Zupi, G. *Neoplasia,* 2004, *6,* 195.

[83]   Uramoto, H.; Izumi, H.; Ise, T.; Tada, M.; Uchiumi, T.; Kuwano, M.; Yasumoto, K.; Funa, K.; Kohno, K. *J. Biol. Chem.,* 2002, *277,* 31694.

[84]   Wolffe, A. P. *Bioessays,* 1994, *16,* 245.

[85]   Kohno, K.; Izumi, H.; Uchiumi, T.; Ashizuka, M.; Kuwano, M. *Bioessays,* 2003, *25,* 691.

[86]   Jurchott, K.; Bergmann, S.; Stein, U.; Walther, W.; Janz, M.; Manni, I.; Piaggio, G.; Fietze, E.; Dietel, M.; Royer, H. D. *J. Biol. Chem.,* 2003, *278,* 27988.

[87]   Fukada, T.; Tonks, N. K. *EMBO J.,* 2003, *22,* 479.

[88]   Izumi, H.; Imamura, T.; Nagatani, G.; Ise, T.; Murakami, T.; Uramoto, H.; Torigoe, T.; Ishiguchi, H.; Yoshida, Y.; Nomoto, M.; Okamoto, T.; Uchiumi, T.; Kuwano, M.; Funa, K.; Kohno, K. *Nucleic Acids Res.,* 2001, *29,* 1200.

[89]   Higashi, K.; Inagaki, Y.; Fujimori, K.; Nakao, A.; Kaneko, H.; Nakatsuka, I. *J. Biol. Chem.,* 2003, *278,* 43470.

[90]   Ashizuka, M.; Fukuda, T.; Nakamura, T.; Shirasuna, K.; Iwai, K.; Izumi, H.; Kohno, K.; Kuwano, M.; Uchiumi, T. *Mol. Cell Biol.,* 2002, *22,* 6375.

[91]   Shnyreva, M.; Schullery, D. S.; Suzuki, H.; Higaki, Y.; Bomsztyk, K. *J. Biol. Chem.,* 2000, *275,* 15498.

[92]   Gaudreault, I.; Guay, D.; Lebel, M. *Nucleic Acids Res.,* 2004, *32,* 316.

[93]   Hayakawa, H.; Uchiumi, T.; Fukuda, T.; Ashizuka, M.; Kohno, K.; Kuwano, M.; Sekiguchi, M. *Biochemistry,* 2002, *41,* 12739.

[94]   Koike, K.; Uchiumi, T.; Ohga, T.; Toh, S.; Wada, M.; Kohno, K.; Kuwano, M. *FEBS Lett.,* 1997, *417,* 390.

[95]   Ohga, T.; Uchiumi, T.; Makino, Y.; Koike, K.; Wada, M.; Kuwano, M.; Kohno, K. *J. Biol. Chem.,* 1998, *273,* 5997.

[96]   Ohga, T.; Koike, K.; Ono, M.; Makino, Y.; Itagaki, Y.; Tanimoto, M.; Kuwano, M.; Kohno, K. *Cancer Res.,* 1996, *56,* 4224.

[97]   Shibahara, K.; Uchiumi, T.; Fukuda, T.; Kura, S.; Tominaga, Y.; Maehara, Y.; Kohno, K.; Nakabeppu, Y.; Tsuzuki, T.; Kuwano, M. *Cancer Sci.,* 2004, *95,* 348.

[98]   Huang, X.; Ushijima, K.; Komai, K.; Takemoto, Y.; Motoshima, S.; Kamura, T.; Kohno, K. *Gynecol. Oncol.,* 2004, *93,* 287.

[99]   Gimenez-Bonafe, P.; Fedoruk, M. N.; Whitmore, T. G.; Akbari, M.; Ralph, J. L.; Ettinger, S.; Gleave, M. E.; Nelson, C. C. *Prostate,* 2004, *59,* 337.

[100]   Shen, Q.; Wu, R.; Leonard, J. L.; Newburger, P. E. *J. Biol. Chem.,* 1998, *273,* 5443.

[101]   Jones, K. A.; Kadonaga, J. T.; Rosenfeld, P. J.; Kelly, T. J.; Tjian, R. *Cell,* 1987, *48,* 79.

[102]   Santoro, C.; Mermod, N.; Andrews, P. C.; Tjian, R. *Nature,* 1988, *334,* 218.

[103]   Nagatani, G.; Nomoto, M.; Takano, H.; Ise, T.; Kato, K.; Imamura, T.; Izumi, H.; Makishima, K.; Kohno, K. *Cancer Res.,* 2001, *61,* 1592.

[104]   Uramoto, H.; Izumi, H.; Nagatani, G.; Ohmori, H.; Nagasue, N.; Ise, T.; Yoshida, T.; Yasumoto, K.; Kohno, K. *Biochem. J.,* 2003, *371,* 301.

[105]   Imamura, T.; Izumi, H.; Nagatani, G.; Ise, T.; Nomoto, M.; Iwamoto, Y.; Kohno, K. *J. Biol. Chem.,* 2001, *276,* 7534.

[106]   He, Q.; Liang, C. H.; Lippard, S. J. *Proc. Natl. Acad. Sci. USA,* 2000, *97,* 5768.

[107]   Arioka, H.; Nishio, K.; Ishida, T.; Fukumoto, H.; Fukuoka, K.; Nomoto, T.; Kurokawa, H.; Yokote, H.; Abe, S.; Saijo, N. *Jpn. J. Cancer Res.,* 1999, *90,* 108.

[108]   Wei, M.; Burenkova, O.; Lippard, S. J. *J. Biol. Chem.,* 2003, *278,* 1769.

[109]   Hai, T.; Hartman, M. G. *Gene,* 2001, *273,* 1.

[110]   Hayes, J. D.; McMahon, M. *Cancer Lett.,* 2001, *174,* 103.

[111]   He, C. H.; Gong, P.; Hu, B.; Stewart, D.; Choi, M. E.; Choi, A. M.; Alam, J. *J. Biol. Chem.,* 2001, *276,* 20858.

[112]   Harding, H. P.; Zhang, Y.; Zeng, H.; Novoa, I.; Lu, P. D.; Calfon, M.; Sadri, N.; Yun, C.; Popko, B.; Paules, R.; Stojdl, D. F.; Bell, J. C.; Hettmann, T.; Leiden, J. M.; Ron, D. *Mol. Cell,* 2003, *11,* 619.

[113]   Averous, J.; Bruhat, A.; Jousse, C.; Carraro, V.; Thiel, G.; Fafournoux, P. *J. Biol. Chem.,* 2004, *279,* 5288.

[114]   Levenson, V. V.; Davidovich, I. A.; Roninson, I. B. *Cancer Res.,* 2000, *60,* 5027.

[115]   Hayakawa, J.; Depatie, C.; Ohmichi, M.; Mercola, D. *J. Biol. Chem.,* 2003, *278,* 20582.

[116]   Tanabe, M.; Izumi, H.; Ise, T.; Higuchi, S.; Yamori, T.; Yasumoto, K.; Kohno, K. *Cancer Res.,* 2003, *63,* 8592.

[117]   Schaub, M.; Myslinski, E.; Schuster, C.; Krol, A.; Carbon, P. *EMBO J.,* 1997, *16,* 173.

[118]   Myslinski, E.; Krol, A.; Carbon, P. *J. Biol. Chem.,* 1998, *273,* 21998.

[119]   Chambers, I.; Frampton, J.; Goldfarb, P.; Affara, N.; McBain, W.; Harrison, P. R. *EMBO J.,* 1986, *5,* 1221.

[120]   Cavdar Koc, E.; Burkhart, W.; Blackburn, K.; Moseley, A.; Spremulli, L. L. *J. Biol. Chem.,* 2001, *276,* 19363.

[121]   Suzuki, T.; Terasaki, M.; Takemoto-Hori, C.; Hanada, T.; Ueda, T.; Wada, A.; Watanabe, K. *J. Biol. Chem.,* 2001, *276,* 33181.

[122]   Droge, W. *Physiol. Rev.,* 2002, *82,* 47.

[123]   Kasai, H.; Crain, P. F.; Kuchino, Y.; Nishimura, S.; Ootsuyama, A.; Tanooka, H. *Carcinogenesis,* 1986, *7,* 1849.

[124]   Parisi, M. A.; Clayton, D. A. *Science,* 1991, *252,* 965.

[125]   Larsson, N. G.; Wang, J.; Wilhelmsson, H.; Oldfors, A.; Rustin, P.; Lewandoski, M.; Barsh, G. S.; Clayton, D. A. *Nat. Genet.*, 1998, *18*, 231.

[126]   Yoshida, Y.; Izumi, H.; Ise, T.; Uramoto, H.; Torigoe, T.; Ishiguchi, H.; Murakami, T.; Tanabe, M.; Nakayama, Y.; Itoh, H.; Kasai, H.; Kohno, K. *Biochem. Biophys. Res. Commun.*, 2002, *295*, 945.

[127]   Yoshida, Y.; Izumi, H.; Torigoe, T.; Ishiguchi, H.; Itoh, H.; Kang, D.; Kohno, K. *Cancer Res.*, 2003, *63*, 3729.

[128]   Eferl, R.; Wagner, E. F. *Nat. Rev. Cancer*, 2003, *3*, 859.

[129]   Bonovich, M.; Olive, M.; Reed, E.; O'Connell, B.; Vinson, C. *Cancer Gene Ther.*, 2002, *9*, 62.

[130]   Pan, B.; Yao, K. S.; Monia, B. P.; Dean, N. M.; McKay, R. A.; Hamilton, T. C.; O'Dwyer, P. J. *Biochem. Pharmacol.*, 2002, *63*, 1699.

[131]   Moorehead, R. A.; Singh, G. *Biochem. Pharmacol.*, 2000, *59*, 337.

[132]   Wang, C. Y.; Mayo, M. W.; Baldwin, A. S. Jr. *Science*, 1996, *274*, 784.

[133]   Yeh, P. Y.; Chuang, S. E.; Yeh, K. H.; Song, Y. C.; Ea, C. K.; Cheng, A. L. *Biochem. Pharmacol.*, 2002, *63*, 1423.

[134]   Phillips, K.; Luisi, B. *J. Mol. Biol.*, 2000, *302*, 1023.

[135]   Zhao, H.; Jin, S.; Fan, F.; Fan, W.; Tong, T.; Zhan, Q. *Cancer Res.*, 2000, *60*, 6276.

[136]   Zwilling, S.; Konig, H.; Wirth, T. *EMBO J.*, 1995, *14*, 1198.

[137]   Black, A. R.; Black, J. D.; Azizkhan-Clifford, J. *J. Cell Physiol.*, 2001, *188*, 143.

[138]   Xu, W.; Liu, L.; Smith, G. C.; Charles, G. *Nat. Cell Biol.*, 2000, *2*, 339.

[139]   Rundlof, A. K.; Carlsten, M.; Arner, E. S. *J. Biol. Chem.*, 2001, *276*, 30542.

[140]   Torigoe, T.; Izumi, H.; Yoshida, Y.; Ishiguchi, H.; Okamoto, T.; Itoh, H.; Kohno, K. *Nucleic Acids Res.*, 2003, *31*, 4523.

[141]   Wang, L.; Wei, D.; Huang, S.; Peng, Z.; Le, X.; Wu, T. T.; Yao, J.; Ajani, J.; Xie, K. *Clin. Cancer Res.*, 2003, *9*, 6371.

[142]   Trimmer, E. E.; Zamble, D. B.; Lippard, S. J.; Essigmann, J. M. *Biochemistry*, 1998, *37*, 352.

[143]   Bruhn, S. L.; Pil, P. M.; Essigmann, J. M.; Housman, D. E.; Lippard, S. J. *Proc. Natl. Acad. Sci. USA*, 1992, *89*, 2307.

[144]   Yaneva, J.; Leuba, S. H.; van Holde, K.; Zlatanova, J. *Proc. Natl. Acad. Sci. USA*, 1997, *94*, 13448.

[145]   Strom, A. C.; Forsberg, M.; Lillhager, P.; Westin, G. *Nucleic Acids Res.*, 1996, *24*, 1981.

[146]   Bouwman, P.; Philipsen, S. *Mol. Cell Endocrinol.*, 2002, *195*, 27.

[147]   D'Addario, M.; Arora, P. D.; Ellen, R. P.; McCulloch, C. A. *J. Biol. Chem.*, 2002, *277*, 47541.

PKCδ and MAPK mediate G₁ arrest induced by PMA in SKBR-3 breast cancer cells

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Abstract

The effects of activating endogenous protein kinase C (PKC) on cell proliferation and the cell cycle were investigated by treating the breast cancer cell line SKBR-3 with phorbol 12-myristate 13 acetate (PMA). This inhibited cell growth in a concentration-dependent manner, causing a marked arrest of cells in G₁. Pre-treatment with GF109203X completely blocked the antiproliferative effect of PMA, and pre-treatment with the PKCδ inhibitor rottlerin partially blocked it. Infecting SKBR-3 cells with an adenovirus vector containing wild-type PKCδ, WTPKCδAdV, had similar effects on PMA. Infecting the cells with a dominant-negative PKCδAdV construct blocked the growth inhibition induced by PMA. Downstream of PKC, PMA treatment inhibited extracellular signal-regulated kinase mitogen-activated protein kinase phosphorylation, up-regulated c-jun NH₂-terminal kinase phosphorylation, and inhibited retinoblastoma (Rb) phosphorylation. These results strongly implicated PKC (mainly PKCδ) in the G₁ arrest induced by PMA and suggested PKC as a target for breast cancer treatment.
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Phorbol esters, such as phorbol 12-myristate 13 acetate (PMA), cause proliferation, differentiation, malignant transformation, and death in many cells [1–3]. These effects are exerted through receptors, which include several protein kinase C (PKC) isozymes and novel non-kinase receptors (α- and β-chimaerins, and Ras-GRP). PKCs are phospholipid-dependent serine/threonine kinases [4] that are involved in regulating basic cell functions, such as proliferation and differentiation. To date, at least 11 isozymes of PKC have been identified [2,5–7] and have been divided into three subgroups. PCKα, -βI, -βII, and -γ are considered to be classical PKC (cPKC) isozymes, which are activated by calcium, diacylglycerol (DAG), and PMA. PKCδ, -ε, -η, and -θ are classified as novel PKC (nPKC) isozymes, which are also activated by DAG and PMA but are not calcium dependent. PKCζ and -λ/ι are atypical PKC (aPKC) isozymes, which are neither calcium dependent nor activated by DAG or PMA.

Some stimuli that activate cPKCs and nPKCs function by activating a G protein that then activates phospholipase C to release DAG from phosphatidylinositol-4, 5-bisphosphate (PIP₂), which is a trace component of the cell membrane; DAG then activates PKCs [7–9]. By contrast, phorbol esters are promoters of carcinogenesis that pass through the cell membrane and activate almost all PKC isozymes by directly binding them. As DAG is metabolized rapidly, it activates PKC

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transiently. By contrast, phorbol esters have long half-lives, and so cause prolonged PKC activation. Several reports have described the biological effects of phorbol esters on cancer cells [10–15]. In the breast cancer cell line, MDA-MB-231, PMA induced the translocation of PKCα from the cytoplasm to the cell membrane, where it inhibited cell spreading and motility by negatively regulating the signaling pathway downstream of EGFR [16]. PMA has been reported to inhibit the growth of MCF7 cells by up-regulating PKCδ and p21 [17]. Another phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), not only acts as a tumor promoter, but also induces apoptosis in breast cancer cell lines by a mechanism that is thought to involve the up-regulation of p21 and Bax, but to be independent of p53 [18].

The distinct functions of different PKC isozymes are gradually being clarified [19–25]. For example, in MCF7 breast cancer cells, PKCη inhibited TNF-α-induced cell death by blocking caspase activation [8], and tamoxifen induced PKCε translocation to the cell membrane and inhibited growth [26]. The atypical PKCζ blocked the phosphorylation of Akt in breast cancer cells [27], while the over-expression of PKCα inhibited the estrogen receptor (ER) [28]. In breast cancer patients, changes in the expression and localization of PKCη [29] and down-regulation of PKCα [30] have been reported. Therefore, the effects of different PKC isozymes on the proliferation, differentiation, and death of cancer cells might also depend on the type of cell. By clarifying the role of PKCs in breast cancer cells, we expected these experiments to contribute to the treatment of breast cancer patients.

Members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase MAPK (ERK MAPK), c-jun NH_2-terminal kinase (JNK), and p38, are crucial regulators of the protein kinase cascades that transfer extracellular signals to the nucleus. ERK MAPK is activated by many growth factors, and this involvement in cell proliferation and differentiation has made it a focus for carcinogenesis research [31,32]. JNK and p38 are thought to play a role in stress responses, inflammatory reactions, and apoptosis [33,34].

Many reports suggest that PKC plays a role in MAPK activation. In PMA-treated lung cancer cells, two PKC isozymes, PKCα and PKCε, were shown to be involved in the activation of JNK [35]. In breast cancer cells, HER2 and PKCδ were shown to be involved in the activation of ERK MAPK by estrogen [36], and PKCα, -β, and -δ translocation preceded the acceleration of ERK MAPK phosphorylation and cell proliferation, induced by angiotensin II [37]. Thus, there appears to be a close relationship between PKC and MAPK; if this could be clarified in breast cancer cells, PKC could be established as a new target for breast cancer therapy.

This study aimed to investigate the effects of activating endogenous PKC with PMA on cell proliferation and the cell cycle in SKBR-3 breast cancer cells. We aimed to identify the PKC isozymes involved in cell proliferation using PKC inhibitors and recombinant adenovirus vectors expressing wild-type and dominant-negative PKCα and -δ genes. The signaling pathways downstream of PKC were investigated by looking at changes in ERK MAPK and JNK phosphorylation.

Materials and methods

Materials. PMA, GF109203X (bisindolylmaleimide I), Gö6976, and rottlerin were purchased from Alexis (San Diego, CA).

Cell culture. The SKBR-3 human breast cancer cell line was purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in 5% CO_2.

Treatment with phorbol esters. SKBR-3 cells were treated with 1, 3 or 10 nM PMA in DMEM with 10% FBS for 1 h and then the medium was changed. This short treatment with low PMA concentrations would not down-regulate PKC. Cells were counted at 24, 48, 72, and 96 h after 0.05% trypsin treatment with a hemocytometer and cell-proliferation curves were prepared.

Flow cytometry. Cells were fixed with 70% ethanol, stained with 1 mg/ml propidium iodide, and the cell-cycle distribution was analyzed by flow cytometry (FACScan; Becton–Dickinson, CA).

Western blot analysis. SKBR-3 cells in sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.000125% bromophenol blue, and 5% β-mercaptoethanol) (10 μg protein) were separated by sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and transferred to nitrocellulose membranes. Membranes were blocked for 24 h with phosphate-buffered saline (PBS) containing 5% milk and 0.1% Tween 20, and then incubated for 24 h with one of the following primary antibodies: anti-PKCα and anti-PKCδ (1:1,000; Transduction Laboratories, Lexington, KY); anti-phospho ERK MAPK, and anti-phospho JNK (1:1,000; Cell Signaling Technology, Beverly, MA); anti-p21 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); anti-cyclin A (1:1000; Upstate Biotechnology, Lake Placid, NY); anti-cyclin D1 and anti-cyclin E (1:1000; PharMingen, San Diego, CA); anti-β-actin (1:1000, Sigma, Saint Louis, MO); or anti-Rb and anti-phospho Rb (Ser807/811, Ser780, and Ser795) (1:1000; Cell Signaling Technology, Beverly, MA). After washing three times with PBS containing 0.1% Tween 20, the membrane was incubated for 60 min with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:3000; Bio-Rad, CA) and bands were detected by enhanced chemiluminescence (ECL; Western blotting detection system; Amersham Biosciences, Buckinghamshire, UK).

Wild-type PKC and dominant-negative PKC adenovirus. Wild-type PKCα adenovirus (WTPKCαAdV), dominant-negative PKCα adenovirus (DNPKCαAdV), wild-type PKCδ adenovirus (WTPKCδAdV), and dominant-negative PKCδ adenovirus (DNPKCδAdV) were obtained from Dr. M. Oba (Showa University, Tokyo) and Dr. T. Kuroki (Gifu University, Gifu), and have been described elsewhere [38,39]. AdVs were amplified in HEK 293 cells using standard techniques [40]. A LacZ-expressing adenovirus (LacZAdV) was used as a control [10,41]. For infection experiments, SKBR-3 cells were seeded into six-well plates, grown to a density of 60–70%, and infected for 14 h at multiplicities of infection (MOI) from 1 to 100 plaque-forming units/cell (pfu/cell). The medium was then changed and the cells were cultured for a further 24 h.

Results

Effect of PMA on SKBR-3 cell growth and cell cycle

When the PKC activator, PMA, was added to SKBR-3 cell cultures at 1, 3, and 10 nM for 1 h, it inhibited cell growth in a concentration-dependent manner (Fig. 1A). After 96 h, cell numbers were reduced to 42% at 3 nM PMA and 60% at 10 nM PMA of the control.

The effect of PMA on the cell cycle was analyzed by flow cytometry. While 56% of the cells in the control group were in the G_0/G_1 phase, 75% of the cells treated with 3 nM PMA, and 78% of those treated with 10 nM PMA, were in G_0/G_1 after 72 h (Fig. 1B). This marked

increase in the number of cells in G_0/G_1 suggested that the role of PKC in growth arrest should be investigated further.

Effect of PMA on cell-cycle regulators

We focused our studies on the cell-cycle regulators that are essential for G_0/G_1 progression, looking at changes in the phosphorylation of Rb protein, and in the expression of p21 and cyclin proteins. SKBR-3 cells were treated with 3 nM PMA for 1 h to activate endogenous PKC and subjected to Western blotting after 0, 24, and 48 h. After 24 h, phosphorylation of Rb protein at Ser 807/811 and Ser 795 was inhibited, but there was no change in the phosphorylation at Ser 780 (Fig. 2). This provided clear evidence that phosphorylation of Rb protein, which has an important role in G_0/G_1 progression, was inhibited by PMA. By contrast, no p21 protein expression was detected, and no changes were seen in the expression of cyclins A, D1, and E (data not shown).

Effect of PKC inhibitors

We next used a PKC inhibitor (GF109203X), a specific PKCδ inhibitor (rottlerin), and a specific classical PKC inhibitor (Gö6976) to confirm that PKC mediated the effect of PMA on SKBR-3 cells. As SKBR-3 cells do not express PKCβ or PKCγ [42], Gö6976 acted as a specific PKCα inhibitor in these cells. Cells were pre-treated with the PKC inhibitors, 1 h before the addition of PMA (3 nM for 1 h), and the effects on cell proliferation were assessed after 24 h. GF109203X (5 μM) almost completely blocked the inhibition of proliferation induced by PMA alone, with a final cell count similar to that of the control without PMA. Rottlerin (0.5 μM) partially blocked the effect of PMA, with a significant difference ($p < 0.05$) between

Fig. 1. Effect of PMA on SKBR-3 cell growth and cell cycle. (A) Different concentrations of PMA (1–10 nM) were added to SKBR-3 cells for 1 h in six-well plates, then washed with PBS and cultured with 10% FBS-DMEM. Cell number was counted after 24, 48, 72, and 96 h. Data are expressed as means ± standard error (SE) of three independent experiments. (B) PMA (3 or 10 nM) was added to cell cultures for 1 h, then washed with PBS and cultured with 10% FBS-DMEM. After 72 h the cells were harvested, fixed, stained with propidium iodide, and analyzed by flow cytometry. The results of a representative experiment are shown. Similar results were obtained in two additional experiments.
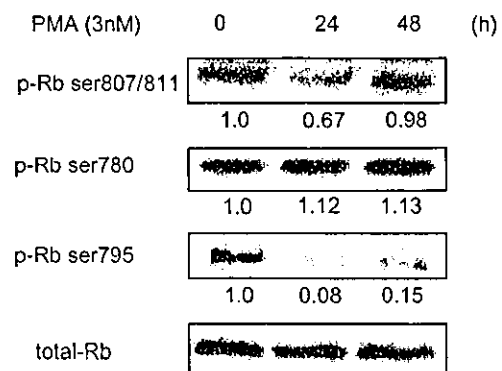

Fig. 2. PMA-induced changes in phosphorylation of Rb protein. Three nanomolar PMA was added to SKBR-3 cells for 1 h and Rb phosphorylation was assessed by Western blot analysis. Values indicate density of the band.

the cell numbers after PMA treatment alone, and after treatment with the PKCδ-specific inhibitor plus PMA. However, Gö6976 (0.2 μM) had no effect on the PMA-induced inhibition of proliferation, with no significant difference observed from the PMA treatment alone, demonstrating that PKCα did not have a role in regulating SKBR-3 cell proliferation. By contrast, the effects of the other PKC inhibitors showed that the inhibition of SKBR-3 cell proliferation by PMA did involve PKC activation, with PKCδ implicated as the isozyme involved (Fig. 3).

(*: p<0.05)
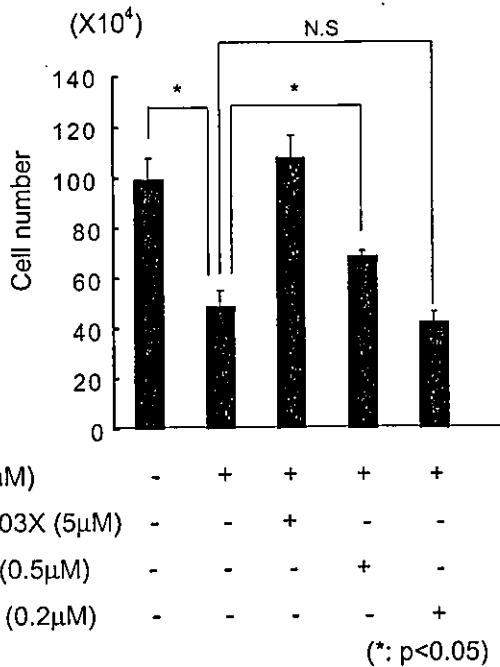
Fig. 3. Effects of PKC inhibitors. SKBR-3 cells were incubated with 5 μM GF109203X, 0.5 μM rottlerin or 0.2 μM Gö6976 1 h before the addition of PMA (3 nM for 1 h). Cell numbers were determined after 24 h. Data are expressed as means ± SE of three independent experiments (NS, not significant).

Effects of over-expressing wild-type PKCα and PKCδ in SKBR-3 cells using adenovirus vectors

To confirm that PKCδ, and not PKCα, has a role in the inhibition of SKBR-3 cell proliferation, we looked at the effects of over-expressing these proteins using adenoviral vectors. When SKBR-3 cells were infected with either WTPKCαAdV or WTPKCδAdV at MOIs between 1 and 100 pfu/cell, dose-dependent increases in the levels of PKCα and PKCδ were observed at 24 h after infection (data not shown). SKBR-3 cells were infected with 3 pfu/cell WTPKCαAdV, WTPKCδAdV or LacZAdV and the cells were counted after 24, 48, and 72 h. No differences were seen between the control and LacZAdV groups. By contrast, in the WTPKCδAdV-infected group, cell proliferation was strongly inhibited (Fig. 4B), but no inhibition was observed in the WTPKCαAdV-infected group (Fig. 4A).

The cell-cycle distribution of SKBR-3 cells infected with 3 pfu/cell WTPKCαAdV or WTPKCδAdV was analyzed by flow cytometry. After infection with WTPKCδAdV, the percentage of cells in G_0/G_1 increased to 84%, compared to 66% in the control cells, and 74% in the PMA-treated cells. By contrast, the percentage of cells in G_0/G_1 after infection with WTPKCαAdV was as low as 56%, which was lower than that in the control group (Fig. 4C). This increase in the percentage of SKBR-3 cells in the G_0/G_1 phase when PKCδ was over-expressed strongly supported the involvement of PKCδ in the induction of G_1 arrest by PMA. The results of over-expressing PKCαAdV excluded the involvement of PKCα.

Effects of dominant-negative PKC adenoviral vectors in SKBR-3 cells

SKBR-3 cells were infected with 3 pfu/cell of DNPKCαAdV, DNPKCδAdV or LacZAdV, and then treated with 3 nM PMA, to look at the effect of blocking
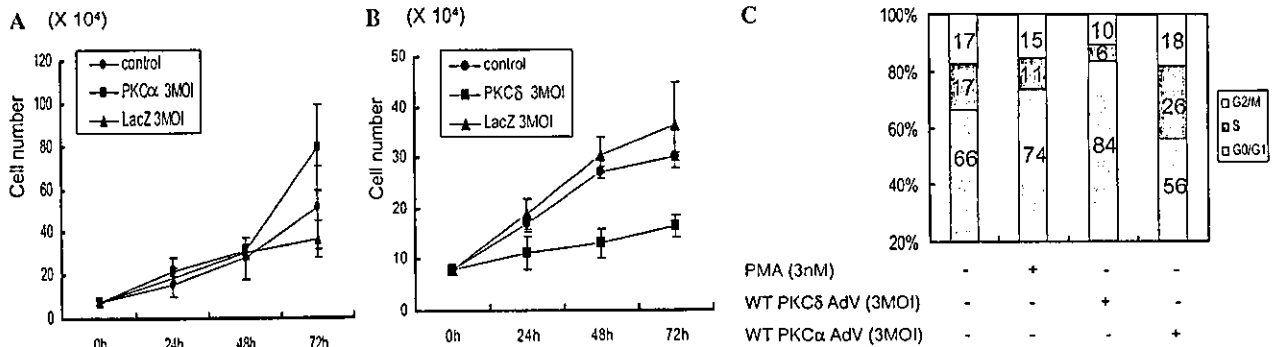

Fig. 4. The effects of WTPKCαAdV and WTPKCδAdV on cell numbers and cell-cycle distribution. (A) Cells were infected with WTPKCαAdV or LacZAdV, or (B) WTPKCδAdV or LacZAdV, at 3 pfu/cell for 14 h. Cell numbers were determined 24, 48, and 72 h later. Data are expressed as means ± SE of three independent experiments. (C) Cells were infected with PKCαAdV or PKCδAdV (MOI = 3 pfu/cell for 14 h), or treated with 3 nM PMA, harvested 72 h later, fixed, stained with propidium iodide, and analyzed by flow cytometry. The results of a representative experiment are shown. Similar results were obtained in two additional experiments.

PKC expression on the inhibition of proliferation by PMA. Cells infected with the control LacZAdV vector showed no difference from PMA-treated uninfected SKBR-3 cells. The proliferation of DNPKCδAdV-infected cells was significantly different ($p < 0.05$) from the cells infected with LacZAdV after PMA treatment, showing that the inhibition of cell proliferation by PMA was blocked by DNPKCδ. However, there was no significant difference in the proliferation of cells infected with DNPKCαAdV or LacZAdV after PMA treatment (Fig. 5). These results provided further evidence that PKCδ, rather than PKCα was involved in the PMA-induced G_1 arrest of SKBR-3 cells.
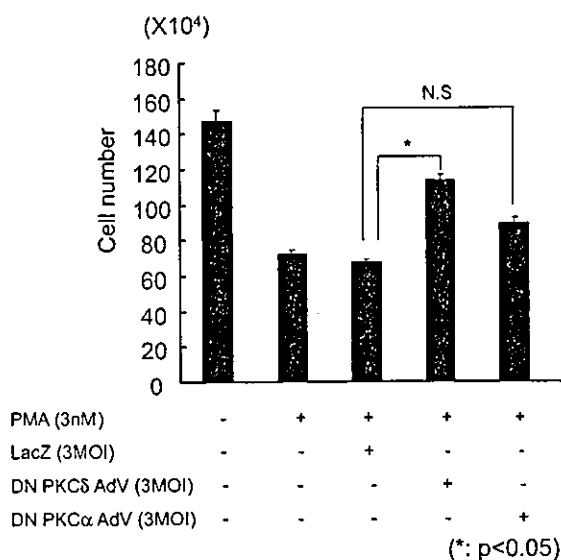
Fig. 5. The effects of DNPKCαAdV and DNPKCδAdV on cell numbers. Cells were infected with DNPKCαAdV, DNPKCδAdV or LacZAdV at 3 pfu/cell for 14 h, and then 3 nM PMA was added to cell cultures for 1 h. Cell numbers were determined 24 h later. Data are expressed as means ± SE of three independent experiments (NS, not significant).
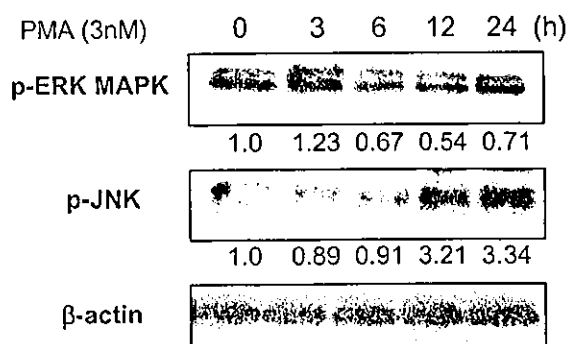

Fig. 6. The effect of PMA on ERK MAPK and JNK phosphorylation. SKBR-3 cells were treated with 3 nM PMA for 1 h and then used in Western blot analysis for phospho-ERK MAPK, phospho-JNK, and β-actin. Similar results were obtained in two additional experiments. Values indicate density of the band.

Effect of PMA on MAPK activation

In the next series of experiments, we used Western blot analysis to investigate the downstream signaling from PKC, looking at changes in the phosphorylation of ERK MAPK and JNK after PMA treatment. Phosphorylation of ERK MAPK was inhibited 6 h after exposure to PMA and remained low for at least 24 h. By contrast, JNK phosphorylation was increased by 12 h after PMA treatment (Fig. 6).

Discussion

The results presented here have clarified the role of PKC in the inhibition of SKBR-3 cell proliferation by PMA. The effects of PKC inhibitors demonstrated that the activation of PKC by PMA was essential to the induction of G_1 arrest, and that the PKC isozyme involved was PKCδ and not PKCα. Experiments that infected SKBR-3 cells with recombinant adenovirus vectors containing wild-type and dominant-negative PKCα and PKCδ supported these conclusions. The over-expression of PKCδ using WTPKCδAdV inhibited cell proliferation and induced G_1 arrest in a similar way to PMA treatment, whereas infection with WTPKCαAdV had no effect. Similarly, when SKBR-3 cells were infected with dominant-negative adenoviral vectors, the blockade of the PMA-induced inhibition of cell proliferation was significant with DNPKCδAdV but not with DNPKCαAdV. Although these results clearly demonstrated the involvement of PKCδ, the PKCδ-specific inhibitor, rottlerin, only partially inhibited the PMA-induced effects, suggesting that other isozymes could also be involved. We have recently shown that over-expression of other novel PKCs, PKCη, and PKCε, inhibited proliferation, although more weakly than PKCδ (unpublished data), suggesting that the effects of PMA were mediated by the activation of several nPKC isozymes.

There have been other reports that PKC regulated the cell cycle. Fima et al. [43] reported that PKCη promoted the proliferation of MCF7 cells, and that expression of PKCη up-regulated the G_1-associated cyclins D and E. Inhibition of PKCα and PKCθ has also been shown to arrest cells in G_1, and to be accompanied by the induction of p21 expression and the inhibition of Rb phosphorylation [44]. These reports, along with our data, suggest that PKC might be an important cell-cycle regulator. However, in this study, PMA did not induce changes in cyclin expression, suggesting that G_1 arrest in these cells might be caused by the reduction of cyclin dependent kinase (cdk) activity or some other unidentified factor.

Other studies with SKBR-3 cells have shown that 10 nM TPA treatment for 48–72 h induced apoptosis by

a mechanism involving the up-regulation of p21 and Bax [18], and that TPA-induced growth arrest, increased the expression of p21, and decreased the expression of c-Myc [45]. Both reports identified the importance of changes in p21 expression, but neither mentioned the role of PKC. We found that PMA induced G_1 arrest when used at a low concentration (3 nM) for a short period (1 h), but we saw no evidence of apoptosis (data not shown). We suggest that when cells are exposed to phorbol esters for long periods, activated PKC is gradually metabolized and disappears, and its activation is down-regulated. Therefore, it appears that phorbol esters have different biological effects under different experimental conditions, even when the same cell line is used, and that p21 is not involved in the inhibition of proliferation under our experimental conditions.

We also looked at the signaling pathways downstream of PKC after PMA treatment, and found that ERK MAPK phosphorylation was inhibited and JNK phosphorylation was increased. We therefore propose that the inhibition of SKBR-3 cell proliferation induced by PMA involves a pathway initiated by the activation of PKC, followed by the inhibition of ERK MAPK phosphorylation for 6 h and the promotion of JNK phosphorylation for 12 h, which then inhibits the phosphorylation of Rb proteins and causes G_1 arrest. Others have reported similar signaling relationships between PKC and JNK [46], and in MCF7 cells that were induced to secrete MMP9 by TPA, which promoted the activation of Ras, the phosphorylation of c-Raf, MEK1/2, and ERK1/2, and also involved the PKCδ Ras/ERK signaling pathway [47].

In conclusion, PKC (mainly PKCδ) causes G_1 arrest in the SKBR-3 breast cancer cell line by a mechanism involving a PKC-ERK MAPK-JNK-Rb protein signaling pathway, suggesting that PKC could be a target for breast cancer treatment.

Acknowledgments

References

[1] P.M. Blumberg, Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture, Cancer Res. 48 (1988) 1–8.

[2] Y. Nishizuka, Intracellular signaling hydrolysis of phospholipids and activation of protein kinase C, Science 258 (1992) 607–614.

[3] G.C. Blobe, L.M. Obeid, Y.A. Hannun, Regulation of protein kinase C and role in cancer biology, Cancer Metastasis Rev. 13 (1994) 411–431.

[4] Y. Takai, A. Kishimoto, M. Inoue, Y. Nishizuka, Studies on acyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an

[5] Y. Nishizuka, The molecular heterogeneity of protein kinase C and its implications for cellular regulation, Nature 334 (1984) 661–665.

[6] Y. Nishizuka, Protein kinase C and lipid signaling for sustained cellular responses, FASEB J. 9 (1995) 484–496.

[7] M. Musashi, The roll of protein kinase C isoform in cell proliferation and apoptosis, Int. J. Hematol. 72 (2000) 12–19.

[8] G.R. Akkaraju, A. Basu, Overexpression of protein kinase C-η attenuates caspase activation and tumor necrosis factor-α-induced cell death, Biochem. Biophys. Res. Commun. 279 (2000) 103–107.

[9] A. Basu, Differential sensitivity of breast cancer cells to tumor necrosis factor-α: involvement of protein kinase C, Biochem. Biophys. Res. Commun. 280 (2001) 883–891.

[10] T. Fujii, M.L. Garcia-Bermej, J.L. Bernabo, J. Caamano, M. Ohba, T. Kuroki, L. Li, S.H. Yuspa, M.G. Kazanietz, Involvement of protein kinase C δ (PKCδ) in phorbol ester-induced apoptosis in LNCaP prostate cancer cells. Lack of proteolytic cleavage of PKCδ, J. Biol. Chem. 275 (2000) 7574–7582.

[11] K. Mizuno, K. Noda, T. Araki, T. Imaoka, Y. Kobayashi, Y. Akita, M. Shimonaka, S. Kishi, S. Ohno, The proteolytic cleavage of protein kinase C isotypes, which generates kinase and regulatory fragments, correlates with Fas-mediated and 12-O-tetradecanoyl-phorbol-13-acetate-induced apoptosis, Eur. J. Biochem. 250 (1997) 7–18.

[12] S.Y. Lin, Y.C. Liang, Y.S. Ho, S.H. Tsai, S. Pan, W.S. Lee, Involvement of both extracellular signal-regulated kinase and c-jun N-terminal kinase pathways in the 12-O-tetradecanoylphorbol-13-acetate-induced upregulation of p21(Cip1) in colon cancer cells, Mol. Carcinog. 35 (2002) 21–28.

[13] H.W. Lee, D.H. Ahn, S.C. Crawley, J.D. Li, J.R. Gum Jr., C.B. Basbaum, N.Q. Fan, D.E. Szymkowski, S.Y. Han, B.H. Lee, M.H. Sleisenger, Y.S. Kim, Phorbol 12-myristate 13-acetate upregulates the transcription of MUC2 intestinal mucin via Ras, ERK, and NF-κB, J. Biol. Chem. 277 (2002) 32624–32631.

[14] U. Vijapurkar, N. Fischbach, W. Shen, C. Brandts, D. Stokoe, H.J. Lawrence, C. Largman, Protein kinase C-mediated phosphorylation of the leukemia-associated HOXA9 protein impairs its DNA binding ability and induces myeloid differentiation, Mol. Cell. Biol. 24 (2004) 3827–3837.

[15] X. Wang, Q. Wang, W. Hu, B.M. Evers, Regulation of phorbol ester-mediated TRAF1 induction in human colon cancer cells through a PKC/RAF/ERK/NF-κB-dependent pathway, Oncogene 23 (2004) 1885–1895.

[16] M.L. Gauthier, C. Torretto, J. Ly, V. Francescutti, D.H. O'Day, Protein kinase Cα negatively regulates cell spreading and motility in MDA-MB-231 human breast cancer cells downstream of epidermal growth factor receptor, Biochem. Biophys. Res. Commun. 307 (2003) 839–846.

[17] M. Shanmugam, N.L. Krett, E.T. Maizels, F.M. Murad, S.T. Rosen, M. Hunzicker-Dunn, A role for protein kinase C δ in the differential sensitivity of MCF-7 and MDA-MB 231 human breast cancer cells to phorbol ester-induced growth arrest and p21(WAFI/CIPI) inductions, Cancer Lett. 172 (2001) 43–53.

[18] Y. Li, M. Bhuiyan, R.M. Mohammad, F.H. Sarkar, Induction of apoptosis in breast cancer cells by TPA, Oncogene 17 (1998) 2915–2920.

[19] G. Griffiths, B. Garrone, E. Deacon, P. Owen, J. Pongracz, G. Mead, A. Bradwell, D. Watters, J. Lord, The polyether bistratene A activates protein kinase C-δ and induces growth arrest in HL60 cells, Biochem. Biophys. Res. Commun. 222 (1996) 802–808.

[20] S. Ohmori, Y. Shirai, N. Sakai, M. Fujii, H. Konishi, U. Kikkawa, N. Saito, Three distinct mechanisms for translocation and activation of the δ subspecies of protein kinase C, Mol. Cell. Biol. 18 (1998) 5263–5271.

[21] L. Ding, H. Wang, W. Lang, L. Xiao, Protein kinase C-ε promotes survival of lung cancer cells by suppressing apoptosis

through dysregulation of the mitochondrial caspase pathway, J. Biol. Chem. 277 (2002) 35305–35313.

[22] N.R. Murray, A.P. Fields, Atypical protein kinase C ι protects human leukemia cells against drug-induced apoptosis, J. Biol. Chem. 272 (1997) 27521–27524.

[23] J. Sonnemann, V. Gekeler, A. Sagrauske, C. Muller, H.P. Hofmann, J.F. Beck, Down-regulation of protein kinase Cη potentiates the cytotoxic effects of exogenous tumor necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells, Mol. Cancer Ther. 3 (2004) 773–781.

[24] Y. Gokmen-Polar, N.R. Murray, M.A. Velasco, Z. Gatalica, A.P. Fields, Elevated protein kinase C βII is an early promotive event in colon carcinogenesis, Cancer Res. 61 (2001) 1375–1381.

[25] N.R. Murray, L. Jamieson, W. Yu, J. Zhang, Y. Gokmen-Polar, D. Sier, P. Anastasiadis, Z. Gatalica, E.A. Thompson, A.P Fields, Protein kinase Cι is required for Ras transformation and colon carcinogenesis in vivo, J. Cell Biol. 164 (2004) 797–802.

[26] Y. Lavie, Z.C. Zhang, H.T. Cao, T.Y. Han, R.C. Jones, Y.Y. Liu, M. Jarman, I.R. Hardcastle, A.E. Giuliano, M.C. Cabot, Tamoxifen induces selective membrane association of protein kinase C ε in MCF-7 human breast cancer cells, Int. J. Cancer 77 (1998) 928–932.

[27] M. Mao, X. Fang, Y. Lu, R. Lapushin, R.C. Bast Jr., G.B. Mills, Inhibition of growth-factor-induced phosphorylation and activation of protein kinase B/Akt by atypical protein kinase C in cancer cells, Biochem. J. 2 (2000) 475–482.

[28] D.A. Tonetti, M.J. Chisamore, W. Grdina, H. Schurz, V.C. Jordan, Stable transfection of protein kinase C α cDNA in hormone-dependent breast cancer cell lines, Br. J. Cancer 83 (2000) 782–791.

[29] P.A. Masso-Welch, J.S. Winston, S. Edge, K.M. Darcy, H. Asch, M.M. Vaughan, M.M. Ip, Altered expression and localization of PKC η in human breast tumors, Breast Cancer Res. Treat. 68 (2001) 211–223.

[30] C. Kerfoot, W. Huang, S.A. Rotenberg, Immunohistochemical analysis of advanced human breast carcinomas reveals downregulation of protein kinase C α, J. Histochem. Cytochem. 52 (2004) 419–422.

[31] S. Cowley, H. Paterson, P. Kemp, C.J. Marshall, Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells, Cell 77 (1994) 841–852.

[32] S.J. Mansour, W.T. Matten, A.S. Hermann, J.M. Candia, S. Rong, K. Fukasawa, G.F. Vande Woude, N.G. Ahn, Transformation of mammalian cells by constitutively active MAP kinase kinase, Science 265 (1994) 966–970.

[33] H. Kawasaki, T. Morooka, S. Shimohama, J. Kimura, T. Hirano, Y. Gotoh, E. Nishida, Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells, J. Biol. Chem. 272 (1997) 18518–18521.

[34] C. Tournier, P. Hess, D.D. Yang, J. Xu, T.K. Turner, A. Nimnual, D. Bar-Sagi, S.N. Jones, R.A. Flavell, R.J. Davis, Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway, Science 288 (2000) 870–874.

[35] W. Lang, H. Wang, L. Ding, L. Xiao, Cooperation between PKC-α and PKC-ε in the regulation of JNK activation in human lung cancer cells, Cell Signal. 16 (2004) 457–467.

[36] V.G. Keshamouni, R.R. Mattingly, K.B. Reddy, Mechanism of 17-β-estradiol-induced Erk1/2 activation in breast cancer cells. A role for HER2 AND PKC-δ, J. Biol. Chem. 277 (2002) 22558–22565.

[37] S. Greco, A. Muscella, M.G. Elia, P. Salvatore, C. Storelli, A. Mazzotta, C. Manca, S. Marsigliante, Angiotensin II activates extracellular signal regulated kinases via protein kinase C and epidermal growth factor receptor in breast cancer cells, J. Cell. Physiol. 196 (2003) 370–377.

[38] M. Ohba, K. Ishino, M. Kashiwagi, S. Kawabe, K. Chida, N.K. Huh, T. Kuroki, Induction of differentiation in normal human keratinocytes by adenovirus-mediated introduction of the η and δ isoforms of protein kinase C, Mol. Cell. Biol. 18 (1998) 5199–5207.

[39] T. Kuroki, M. Kashiwagi, K. Ishino, N. Huh, M. Ohba, Adenovirus-mediated gene transfer to keratinocytes, J. Investig. Dermatol. Symp. Proc. 4 (1999) 153–157.

[40] K.L. Berkner, Expression of heterologous sequences in adenoviral vectors, Curr. Top. Microbiol. Immunol. 158 (1992) 39–66.

[41] M.L. Garcia-Bermejo, F.C. Leskow, T. Fujii, Q. Wang, P.M. Blumberg, M. Ohba, T. Kuroki, K.C. Han, J. Lee, V.E. Marquez, M.G. Kazanietz, Diacylglycerol (DAG)-lactones, a new class of protein kinase C (PKC) agonists, induce apoptosis in LNCaP prostate cancer cells by selective activation of PKC α, J. Biol. Chem. 277 (2002) 645–655.

[42] X.F. Le, M. Marcelli, A. McWatters, B. Nan, G.B. Mills, C.A. O'Brian Jr., R.C. Bast, Heregulin-induced apoptosis is mediated by down-regulation of Bcl-2 and activation of caspase-7 and is potentiated by impairment of protein kinase C α activity, Oncogene 20 (2001) 8258–8269.

[43] E. Fima, M. Shtutman, P. Libros, A. Missel, G. Shahaf, G. Kahana, E. Livneh, PKCη enhances cell cycle progression, the expression of G1 cyclins and p21 in MCF-7 cells, Oncogene 20 (2001) 6794–6804.

[44] L. Deeds, S. Teodorescu, M. Chu, Q. Yu, C.Y. Chen, A p53-independent G1 cell cycle checkpoint induced by the suppression of protein kinase C α and θ isoforms, J. Biol. Chem. 278 (2003) 39782–39793.

[45] K.O. Mitchell, W.S. El-Deiry, Overexpression of c-Myc inhibits p21WAF1/CIP1 expression and induces S-phase entry in 12-O-tetradecanoylphorbol-13-acetate (TPA)-sensitive human cancer cells, Cell Growth Differ. 10 (1999) 223–230.

[46] I. Brändlin, S. Hubner, T. Eiseler, M. Martinez-Moya, A. Horschinek, A. Hausser, G. Link, S. Rupp, P. Storz, K. Pfizenmaier, F.J. Johannes, Protein kinase C (PKC)η-mediated PKCμ activation modulates ERK and JNK signal pathways, J. Biol. Chem. 277 (2002) 6490–6496.

[47] J.F. Liu, M. Crepin, J.M. Liu, D. Barritault, D. Ledoux, FGF-2 and TPA induce matrix metalloproteinase-9 secretion in MCF-7 cells through PKC activation of the Ras/ERK pathway, Biochem. Biophys. Res. Commun. 293 (2002) 1174–1182.

Phytoestrogens/Flavonoids Reverse Breast Cancer Resistance Protein/ABCG2-Mediated Multidrug Resistance

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ABSTRACT

Breast cancer resistance protein (BCRP), also called ABCG2, confers resistance to anticancer agents such as 7-ethyl-10-hydroxycamptothecin (SN-38), mitoxantrone, and topotecan. We found previously that sulfated estrogens are physiologic substrates of BCRP. Flavonoids with weak estrogenic activities are called phytoestrogens. In this study, we show that phytoestrogens/flavonoids, such as genistein, naringenin, acacetin, and kaempferol, potentiated the cytotoxicity of SN-38 and mitoxantrone in BCRP-transduced K562 (K562/BCRP) cells. Some glycosylated flavonoids, such as naringenin-7-glucoside, also effectively inhibited BCRP. These flavonoids showed marginal effect on the drug sensitivity of K562 cells. Genistein and naringenin reversed neither P-glycoprotein-mediated vincristine resistance nor multidrug resistance-related protein 1-mediated VP-16 resistance. Genistein and naringenin increased cellular accumulation of topotecan in K562/BCRP cells. K562/BCRP cells also accumulated less [3H]genistein than K562 cells. [3H]genistein transport in the basal-to-apical direction was greater in BCRP-transduced LLC-PK1 (LLC/BCRP) cells, which express exogenous BCRP in the apical membrane, than in parental cells. Fumitremorgin C abolished the increased transport of [3H]genistein in LLC/BCRP cells compared with parental cells. TLC analysis revealed that genistein was transported in its native form but not in its metabolized form. These results suggest that genistein is among the natural substrates of BCRP and competitively inhibits BCRP-mediated drug efflux. The results have two important clinical implications: (a) flavonoids and glycosylated flavonoids may be useful in overcoming BCRP-mediated drug resistance in tumor cells; and (b) coadministration of flavonoids with BCRP-substrate antitumor agents may alter the pharmacokinetics and consequently increase the toxicity of specific antitumor agents in cancer patients.

INTRODUCTION

Multidrug-resistance (MDR; Ref. 1) is a phenomenon in which cancer cells display cross-resistance to structurally unrelated drugs (2). During chemotherapy, cancer cells displaying an MDR phenotype gradually appear in the course of repeated chemotherapeutic drug regimens, and patients displaying MDR phenotype eventually become nonresponsive to these treatments. Breast cancer resistance protein (BCRP), also called ABCG2, is a half-transporter with a molecular weight of M_r 70,000 and is a member of the ATP-binding cassette transporters (1, 3, 4). BCRP mediates concurrent resistance to chemotherapeutic agents, such as SN-38 (an active metabolite of CPT-11), mitoxantrone, and topotecan, presumably by pumping these compounds out of the cell and thus decreasing their cytotoxic effects (1, 3–6). We reported previously that estrone and 17β-estradiol circumvented BCRP-mediated drug resistance (7), and we have demonstrated recently that BCRP transports sulfated estrogens as physiologic substrates (8). In light of the findings that BCRP interacts with

estrogens and sulfated estrogens, we then screened synthesized estrogen agonists and antagonists for BCRP inhibitors and found that tamoxifen derivatives effectively circumvented BCRP-mediated drug resistance (9). These tamoxifen derivatives showed weaker affinity for estrogen receptors than 17β-estradiol, which might serve for development of BCRP inhibitors with fewer clinical side effects.

In the present study, we examined the possible effects of phytoestrogens and other flavonoids in BCRP-mediated MDR. The chemical structures of isoflavones resemble those of estrogens, and their weak estrogenic activities have been reported previously (10). Isoflavones constitute a group of flavonoids that are particularly abundant in soybean, and genistein, a member of the isoflavones, revealed stronger BCRP-inhibitory effects than estrone. Naringenin, a member of the flavanones that is contained in grapefruit juice, also showed BCRP-inhibitory effects. In addition, many other flavonoids, especially flavones, were found to strongly reverse BCRP-mediated drug resistance with few growth-inhibitory effects on cells. The BCRP-inhibitory effect of flavonoids might be explained, in part, by competitive inhibition of the BCRP-mediated efflux of anticancer agents because genistein was found to be a natural substrate that is transported by BCRP. The mechanisms by which isoflavones and other flavonoids inhibit drug export by BCRP currently are under investigation.

MATERIALS AND METHODS

Reagents. Flavonoids used in these experiments were purchased from Funakoshi (Tokyo, Japan). Anti-P-glycoprotein monoclonal antibody C219 was purchased from Centocor (Malvern, PA), and anti-MRP1 monoclonal antibody MRPm6 was obtained from Nichirei (Tokyo, Japan). [3H]genistein (5 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Establishment of K562/BCRP, LLC/BCRP, K562/MDR, and KB/MRP Cell Lines. K562 human leukemic cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. K562/BCRP cells were established by transduction of K562 cells with HaBCRP retrovirus, bearing human BCRP cDNA, and subsequent selection with 20 ng/ml SN-38 for 5 days. LLC-PK1 cells, epithelial cells of the porcine kidney, were cultured in M199 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. LLC/BCRP cells were established by the transduction of LLC-PK1 cells with HaBCRP retrovirus and subsequent selection with increasing doses of mitoxantrone (2, 4, and 8 nM) for 17 days. The resulting mixed population of drug-resistant cells was used in this study as described previously (8, 11). K562/MDR cells were established by transduction of K562 cells with HaMDR retrovirus containing human MDR1 cDNA, and this was followed by selection using 4 ng/ml vincristine for 7 days (12). KB-3-1 human epidermoid carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. KB/MRP cells were established by introduction of the pJ3U-MRP1 construct bearing human MRP1 cDNA into KB-3-1 cells, followed by selection with increasing concentrations of doxorubicin (13). Expression of BCRP in K562/BCRP and LLC/BCRP cells, expression of P-glycoprotein in K562/MDR, and expression of MRP1 in KB/MRP cells were confirmed by Western blot analysis with the anti-BCRP polyclonal antibody 3488, anti-P-glycoprotein monoclonal antibody C219, and anti-MRP1 monoclonal antibody MRPm6, respectively. The Western blot analysis procedure is described elsewhere (11).

Cell Growth Inhibition Assay. The effects of specific compounds on the sensitivity of cells to SN-38 and mitoxantrone were evaluated by measuring

cell growth inhibition after incubation at 37°C for 5 days in the absence or presence of various concentrations of anticancer drugs in combination with the specific chemicals being examined. Cell numbers were determined using a cell counter (Sysmex, Kobe, Japan). IC_{50} values (drug dosages that cause 50% inhibition of cell growth) were determined from growth inhibition curves.

Intracellular Topotecan Uptake. The effects of specific compounds on the cellular accumulation of topotecan were determined by flow cytometry. Cells (5×10^5) were incubated with 20 μM topotecan for 30 min at 37°C in the absence or presence of modifying agents, washed in ice-cold PBS, and subjected to fluorescence analysis using FACSCalibur (Becton-Dickinson, San Jose, CA).

Cellular [³H]Genistein Accumulation in K562/BCRP Cells. Either K562 or K562/BCRP cells (2×10^6) were incubated with 30 nM [³H]genistein for 0, 1, 2, or 4 h at 37°C. The cells then were washed with ice-cold PBS, dissolved in 100 μl PBS plus 400 μl Soluene-350 (Packard, Downer's Grove, IL), and mixed with 5 ml ACS II scintillation mixture (Amersham, Piscataway, NJ). Radioactivity levels were measured using a scintillation counter (Beckman, Fullerton, CA).

Transcellular Transport Assay of [³H]Genistein and Silica Gel TLC of Transported Compounds. Details of the experimental procedure are described previously (8). Briefly, exponentially growing LLC-PK1 and/or LLC/BCRP cells were plated on 3-μm pore Transwell 3414 filters (Corning Costar, Cambridge, MA) at a density of 2.4×10^6 cells/well and cultured for 3 days. Culture medium in the upper and lower wells was replaced with 2 ml of serum-free M199 medium 1.5 h before beginning the experiments. When needed, fumitremorgin C was added to the apical and basal side medium at this time (14). The medium in either the upper or lower well then was replaced with 2 ml of medium containing ¹⁴C-labeled inulin and/or ³H-labeled genistein. The cells were incubated at 37°C in 5% CO_2, and 50 μl of the medium from the opposite side were sampled at 1, 2, and 4 h following the addition of radiolabeled compounds. The radioactivity of each sample was measured by liquid scintillation counting and expressed as a percentage fraction of the total radioactivity before incubation. All of the data were presented as mean values with SD of triplicate determinations from three different cultures.

For silica gel TLC, 50 μl of medium in the opposite side of the chamber following incubation were mixed with 100 μl of methanol, spotted, and run on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) in chloroform/methanol/acetic acid (8:3:1). Separated zones were excised, and their radioactivities were measured using a liquid scintillation counter. The radioactivities were expressed as a percentage fraction of the total radioactivity before incubation. Each point represents a mean value with SD of triplicate determinations.

Statistical Analysis. The two-sided unpaired Student's t test was used to evaluate the statistical significance of the differences between the two sets of data. The differences were considered significant when $P < 0.05$.

RESULTS

Characteristics of K562/BCRP, LLC/BCRP, K562/MDR, and KB/MRP Cells. Among the four drug-resistant cell lines used in this study, K562/BCRP cells expressed BCRP but not P-glycoprotein or
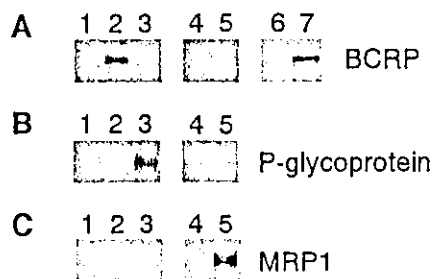

Fig. 1. Analysis of expression levels of breast cancer resistance protein (BCRP), P-glycoprotein, and MRP1 in transfected cells by Western blot analysis. Cell lysates (20 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. A, BCRP expression. Blots were treated with anti-BCRP polyclonal antibody 3488. B, P-glycoprotein expression. Blots were treated with anti-P-glycoprotein antibody C219. C, MRP1 expression. Blots were treated with anti-MRP1 monoclonal antibody MRPm6. 1, K562 cells. 2, K562/BCRP cells. 3, K562/MDR cells. 4, KB-3-1 cells. 5, KB/MRP cells. 6, LLC-PK1 cells. 7, LLC/BCRP cells.

Table 1 *Drug resistances of K562/BCRP, K562/MDR, and KB/MRP cells*

Cells were cultured for 5 days with increasing concentrations of anticancer drugs. Cell numbers were measured with a Coulter counter and IC_{50} values were then determined. The degree of drug resistance is calculated as the IC_{50} ratio of resistant cells divided by that of the parental cells. The data are represented as mean values ± SD from triplicate determinations.

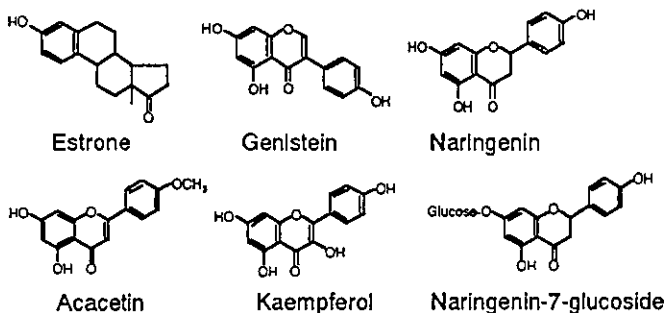| Parental cell	Resistant cell	Drug	Degree of resistance
K562	K562/BCRP	SN-38	24.8 ± 0.63
K562	K562/BCRP	Mitoxantrone	10.1 ± 0.27
K562	K562/MDR	Vincristine	173 ± 18.7
KB-3-1	KB/MRP	VP-16	10.3 ± 0.60

Fig. 2. Chemical structures of estrone and the indicated phytoestrogens/flavonoids.

MRP1. LLC/BCRP cells expressed BCRP. K562/MDR cells expressed P-glycoprotein but not BCRP or MRP1, and KB/MRP cells expressed MRP1 but not BCRP or P-glycoprotein (Fig. 1). Expression of BCRP, P-glycoprotein, or MRP1 was not detected in parental K562 and KB-3-1 cells.

K562/BCRP cells showed significantly higher resistance to SN-38 and mitoxantrone than K562 cells (Table 1). LLC/BCRP cells were five to six times more resistant to SN-38 and mitoxantrone than parental LLC-PK1 cells as described previously (8). K562/MDR cells showed significantly higher resistance to vincristine than K562 cells, and KB/MRP cells were significantly more resistant to VP-16 than KB-3-1 cells (Table 1). Protein expression and drug-resistance levels in each resistant cell line were stable for at least 2 months.

Reversal of BCRP-Mediated Drug Resistance by Flavonoids. Estrone, 17β-estradiol, estrogen agonists, and estrogen antagonists reverse BCRP-mediated drug resistance. In the present study, we examined the potential reversal effects of phytoestrogens/flavonoids because they have been shown to have weak estrogenic activities (10). Structures of representative flavonoids are shown in Fig. 2. We first examined the effects of representative phytoestrogens, genistein and naringenin, on drug resistance in K562/BCRP cells (Fig. 3, A–C). Reversal indexes (ratios of IC_{50} measurements in the absence of reversing agents divided by levels in the presence of reversing agents) of 3 μM (10 μM) genistein for SN-38 and mitoxantrone were 7.23 ± 0.35 (16.4 ± 0.56) and 6.28 ± 0.51 (11.7 ± 0.40), respectively. In addition, reversal indexes of 3 μM (10 μM) naringenin for SN-38 and mitoxantrone were 5.94 ± 0.26 (15.2 ± 0.92) and 3.42 ± 0.27 (10.6 ± 0.30), respectively. The reversing effects of genistein and naringenin proved to be greater than estrone. Analysis then was extended to other flavonoids, many of which reversed BCRP-mediated SN-38 resistance at a fixed concentration of 3 μM (Fig. 4). The flavones acacetin, apigenin, chrysin, diosmetin, and luteolin and the flavonols kaempferide and kaempferol displayed strong reversal effects (Fig. 3, D and E, and Fig. 4). Reversal indexes of 1 μM (3 μM) acacetin for SN-38 and mitoxantrone were 15.2 ± 1.10 (21.4 ± 0.34) and 9.89 ± 0.27 (9.71 ± 0.81), respectively. Reversal indexes of 1 μM (3 μM) kaempferol for SN-38 and