News and views

Novel insights in clinical trials with preoperative systemic therapy for primary breast cancer

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Abstract

Preoperative systemic therapy has provided novel insights into the treatment of primary breast cancer. The knowledge of the response to the treatment helps to predict long-term survival outcome, which indicated that the patients who benefit from the treatment are identifiable at individual level. The subgroup with pathological complete response (pCR) to the preoperative chemotherapy (Pre-CT) shows a favorable prognosis, whereas the subgroup with non-pCR shows an unfavorable prognosis. These identifications enable to classify the patients into multiple categories and to consider novel therapeutic implication more efficiently and more individually. Theoretically in the preoperative treatment system it is possible to distinguish the patients who require novel therapy beyond the conventional therapy from those require no further systemic chemotherapy except the conventional therapy or those who need no systemic chemotherapy. Novel therapy development specific for non-pCR patients should be considered in the future clinical trials. In this review, we will focus on these insights elicited by Pre-CT in primary breast cancer treatment.

Keywords: Breast cancer; Individualized treatment; Pathological response; Preoperative chemotherapy

1. Introduction

The number of studies and clinical trials on preoperative systemic therapy has increased sharply in recent years. According to the literature research by *PubMed*, chemotherapy took off in early 90s and hormone therapy took off around 2000, respectively. Publications on translational researches have also increased in parallel. Why the vigorous attentions have been paid to preoperative systemic therapy? Two major reasons could be raised. One is the increment of the chances for breast-conserving therapy (BCT) and another is the knowledge of the sensitivity or resistance to the treatment at individual levels [1–6]. According to National Surgical Adjuvant Breast and Bowel Project (NSABP) B-18 trial result, preoperative 4-cycle adriamycin plus cyclophosphamide (AC) therapy drove an

additional 8% chance of BCT as compared with surgical treatment at front for primary breast cancer patients [1]. Many other investigations have also confirmed a remarkable increase in BCT rate by preoperative chemotherapy (Pre-CT) [4]. It is clear that the shift in the timing of the treatment beyond the surgery brings a significant advance to the treatment of primary breast cancer.

In NSABP B-18 trial it was also revealed that tumor response, especially pathological complete response (pCR), promises a favorable prognosis whereas non-pCR predicts a poor prognosis at 8-year follow-up time [4] In addition, it is also speculated that node-negative conversion, from histologically node-positive to node-negative in the axilla, could be caused by preoperative AC chemotherapy in about 30% of the cases. Recent 8-cycle sequential regimens such as 4-cycle anthracycline followed by 4-cycle docetaxel are noted to have achieved even higher pCR rates from 25% to 34% [7]. The estimated node-negative conversion rate seems to have also

increased proportionally. For these backgrounds, a novel consensus has been formed that most of primary breast cancer patients who require postoperative adjuvant chemotherapy (Post-CT) are the potential candidates for Pre-CT [8].

2. Clinical phenotypes emerged by Pre-CT

Recurrence or death is the ultimate marker for the assessment of adjuvant treatments, in either postoperative or preoperative setting. In the postoperative setting, the patients are categorized into two subgroups consisted of a group that failed to the treatment and another group that did not fail, however, in the preoperative setting, particularly in the Pre-CT, the patients can be classified into further multiple categories due to the sensitivity to the treatment like Figs. 1 and 2. These classifications raise novel therapeutic implication that conventional chemotherapy is recommended to the patients categorized as therapy-sensitive and curable property (Category A: therapy-relevant favorable prognosis group), howev-

er, new therapeutic approaches should be considered for the patients categorized as therapy-resistant and incurable property (Category D: therapy-irrelevant unfavorable prognosis group) (Table 1). The patients categorized as therapy-resistant but curable (Category C: therapy-irrelevant favorable prognosis group) might not require any type of chemotherapy. A controversy may exist in the interpretation of the patients categorized as therapy-sensitive but incurable. It might be possible to raise two types of explanation. Firstly, Pre-CT might have killed all invasive tumor cells in the breast but may not have killed all the metastatic tumor cells in the distant organs. Secondly, the killing of invasive tumor cells in primary site might, inversely, facilitate the growth of metastatic tumor cells in the secondary site that has been already indicated in animal experimental models [9]. From therapeutic point of view, the former hypothesis may suggest that new approaches based upon the conventional therapy is considerable for the future, whereas the latter hypothesis indicates that novel therapeutic approaches based upon totally new therapeutic concept are required to obtain the better outcome.

Table 1
Tumor properties relating to the treatment and therapeutic implication

| Subgroup	Phenotype	Properties of treatment	Implication	Therapeutic approach
A	Responder non-recurrence	Sensitive/curable	Therapy-relevant favorable prognosis	Conventional therapy
B	Responder recurrence	Sensitive/curable	Therapy-irrelevant unfavorable prognosis	Novel approach or modification of conventional therapy
C	Non-responder non-recurrence	Resistant/curable	Therapy-irrelevant favorable prognosis	No need for therapy
D	Non-responder recurrence	Resistant/incurable	Therapy-irrelevant unfavorable prognosis	Novel approach with totally novel mechanisms
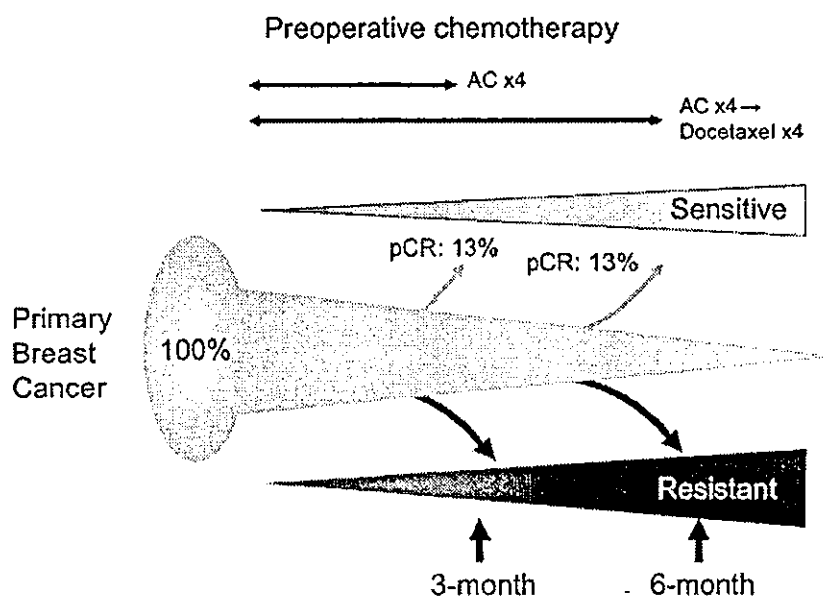
Fig. 1. A preoperative chemotherapy model simulated from NSABP B-18 and NSABP B-27 trial data. Pathological complete response (pCR) was achievable in 13% of operable primary breast cancer patients by preoperative AC (adriamycin + cyclophosphamide) 4-cycle in NSABP B-18 trial. The remaining 87% non-pCR patients were basically resistant to the AC 4-cycle treatment. According to NSABP B-27 data, pCR was achievable in further 13% of primary breast cancer patients by the additional 4-cycle treatment of docetaxel.
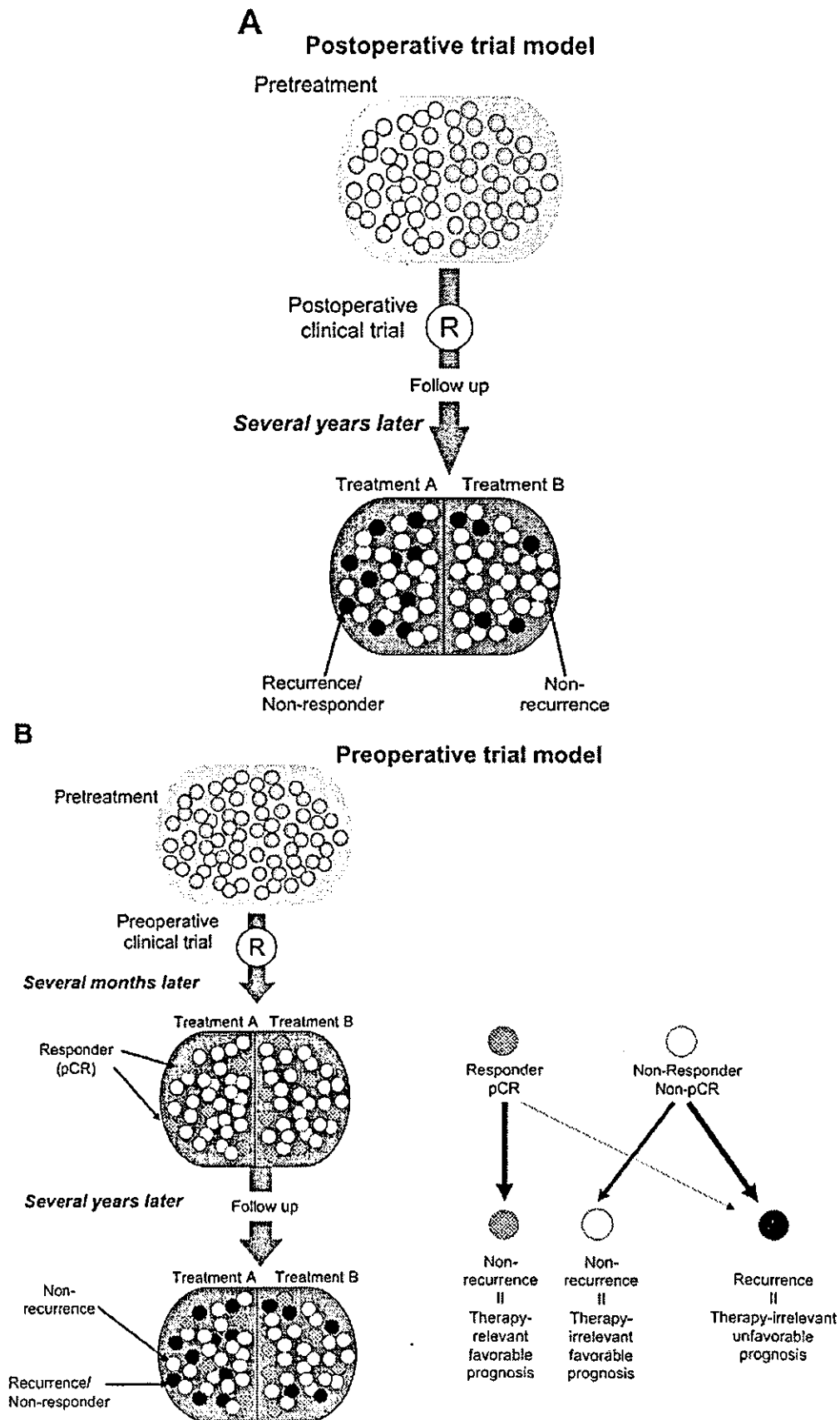
Fig. 2. A postoperative trial model and a preoperative trial model. The data sets of short-term therapy-sensitivity and long-term survival provide novel insights to clinical trials for primary breast cancer patients.

3. Simulation of the proportion of patients classified into the categories

As simulated the proportion of patients for each category according to an 8-year follow-up data of NSABP B-18, the Category A should be roughly 10%, the Category B should be several percent, the Category C should be about 50% and the Category D should be about 35% [1,2]. Therefore, we can take the conventional chemotherapy, AC 4-cycle, for about 60% of primary breast cancer patients, unless we care the toxicity. Nevertheless, we need novel therapy for the remaining 40%. As applied the tumor response data of NSABP B-27, where pCR rate was approximately 26%, a simulation exhibits that the Category A should be about 20%, the Category C should be about 50%, and the Category B and the Category D should be about 30% in total. Approximately, 30% of the patients would require novel therapeutic approaches. Only preoperative treatment fashion enables to provide such information as who do not require any type of chemotherapy, who do not require additional chemotherapy and who do require novel therapy. In the future clinical trials with new therapeutic tools, the shift of the patients from Category B or Category D to Category A would occur.

4. Translational research

These classifications according to tumor response and survival outcome are also useful for conducting efficient translational researches. As illustrated by Hayes et al., it is important to distinguish predictive markers form prognostic markers [10]. In the postoperative situation, no treatment control (local therapy ± hormone therapy alone) is indispensable for analyzing pure prognostic value or pure predictive value of the 'so-called'

prognostic markers. Nevertheless, in the situation of preoperative therapy, the study for Category A could reveal the factors relating pure predictive value of the treatment and the study for Category C could reveal the factors relating pure prognostic value even in one-arm study. Studies of subgroups of Category B and Category D would be useful for exploring new therapeutic targets. In a setting of clinical trials comparing new treatment arm with conventional treatment arm, the pure prognostic value emerged from the study of Category C could be validated during the analysis in a clinical trial, because the therapy-irrelevant favorable prognosis group would be same between the two arms.

5. Response-based individualized treatment system

It has been demonstrated that postoperative hormone therapy drives a significant survival benefit to hormone-receptor (HR) positive patients but not to HR negative patients [11–13]. Therefore, there is no doubt about that tumor phenotyping on HR ought to be done before starting any type of therapy [8]. Her-2 status should be also determined before starting the therapy because of a tight association with the effect of treatment trastuzumab that targets Her-2 molecule specifically [14].

After the exclusion of minimal risk patients those would not require any type of systemic chemotherapy, most of high-risk primary breast cancer patients could be treated by Pre-CT [15]. After confirming the pathological tumor response, the choice of postoperative systemic therapy might be divers. For pCR patients, cytostatic systemic therapy such as hormone therapy or no systemic therapy might be considered, however for non-pCR patients, additional treatments including other types of chemotherapy may have to be taken into consideration (Fig. 3).
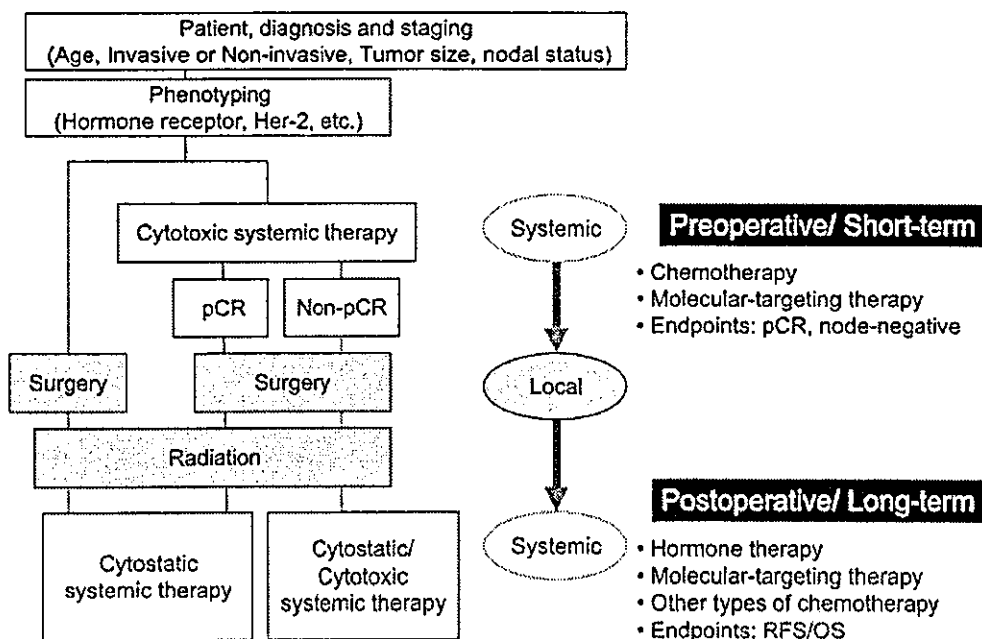

Fig. 3. A strategy with preoperative systemic treatment.

Tests of new therapeutic approaches will be targeted for non-pCR patients in order to avoid over-treatment. Translational researches will help to clarify the subpopulation that requires the new types of therapy more precisely and to explore new therapeutic target [15]. These response-based treatment strategy seems to be promising to improve the survival outcome of primary breast cancer patients efficiently and individually.

References

[1]   Fisher B, Bryant J, Wolmark N, et al. Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. J Clin Oncol 1998;16:2672–85.

[2]   Wolmark N, Wang J, Mamounas E, et al. Preoperative chemotherapy in patients with operable breast cancer: nine-year results from National Surgical Adjuvant Breast and Bowel Project B-18. J Natl Cancer Inst Monogr 2001;30:96–102.

[3]   Miller WR, Dixon JM, Cameron DA, Anderson TJ. Biological and clinical effects of aromatase inhibitors in neoadjuvant therapy. J Steroid Biochem Mol Biol 2001;79:103–7.

[4]   Smith IE, Lipton L. Preoperative/neoadjuvant medical therapy for early breast cancer. Lancet Oncol 2001;2:561–70.

[5]   Toi M, Bando H, Saji S. Decision tree and paradigms of primary breast cancer: changes elicited by preoperative therapy. Med Sci Monit 2003;9:RA 90–5.

[6]   Smith IC, Heys SD, Hutcheon AW, et al. Neoadjuvant chemotherapy in breast cancer: significantly enhanced response with docetaxel. J Clin Oncol. 2002;20:1456–66.

[7]   Bear HD, Anderson S, Brown A, et al. The effect of tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project protocol B-27. J Clin Oncol 2003.

[8]   Goldhirsch A, Wood WC, Gelber RD, et al. Meeting highlights: updated international expert consensus on the primary therapy of early breast cancer. J Clin Oncol 2003;21:3357–65.

[9]   Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995;1:27–31.

[10] Yamauchi H, Stearns V, Hayes DF. When is a tumor marker ready for prime time? A case study of c-erbB-2 as a predictive factor in breast cancer. J Clin Oncol 2001;19:2334–56.

[11] Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer. Cochrane Database Syst Rev 2001;1:CD000486.

[12] Johnston SRD, Dowsett M. Aromatase inhibitors for breast cancer. Lessons from the laboratory. Nature Rev 2003;3:821–31.

[13] Goss PE, Ingle JN, Martino S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. N Engl J Med 2003;349:1793–802.

[14] Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med, 2001;344:783–92.

[15] Buchholz TA, Hunt KK, Whitman GJ, et al. Neoadjuvant chemotherapy for breast carcinoma: multidisciplinary considerations of benefits and risks. Cancer 2003;98:1150–60.

Molecular Cancer Therapeutics
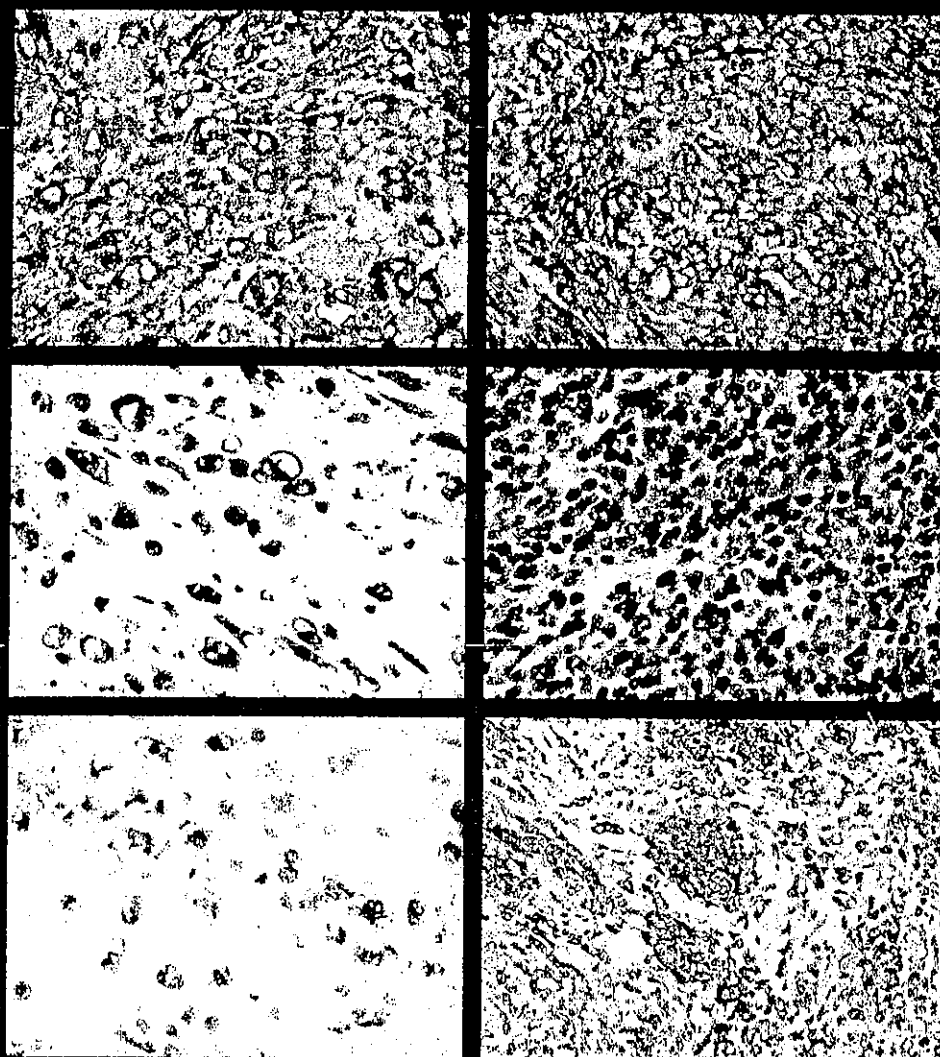
Minireview

The role of nuclear Y-box binding protein 1 as a global marker in drug resistance

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Abstract

Gene expression can be regulated by nuclear factors at the transcriptional level. Many such factors regulate *MDR1* gene expression, but what are the sequence elements and transcription factors that control the basal and inducible expression of this gene? The general principles through which transcription factors participate in drug resistance are now beginning to be understood. Here, we review the factors involved in the transcriptional regulation of the *MDR1* gene. In particular, we focus on the transcription factor Y-box binding protein 1 and discuss the possible links between Y-box binding protein 1 expression and drug resistance in cancer, which are mediated by the transmembrane P-glycoprotein or non–P-glycoprotein. [Mol Cancer Ther 2004;3(11):1485–92]

Introduction

Drug export from cells is mediated through a group of proteins belonging to the ATP binding cassette family of transporters. The 170-kDa transmembrane protein P-glycoprotein (PGP), which is encoded by the multidrug resistance 1 (*MDR1*) gene, is a representative example of

an ATP binding cassette transporter. PGP consists of two membrane-spanning domains and two nucleotide binding domains and has been reported to affect the pharmacokinetics of drugs by limiting the rate at which they are absorbed (1–5). Various molecules are targeted by drug treatments for cancer; however, PGP expression is responsible for resistance to the widest range of anticancer drugs (6, 7).

The expression of *MDR1*/PGP in human malignant cancers is expected to play a critical role in limiting their sensitivity to anticancer agents. Therefore, the determination of *MDR1* gene expression levels, along with studies of the regulatory mechanisms of this gene, will be useful in developing tailor-made therapeutic strategies for cancer patients.

The partial sequence of the human *MDR1* gene was first reported in the 1980s (8), and its complete sequence, including clustered CpG sites that are not associated with a TATA box, is now known (9). Within the *MDR1* promoter sequence, a GC box forming a Sp1 site and an inverted CCAAT (ATTGG) site for Y-box binding protein 1 (YB-1) or nuclear factor Y (NF-Y) binding both play key roles in *MDR1* gene expression (10).

MDR1 gene expression is often observed in recurrent cancers and appears after the chemotherapeutic treatment of various human malignancies. In cultured human cancer cells, the *MDR1* promoter was activated by both PGP targeting drugs (vincristine and doxorubicin) and non-PGP-targeting drugs (5-fluorouracil and etoposide; ref. 11). In addition, treatment with retinoic acids and other differentiating agents resulted in enhanced expression of the *MDR1* gene product PGP (12). Expression of the *MDR1* gene was also up-regulated by heat shock, arsenate, and serum starvation in cultured human cancer cells (13–16). Consistent with these findings, *MDR1* gene expression was markedly induced by anticancer agents (17); the gene promoter was also activated in response to both anticancer agents and UV light (18, 19). These results show that *MDR1* gene expression is highly susceptible to various environmental stimuli (Table 1) and might therefore be stress responsive (11).

This review focuses on the molecular mechanism of the transcriptional regulation of human *MDR1*/PGP and the role of YB-1 expression in the acquisition of drug resistance.

Transcriptional Regulation of the Human *MDR1* Gene

Many studies have shown the involvement of various *cis*-acting elements in *MDR1* gene expression, suggesting

Table 1. Transcriptional regulation of the *MDR1* gene in human cell lines

Transcription factor	Inducers	References
NF-Y	None	(40)
	Sodium butylate	(43)
	Trichostatin A	(42)
Sp1	None	(45)
YB-1	UV light	(30, 32)
	Anticancer agents	(31)
Nuclear factor-interleukin-6	Phorbol ester	(52, 78)
EGR1	Phorbol ester	(45, 46)
HSF1	Heat shock	(15, 55)
20-kDa protein	Serum starvation	(16)
Transcription factor 4/β-catenin	None	(56)
Human T-cell lymphotrophic virus-1 Tax	Virus infection	(79)
SXR	Digoxin	(80)
MDR1 promoter-enhancing factor 1/RNA helicase A	None	(59, 60)
Nuclear factor-κB	Daunomycin	(58)
p53	None	(49–51)

pleiotropic mechanisms (10). As shown in Table 1, several transcription factors are expected to play critical roles in the basal expression of the *MDR1* promoter in addition to stimulus-induced activation.

Y-Box Binding Protein 1

Many reports on the factors associated with drug resistance have shown a plausible association of YB-1 with drug resistance both in cultured cancer cells and in numerous clinical human tumor samples.

YB-1 is a member of the cold shock domain (CSD) protein family, which is found in the cytoplasm and nucleus of mammalian cells. It has pleiotropic functions in the regulation of gene transcription and translation, DNA repair, drug resistance, and cellular responses to environmental stimuli (20–22). The structures of YB-1 and two other members of the CSD family, hdbpA (23) and Contrin/hdbpC (24), are presented in Fig. 1A. The *YB-1* gene, which is located on chromosome 1p34 (25, 26), contains eight exons spanning 19 kb of genomic DNA (Fig. 1B). The 1.5-kb mRNA encodes a 43-kDa protein comprising three domains: a variable NH_2-terminal tail domain (A/P domain), a highly conserved nucleic acid binding CSD, and a COOH-terminal tail domain (B/A repeat; refs. 27–29). The A/P domain (amino acids 1-51) seems to be involved in transcriptional regulation, whereas the CSD domain and part of the B/A repeat (amino acids 51-205) function in binding the Y-box (inverted CCAAT box) or double-stranded DNA. Most of the COOH-terminal region of the B/A repeat domain (amino acids 129-324) is thought to bind ssDNA or RNA, and part of this region (amino acids 129-205) is involved in dimerization.

We identified YB-1 as a transcription factor that binds to the inverted CCAAT box of the *MDR1* promoter (30).

Decreased expression of YB-1, resulting from the introduction of YB-1 antisense expression constructs into cancer cells, markedly reduced the activation of the *MDR1* gene by DNA-damaging agents (31).

YB-1 is normally present in the cytoplasm, although it is translocated to the nucleus when cells are exposed to anticancer agents, hyperthermia, or UV light irradiation (19, 32, 33). YB-1 is often overexpressed in malignant cells and its expression is regulated by both the proto-oncogene product c-Myc and the tumor suppression gene product p73 (25, 34). The COOH-terminal tail domain seems to play a key role in the localization of YB-1 to either the cytoplasm or the nucleus (32). Studies have shown that cell cycle–specific nuclear translocation is mediated by cooperation of the CSD and COOH-terminal tail domain (35) and that the nuclear translocation of YB-1 requires wild-type p53 (36). The introduction of antisense RNA into human cancer cell lines,[10] and the targeted disruption of one Y-box allele in chicken DT40 cells (37) both inhibited growth. By contrast, the targeted disruption of one allele of the *YB-1* gene in mouse ES-1 cells had no effect on the growth rate (38).

Nuclear Factor Y

The CCAAT box is among the most ubiquitous DNA elements in both forward and reverse orientation. NF-Y is the major transcription factor recognizing the CCAAT box (39). This heteromeric protein is composed of three subunits, NF-YA, NF-YB, and NF-YC, all of which are necessary for DNA binding (Fig. 1A). Mutation and/or deletion of the CCAAT box have been shown to result in a significant loss of *MDR1* promoter activity (40). It has been reported that both the inverted CCAAT box and the GC box are required for activation of the *MDR1* promoter by UV light, and NF-Y, not YB-1, is thought to be the factor regulating the *MDR1* gene (41). However, these findings are not consistent with the results discussed above. The YB-1 protein is abundant and localized in the cytoplasm; however, when the effect of YB-1 overexpression on *MDR1* promoter activity was evaluated in human cancer KB cells, it was unclear whether the nuclear YB-1 content was increased. As YB-1 is known to repress translation, increased levels of cytoplasmic YB-1 might inhibit the translation of luciferase mRNA. Further studies are required to resolve this issue. Treatment with a histone deacetylase inhibitor (trichostatin) induced a marked increase in the amount of *MDR1* mRNA, although this drug-induced increase was inhibited in dominant-negative NF-Y mutants (42). NF-Y therefore seems to regulate *MDR1* gene expression through an interaction with p300/CBP-associated factor, which shows histone acetylation activity. NF-Y might also be responsible for the sodium butyrate–induced *MDR1* gene up-regulation in colon cancer cells (43). This transcription factor therefore plays a pivotal role in *MDR1* gene expression. Recently, the antitumor agent HMN-176, which interacts with NF-YB, has been shown to inhibit *MDR1* gene expression and to restore chemosensitivity to MDR cells (44).

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[10] K. Kohno and M. Kuwano, unpublished data.

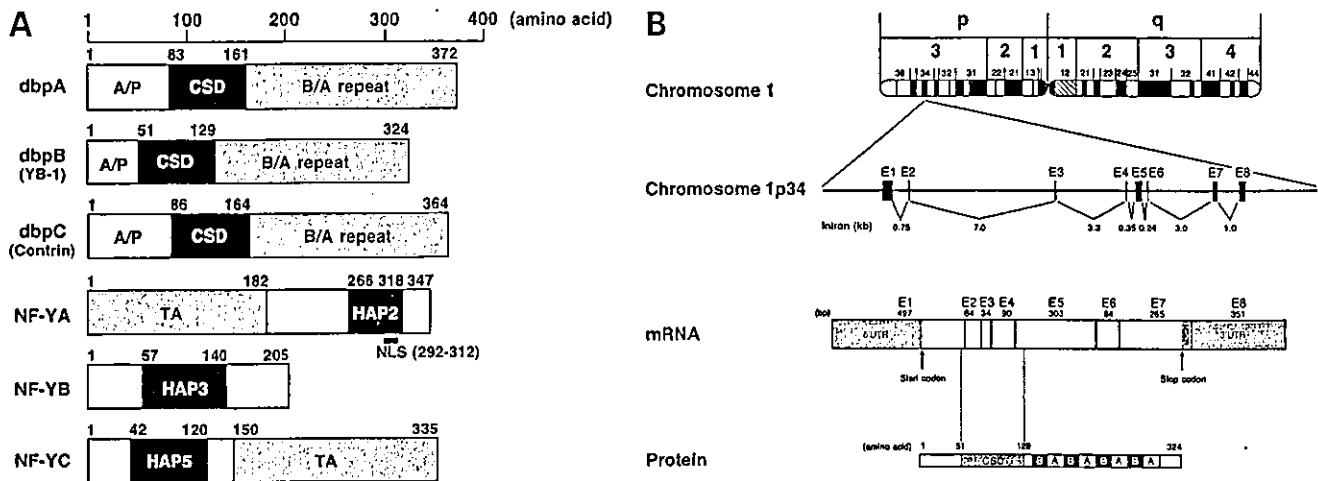**Figure 1.** **A,** protein structure and functional domains of hdbpB/YB-1, hdbpA, hdbpC, NF-YA, NF-YB, and NF-YC. *A/P,* alanine and proline domain, residues 1-82, 1-50, and 1-85 in hdbpA, hdbpB/YB-1, and Contrin/hdbpC, respectively. *CSD,* residues 83-161, 51-129, and 86-164. *B/A repeat,* basic and acidic amino acid, residues 162-372, 130-324, and 165-364. The CSD domains of the three genes are highly homologous. Of the three subunits of NF-Y, NF-YB and NF-YC contain histone folding motifs homologous to the yeast transcription factors *HAP3* and *HAP5,* respectively. NF-YA contains a domain homologous to *HAP2,* which interacts with NF-YB and NF-YC, and the heterotrimer of NF-Y binds to DNA. Both NF-YA and NF-YC contain glutamine-rich domains and activate transcription. **B,** general structure of the genomic DNA, mRNA, and protein product of *YB-1.* The gene is mapped at chromosome 1p34 and has eight exons (*E1, E2, E3, E4, E5, E6, E7,* and *E8*). The YB-1 protein consists of 324 amino acids. *B,* basic amino acid clusters; *A,* acidic amino acid clusters.

Sp1 and Early Growth Response Element 1

The introduction of mutations in the GC-rich region −59 to −45 (G region) of the *MDR1* promoter markedly decreased its activity as a result of the transcription factor Sp1 (40, 45). Sp1 was first cloned and identified as a transcription factor specifically bound to the GC box of the SV40 promoter. A GC box is found in the promoter region of many eukaryotic genes. The Sp1 family is involved in various cellular functions including proliferation, apoptosis, differentiation, and neoplastic changes. As the early growth response element 1 (EGR1) binding motif partially overlaps with the Sp1 binding sites, it is conceivable that they mutually influence *MDR1* gene expression in a competitive manner (45). Treatment with phorbol ester induced the expression of both *EGR1* and *MDR1* genes in human leukemia cells (46). However, the expression of *EGR1* alone did not enhance *MDR1* promoter activity. Coexpression of the oncosuppressor gene *WT1* resulted in the inhibition of *MDR1* promoter activation by EGR1 or phorbol ester (47). Therefore, the direct binding of WT1 to the GC box might compete with Sp1 to down-regulate the *MDR1* gene. These findings suggest that interactions between EGR1 and WT1 might play a key role in *MDR1* promoter activation.

p53

Mutant p53 has been shown to enhance *MDR1* promoter activity in mouse cells; this was reversed by wild-type p53 (14, 48). By contrast, stimulation of the *MDR1* promoter by wild-type, but not mutant, p53 was shown in several human p53-null cancer cell lines. The *MDR1* promoter region −39 to +53 is responsible for this p53-mediated activation (49), whereas the region −189

to +133 is thought to be responsible for negative regulation by wild-type p53 (50). In addition, p53 has been reported recently to bind directly to a novel binding element (−72 to −40) within the *MDR1* core promoter and to repress its promoter activity (51).

Nuclear Factor-Interleukin-6

The treatment of human monocytic cells with phorbol ester enhanced *MDR1* promoter activity through interaction with nuclear factor-interleukin-6, which is a CCAAT/enhancer binding protein family member. This study also revealed that the mitogen-activated protein kinase pathway activates nuclear factor-interleukin-6 (52). In addition, CCAAT/enhancer binding protein β has been shown recently to transactivate the *MDR1* promoter by interaction with the Y-box (53).

Heat Shock Factor

MDR1 promoter activation in response to arsenate or heat shock seems to be mediated through a heat shock element in the −178 to −165 region. An additional region at −136 to −76 has also been proposed as a critical heat shock element for the heat shock response (15, 54), although no direct binding of heat shock factor to this region has been shown. Recently, Vilaboa et al. (55) reported that infection with adenovirus carrying heat shock transcription factor 1 cDNA increased the levels of *MDR1* mRNA and PGP.

Transcription Factor 4/β-Catenin

Transcriptional profiles produced using cDNA microarrays in human colon cancer cell lines identified the *MDR1* gene as the target of transcription factor 4/β-catenin. Seven transcription factor 4/β-catenin binding sites were in the promoter region between −2,030 and +31 (56).

Nuclear Factor-κB

The hepatocarcinogen 2-acetylaminofluorene was shown to activate the *MDR1* gene in human hepatoma cells and the induction of *MDR1* by 2-acetylaminofluorene was mediated by a nuclear factor-κB binding site located around −6 kb (57). Another group showed that the inhibition of nuclear factor-κB reduced levels of *MDR1* mRNA and PGP expression and that nuclear factor-κB transactivated the *MDR1* promoter in human colon cancer HCT15 cells (58). This study identified a nuclear factor-κB binding site in the first intron.

MDR1 Promoter-Enhancing Factor 1/RNA Helicase A

MDR1 promoter-enhancing factor 1 has been shown to bind to the CCAAT sequence causing up-regulation of the *MDR1* gene (59). RNA helicase A has also been reported to bind to the CCAAT box as a member of the *MDR1* promoter-enhancing factor 1 complex (60). Overexpression of RNA helicase A enhanced the expression of both the *MDR1* promoter-reporter construct and endogenous PGP.

Clinical Implications of PGP Expression and Nuclear Translocation of YB-1

PGP triggers resistance to a wide range of anticancer agents including *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and taxols (7). In addition, YB-1 plays a role in limiting the drug sensitivity of cancer cells by increasing the expression of PGP and other proteins. Immunohistochemical studies of YB-1 expression in the nuclei of untreated primary breast cancers showed an almost complete association between nuclear YB-1 and PGP expression in 9 of 27 cases (Table 2; ref. 61). Studies of clinical specimens have also shown an association between YB-1 and PGP in osteosarcoma (62), synovial sarcoma (63), breast cancer (64, 65), ovarian cancer (66–68), and prostate cancer (Table 2; ref. 69). Figure 2 shows examples of the presence and absence of YB-1 and PGP in clinical samples of osteosarcoma and synovial sarcoma based on the results of immunohistochemical analyses with anti-YB-1 and anti-PGP antibodies.

Table 2. The association of nuclear expression of YB-1 with PGP-mediated and/or non-PGP-mediated drug resistance in human malignancies

Tumor type	Malignant characteristics	References
Ovarian cancer	PGP* ↑	(66)
	PGP* ↑	(67)
	Cisplatin resistance	(68)
Breast cancer	PGP ↑	(61)
	PGP* ↑	(64)
	Drug resistance	(65)
Osteosarcoma	PGP ↑	(62)
Synovial sarcoma	PGP* ↑	(63)
Prostate cancer	PGP* ↑	(69)

*These studies also reported a significant correlation between nuclear YB-1 expression and disease progression or prognosis.
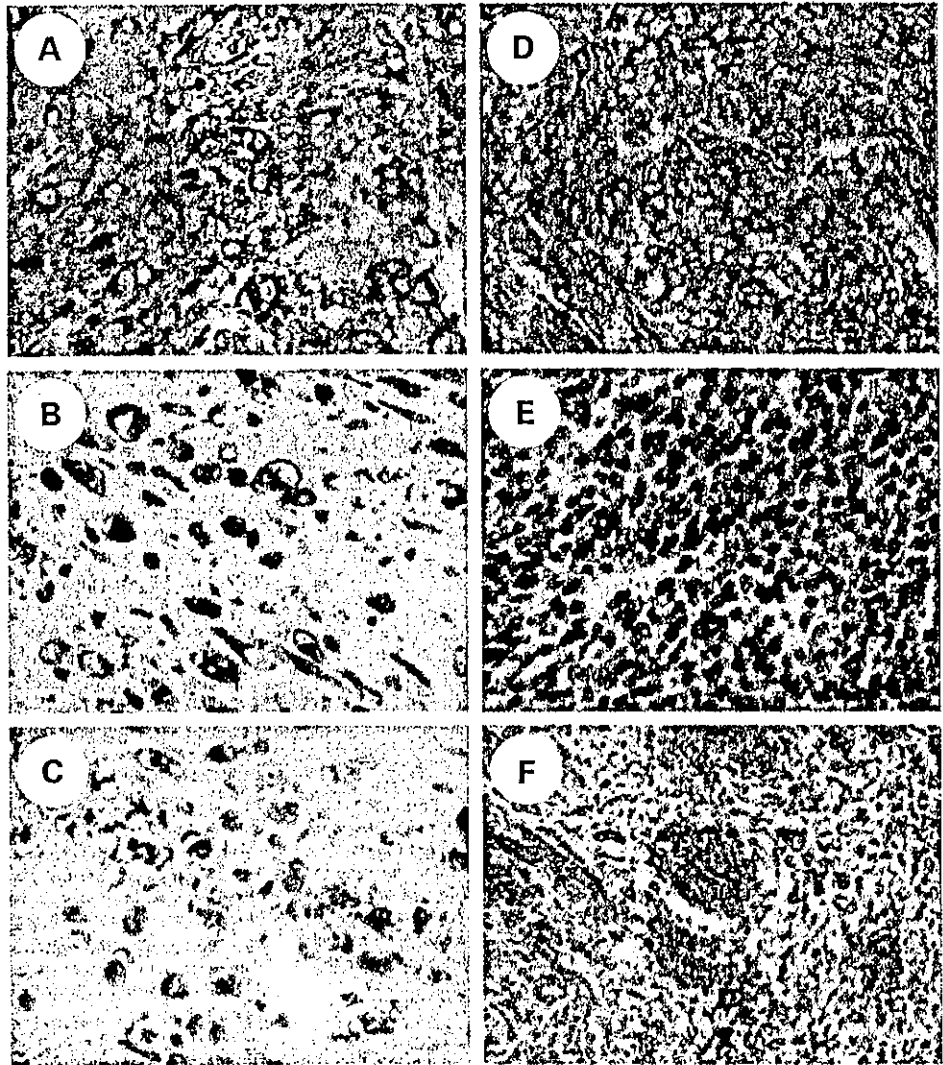
There was a significant correlation between the nuclear expression of YB-1 and the presence of PGP in 69 cases of osteosarcomas (62). A recent study confirmed that YB-1 expression was specifically associated with the overexpression of PGP rather than with three other ATP binding cassette transporters: MRP1, MRP2, and MRP3 (63). By contrast, no association was observed between YB-1 and PGP expression in colon cancers (70). It remains unclear whether YB-1 is directly involved in the transcriptional regulation of PGP in human malignancies. Nevertheless, measurements of the expression of YB-1 and PGP could suggest treatment modalities for individual cancer patients. Recently, we showed that coexpression of YB-1 and PGP correlated with poor prognosis in epithelial ovarian cancer (67). The expression of *MDR1* is augmented in cancerous areas in breast cancer and other tumors, resulting in drug resistance. Furthermore, the presence of YB-1 in the nuclei of cancer cells is closely associated with the clinical outcome. YB-1 could therefore be a useful indicator of malignancy as well as a promising target for cancer therapy. We recommend the use of non-PGP-targeting drugs against malignant tumors with nuclear YB-1 expression.

Clinical Implications of Nuclear Localization of YB-1: Drug Resistance to non-PGP-Targeting Drugs

As described above, YB-1 is translocated to the nucleus in response to various environmental stresses including UV light, anticancer agents, heat, and infection in cultures of cancer cells (21). YB-1 was shown to be overexpressed in cisplatin-resistant cell lines, and antisense YB-1 RNA triggered the augmentation of sensitivity to cisplatin, mitomycin C, UV light, and hydrogen peroxide (30, 38). YB-1 associates with p53 (71) and proliferating cell nuclear antigen (72), both of which modulate DNA repair, cell cycle, transcription, and drug sensitivity. Moreover, wild-type p53 is required for the nuclear translocation of YB-1, which in turn inhibits p53-induced cell death (36). However, it remains unclear how reduced YB-1 expression increases resistance to non-PGP-targeting DNA-damaging agents such as cisplatin and mitomycin C. Potential mechanisms might include a reduction in the YB-1 interaction with proliferating cell nuclear antigen, which is necessary for nucleotide excision repair, or in the interaction with p53. However, pleiotropic drug resistance to DNA-interacting drugs (e.g., aphidicolin, hydroxyurea, cytarabine, etoposide, doxorubicin, and mafosfamide) is associated with the increased expression of YB-1 and 19 other genes that are involved in DNA replication, repair, and stress responses (73).

Nuclear expression of YB-1 was reported to be a prognostic factor in ovarian serous adenocarcinoma (66). It was also associated with cisplatin resistance in ovarian cancer cell lines, and expression levels were increased at some sites of ovarian cancer recurrence (68). This pattern was seen in 7 of 21 serous adenocarcinomas, 2 of 7 clear cell

Figure 2. Immunohistochemical detection of nuclear and cytoplasmic YB-1 in osteosarcoma and synovial sarcoma. Antibodies were used against YB-1 (A, B, D, and E) or PGP (C and F). Osteosarcoma is shown with cytoplasmic YB-1 expression (A), nuclear YB-1 expression (B), and PGP expression (C). Synovial sarcoma is shown with cytoplasmic YB-1 expression (D), nuclear YB-1 expression (E), and PGP expression (F). The patient in D showed no evidence of disease 131 months after surgery. The patient in F died of lung metastasis 8 months after the initial surgery.

adenocarcinomas, and 1 of 4 mucinous adenocarcinomas (Table 2). There was also a positive correlation between the nuclear expression of YB-1 and poor prognosis in synovial sarcoma (63).

Analysis of the clinical relevance of YB-1 expression in the cytoplasm or nucleus in 83 cases of breast cancer, after a median follow-up of 61 months, revealed that the 5-year relapse rate was 66% in patients with high YB-1 expression who received postoperative chemotherapy (65). By contrast, none of the patients with low YB-1 expression experienced relapse. Taken together, these findings indicate that the overexpression and nuclear expression of YB-1 have a predictive value in some human malignancies, both with and without postoperative chemotherapy.

An investigation of 588 genes associated with mouse lung tumor progression revealed that 19 were differentially expressed between lung adenoma and adenocarcinoma; YB-1 was one of these candidate lung tumor progression genes (74). Overexpression of YB-1 was observed in >90% of anaplastic thyroid carcinomas,

whereas it was absent in normal follicles and other pathologic tumor types. These findings suggested the involvement of YB-1 in the anaplastic transformation of thyroid carcinoma (75). YB-1 expression induced a strong cellular resistance to malignant transformation through the phosphatidylinositol 3-kinase pathway possibly through the inhibition of protein synthesis that is required for the phosphatidylinositol 3-kinase– or Akt-induced oncogenic transformation (76).

Conclusion

The ancestral protein YB-1 modulates cell growth, apoptosis, drug resistance, DNA repair, transcription, and translation as a pleiotropic regulator. YB-1 overexpression or nuclear YB-1 expression might play a key role not only in the acquirement of PGP-mediated drug resistance but also in sensitivity to non-PGP-targeting chemotherapeutic agents. YB-1 in the nucleus modulates drug resistance to PGP-targeting and non-PGP-targeting drugs in cancer cells
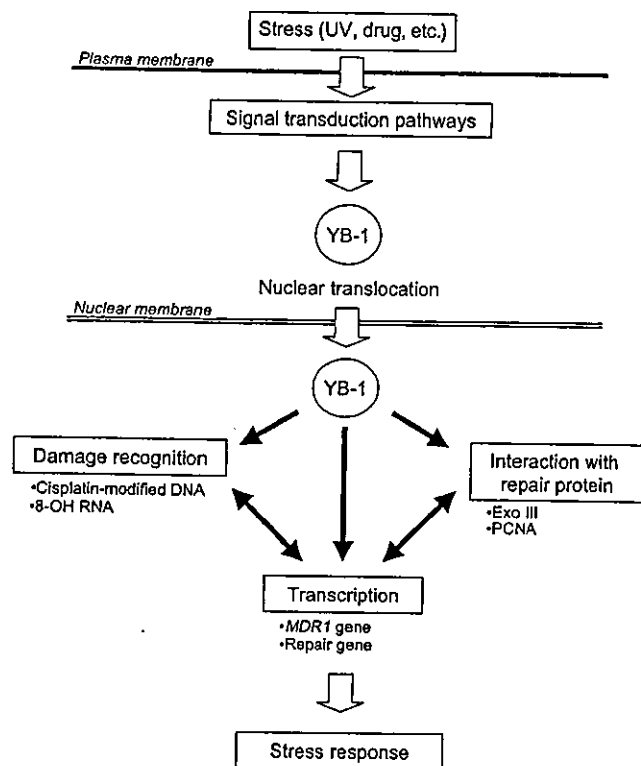
Figure 3. Schematic summary of MDR mediated by PGP or non-PGP. YB-1 is normally present in the cytoplasm but is translocated to the nucleus by treatment with anticancer agents, hyperthermia, or UV light irradiation. YB-1 in the nucleus functions as a transcription factor, which can bind to the Y-box and transactivate promoters, such as the *MDR1* gene or repair genes. By contrast, YB-1 can bind directly to cisplatin-modified DNA and interact with repair proteins including NTH1 (*Exo III*) and proliferating cell nuclear antigen (*PCNA*). These functions might be advantageous for the acquisition of drug resistance.

that are exposed to anticancer and other cytotoxic DNA-damaging agents (Fig. 3). In one response pathway to environmental stimuli, YB-1 is translocated to the nucleus and up-regulates *MDR1* gene expression through binding to the Y-box on the promoter. Alternatively, YB-1 might operate its DNA repair pathway through interactions with p53 (71), proliferating cell nuclear antigen (72), and other molecules (77) when DNA is damaged (Fig. 3). Further research is needed to fully understand the role of YB-1 in cancer and drug resistance.

References

1. Schinkel AH, Mayer U, Wagenaar E, et al. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. Proc Natl Acad Sci U S A 1997;15:4028 – 33.

2. Schinkel AH. Pharmacological insights from P-glycoprotein knockout mice. Int J Clin Pharmacol Ther 1998;36:9 – 13.

3. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 1999;39:361 – 98.

4. Watkins PB. The barrier function of CYP3A4 and P-glycoprotein in the small bowel. Adv Drug Deliv Rev 1997;15:161 – 70.

5. Fromm MF. The influence of *MDR1* polymorphisms on P-glycoprotein expression and function in humans. Adv Drug Deliv Rev 2002; 18:1295 – 310.

6. Scherf U, Ross DT, Waltham M, et al. A gene expression database for the molecular pharmacology of cancer. Nat Genet 2000;24:236 – 44.

7. Alvarez M, Paull K, Monks A, et al. Generation of a drug resistance profile by quantitation of mdr-1/P-glycoprotein in the cell lines of the National Cancer Institute Anticancer Drug Screen. J Clin Invest 1995; 95:2205 – 14.

8. Ueda K, Clark DP, Chen CJ, Roninson IB, Gottesman MM, Pastan I. The human multidrug resistance (*MDR1*) gene. cDNA cloning and transcription initiation. J Biol Chem 1987;262:505 – 8.

9. Kohno K, Sato S, Uchiumi T, Takano H, Kato S, Kuwano M. Tissue-specific enhancer of the human multidrug-resistance (*MDR1*) gene. J Biol Chem 1990;15:19690 – 6.

10. Labialle S, Gayet L, Marthinet E, Rigal D, Baggetto LG. Transcriptional regulators of the human multidrug resistance 1 gene: recent views. Biochem Pharmacol 2002;64:943 – 8.

11. Kohno K, Sato S, Takano H, Matsuo K, Kuwano M. The direct activation of human multidrug resistance gene (*MDR1*) by anticancer agents. Biochem Biophys Res Commun 1989;165:1415 – 21.

12. Bates SE, Mickley LA, Chen YN, et al. Expression of a drug resistance gene in human neuroblastoma cell lines: modulation by retinoic acid-induced differentiation. Mol Cell Biol 1989;9:4337 – 44.

13. Chin KV, Tanaka S, Darlington G, Pastan I, Gottesman MM. Heat shock and arsenite increase expression of the multidrug resistance (*MDR1*) gene in human renal carcinoma cells. J Biol Chem 1990;265:221 – 6.

14. Chin KV, Ueda K, Pastan I, Gottesman MM. Modulation of activity of the promoter of the human *MDR1* gene by Ras and p53. Science 1992;255:459 – 62.

15. Miyazaki M, Kohno K, Uchiumi T, et al. Activation of human multidrug resistance-1 gene promoter in response to heat shock stress. Biochem Biophys Res Commun 1992;187:677 – 84.

16. Tanimura H, Kohno K, Sato S, et al. The human multidrug resistance 1 promoter has an element that responds to serum starvation. Biochem Biophys Res Commun 1992;183:917 – 24.

17. Chaudhary PM, Roninson IB. Induction of multidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. J Natl Cancer Inst 1993;85:632 – 9.

18. Asakuno K, Kohno K, Uchiumi T, et al. Involvement of a DNA binding protein, MDR-NF1/YB-1, in human *MDR1* gene expression by actinomycin D. Biochem Biophys Res Commun 1994;199:1428 – 35.

19. Uchiumi T, Kohno K, Tanimura H, et al. Enhanced expression of the human multidrug resistance 1 gene in response to UV light irradiation. Cell Growth Differ 1993;4:147 – 57.

20. Izumi H, Imamura T, Nagatani G, et al. Y box-binding protein-1 binds preferentially to single-stranded nucleic acids and exhibits 3'→5' exonuclease activity. Nucleic Acids Res 2001;29:1200 – 7.

21. Kohno K, Izumi H, Uchiumi T, Ashizuka M, Kuwano M. The pleiotropic functions of the Y-box-binding protein, YB-1. Bioessays 2003;25:691 – 8.

22. Kuwano M, Uchiumi T, Hayakawa H, et al. The basic and clinical implications of ABC transporters, Y-box-binding protein-1 (YB-1) and angiogenesis-related factors in human malignancies. Cancer Sci 2003; 94:9 – 14.

23. Kudo S, Mattei MG, Fukuda M. Characterization of the gene for dbpA, a family member of the nucleic-acid-binding proteins containing a cold-shock domain. Eur J Biochem 1995;231:72 – 82.

24. Tekur S, Pawlak A, Guellaen G, Hecht NB. Contrin, the human homologue of a germ-cell Y-box-binding protein: cloning, expression, and chromosomal localization. J Androl 1999;20:135 – 44.

25. Makino Y, Ohga T, Toh S, et al. Structural and functional analysis of the human Y-box binding protein (YB-1) gene promoter. Nucleic Acids Res 1996;24:1873 – 8.

26. Toh S, Nakamura T, Ohga T, et al. Genomic organization of the human Y-box protein (YB-1) gene. Gene 1998;206:93 – 7.

27. Wolffe AP. Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. Bioessays 1994; 16:245 – 51.

28. Ladomery M, Sommerville J. A role for Y-box proteins in cell proliferation. Bioessays 1995;17:9 – 11.

29. Graumann PL, Marahiel MA. A superfamily of proteins that contain the cold-shock domain. Trends Biochem Sci 1998;23:286 – 90.

30. Ohga T, Koike K, Ono M, et al. Role of the human Y box-binding protein YB-1 in cellular sensitivity to the DNA-damaging agents cisplatin, mitomycin C, and ultraviolet light. Cancer Res 1996;56:4224 – 8.

31. Ohga T, Uchiumi T, Makino Y, et al. Direct involvement of the Y-box binding protein YB-1 in genotoxic stress-induced activation of the human multidrug resistance 1 gene. J Biol Chem 1998;273:5997 – 6000.

32. Koike K, Uchiumi T, Ohga T, et al. Nuclear translocation of the Y-box binding protein by ultraviolet irradiation. FEBS Lett 1997;417:390 – 4.

33. Stein U, Jurchott K, Walther W, Bergmann S, Schlag PM, Royer HD. Hyperthermia-induced nuclear translocation of transcription factor YB-1 leads to enhanced expression of multidrug resistance-related ABC transporters. J Biol Chem 2001;276:28562 – 9.

34. Uramoto H, Izumi H, Ise T, et al. p73 Interacts with c-Myc to regulate Y-box-binding protein-1 expression. J Biol Chem 2002;277:31694 – 702.

35. Jurchott K, Bergmann S, Stein U, et al. YB-1 as a cell cycle-regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. J Biol Chem 2003;278:27988 – 96.

36. Zhang YF, Homer C, Edwards SJ, et al. Nuclear localization of Y-box factor YB-1 requires wild-type p53. Oncogene 2003;22:2782 – 94.

37. Swamynathan SK, Varma BR, Weber KT, Guntaka RV. Targeted disruption of one allele of the Y-box protein gene, Chk-YB-1b, in DT40 cells results in major defects in cell cycle. Biochem Biophys Res Commun 2002;296:451 – 7.

38. Shibahara K, Uchiumi T, Fukuda T, et al. Targeted disruption of one allele of Y-box binding protein-1 (YB-1) gene in mouse embryonic stem cells and increased sensitivity to cisplatin and mitomycin C. Cancer Sci 2004;95:348 – 53.

39. Mantovani R. The molecular biology of the CCAAT-binding factor NF-Y. Gene 1999;239:15 – 27.

40. Sundseth R, MacDonald G, Ting J, King AC. DNA elements recognizing NF-Y and Sp1 regulate the human multidrug-resistance gene promoter. Mol Pharmacol 1997;51:963 – 71.

41. Hu Z, Jin S, Scotto KW. Transcriptional activation of the MDR1 gene by UV irradiation. Role of NF-Y and Sp1. J Biol Chem 2000;275:2979 – 85.

42. Jin S, Scotto KW. Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y. Mol Cell Biol 1998;18:4377 – 84.

43. Morrow CS, Nakagawa M, Goldsmith ME, Madden MJ, Cowan KH. Reversible transcriptional activation of mdr1 by sodium butyrate treatment of human colon cancer cells. J Biol Chem 1994;269:10739 – 46.

44. Tanaka H, Ohshima N, Ikenoya M, Komori K, Katoh F, Hidaka H. HMN-176, an active metabolite of the synthetic antitumor agent HMN-214, restores chemosensitivity to multidrug-resistant cells by targeting the transcription factor NF-Y. Cancer Res 2003;63:6942 – 7.

45. Cornwell MM, Smith DE. Sp1 activates the MDR1 promoter through one of two distinct G-rich regions that modulate promoter activity. J Biol Chem 1993;268:19505 – 11.

46. McCoy C, Smith DE, Cornwell MM. 12-O-tetradecanoylphorbol-13-acetate activation of the MDR1 promoter is mediated by EGR1. Mol Cell Biol 1995;15:6100 – 8.

47. Madden SL, Cook DM, Morris JF, Gashler A, Sukhatme VP, Rauscher FJ III. Transcriptional repression mediated by the WT1 Wilms tumor gene product. Science 1991;253:1550 – 3.

48. Thottassery JV, Zambetti GP, Arimori K, Schuetz EG, Schuetz JD. p53-dependent regulation of MDR1 gene expression causes selective resistance to chemotherapeutic agents. Proc Natl Acad Sci U S A 1997; 94:11037 – 42.

49. Goldsmith ME, Gudas JM, Schneider E, Cowan KH. Wild type p53 stimulates expression from the human multidrug resistance promoter in a p53-negative cell line. J Biol Chem 1995;270:1894 – 8.

50. Strauss BE, Haas M. The region 3' to the major transcriptional start site of the MDR1 downstream promoter mediates activation by a subset of mutant P53 proteins. Biochem Biophys Res Commun 1995; 217:333 – 40.

51. Johnson RA, Ince TA, Scotto KW. Transcriptional repression by p53 through direct binding to a novel DNA element. J Biol Chem 2001;276: 27716 – 20.

52. Combates NJ, Kwon PO, Rzepka RW, Cohen D. Involvement of the transcription factor NF-IL6 in phorbol ester induction of P-glycoprotein in U937 cells. Cell Growth Differ 1997;8:213 – 9.

53. Chen GK, Sale S, Tan T, Ermoian RP, Sikic, BI. CCAAT/enhancer-binding protein β (nuclear factor for interleukin 6) transactivates the human MDR1 gene by interaction with an inverted CCAAT box in human cancer cells. Mol Pharmacol 2004;65:906 – 16.

54. Kioka N, Yamano Y, Komano T, Ueda K. Heat-shock responsive elements in the induction of the multidrug resistance gene (MDR1). FEBS Lett 1992;301:37 – 40.

55. Vilaboa NE, Galan A, Troyano A, de Blas E, Aller P. Regulation of multidrug resistance 1 (MDR1)/P-glycoprotein gene expression and activity by heat-shock transcription factor 1 (HSF1). J Biol Chem 2000; 275:24970 – 6.

56. Yamada T, Takaoka AS, Naishiro Y, et al. Transactivation of the multidrug resistance 1 gene by T-cell factor 4/β-catenin complex in early colorectal carcinogenesis. Cancer Res 2000;60:4761 – 6.

57. Kuo MT, Liu Z, Wei Y, et al. Induction of human MDR1 gene expression by 2-acetylaminofluorene is mediated by effectors of the phosphoinositide 3-kinase pathway that activate NF-κB signaling. Oncogene 2002;21:1945 – 54.

58. Bentires-Alj M, Barbu V, Fillet M, et al. NF-κB transcription factor induces drug resistance through MDR1 expression in cancer cells. Oncogene 2003;22:90 – 7.

59. Ogretmen B, Safa AR. Identification and characterization of the MDR1 promoter-enhancing factor 1 (MEF1) in the multidrug resistant HL60/VCR human acute myeloid leukemia cell line. Biochemistry 2000;39:194 – 204.

60. Zhong X, Safa AR. RNA helicase A in the MEF1 transcription factor complex up-regulates the MDR1 gene in multidrug-resistant cancer cells. J Biol Chem 2004;279:17134 – 41.

61. Bargou RC, Jurchott K, Wagener C, et al. Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic MDR1 gene expression. Nat Med 1997;3:447 – 50.

62. Oda Y, Sakamoto A, Shinohara N, et al. Nuclear expression of YB-1 protein correlates with P-glycoprotein expression in human osteosarcoma. Clin Cancer Res 1998;4:2273 – 7.

63. Oda Y, Ohishi Y, Saito T, et al. Nuclear expression of Y-box-binding protein-1 correlates with P-glycoprotein and topoisomerase IIα expression, and with poor prognosis in synovial sarcoma. J Pathol 2003;199: 251 – 8.

64. Saji H, Toi M, Saji S, Koike M, Kohno K, Kuwano M. Nuclear expression of YB-1 protein correlates with P-glycoprotein expression in human breast carcinoma. Cancer Lett 2003;190:191 – 7.

65. Janz M, Harbeck N, Dettmar P, et al. Y-box factor YB-1 predicts drug resistance and patient outcome in breast cancer independent of clinically relevant tumor biologic factors HER2, uPA and PAI-1. Int J Cancer 2002; 97:278 – 82.

66. Kamura T, Yahata H, Amada S, et al. Is nuclear expression of Y box-binding protein-1 a new prognostic factor in ovarian serous adenocarcinoma? Cancer 1999;85:2450 – 4.

67. Huang X, Ushijima K, Komai K, et al. Co-expression of Y box-binding protein-1 and P-glycoprotein as a prognostic marker for survival in epithelial ovarian cancer. Gynecol Oncol 2004;93:287 – 91.

68. Yahata H, Kobayashi H, Kamura T, et al. Increased nuclear localization of transcription factor YB-1 in acquired cisplatin-resistant ovarian cancer. J Cancer Res Clin Oncol 2002;128:621 – 6.

69. Gimenez-Bonafe P, Fedoruk MN, Whitmore TG, et al. YB-1 is upregulated during prostate cancer tumor progression and increases P-glycoprotein activity. Prostate 2004;59:337 – 49.

70. Shibao K, Takano H, Nakayama Y, et al. Enhanced coexpression of YB-1 and DNA topoisomerase IIα genes in human colorectal carcinomas. Int J Cancer 1999;83:732 – 7.

71. Okamoto T, Izumi H, Imamura T, et al. Direct interaction of p53 with the Y-box binding protein, YB-1: a mechanism for regulation of human gene expression. Oncogene 2000;19:6194 – 202.

72. Ise T, Nagatani G, Imamura T, et al. Transcription factor Y-box binding protein 1 binds preferentially to cisplatin-modified DNA and interacts with proliferating cell nuclear antigen. Cancer Res 1999;59:342 – 6.

73. Levenson VV, Davidovich IA, Roninson IB. Pleiotropic resistance to DNA-interactive drugs is associated with increased expression of genes involved in DNA replication, repair, and stress response. Cancer Res 2000; 60:5027 – 30.

74. Yao R, Wang Y, Lubet RA, You M. Differentially expressed genes associated with mouse lung tumor progression. Oncogene 2002;21: 5814–21.

75. Ito Y, Yoshida H, Shibahara K, et al. Y-box binding protein expression in thyroid neoplasms: its linkage with anaplastic transformation. Pathol Int 2003;53:429–33.

76. Bader AG, Felts KA, Jiang N, Chang HW, Vogt PK. Y box-binding protein 1 induces resistance to oncogenic transformation by the phosphatidylinositol 3-kinase pathway. Proc Natl Acad Sci U S A 2003; 100:12384–9.

77. Marenstein DR, Ocampo MT, Chan MK, et al. Stimulation of human endonuclease III by Y box-binding protein 1 (DNA-binding protein B).

Interaction between a base excision repair enzyme and a transcription factor. J Biol Chem 2001;276:21242–9.

78. Combates NJ, Rzepka RW, Chen YN, Cohen D. NF-IL6, a member of the C/EBP family of transcription factors, binds and trans-activates the human *MDR1* gene promoter. J Biol Chem 1994;269:29715–9.

79. Chuang SE, Doong SL, Lin MT, Cheng AL. Tax of the human T-lymphotropic virus type I transactivates promoter of the *MDR-1* gene. Biochem Biophys Res Commun 1997;238:482–6.

80. Takara K, Takagi K, Tsujimoto M, Ohnishi N, Yokoyama T. Digoxin up-regulates multidrug resistance transporter (*MDR1*) mRNA and simultaneously downregulates steroid xenobiotic receptor mRNA. Biochem Biophys Res Commun 2003;306:116–20.

Targeting of Nuclear Factor κB Pathways by Dehydroxymethylepoxyquinomicin, a Novel Inhibitor of Breast Carcinomas: Antitumor and Antiangiogenic Potential *In vivo*

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ABSTRACT

We previously designed and synthesized the new nuclear factor κB (NF-κB) inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) derived from the structure of the antibiotic epoxyquinomicin C. We looked into the effect of DHMEQ on cellular phenotypes and tumor growth in mice injected with human breast carcinoma cell line MDA-MB-231 or MCF-7. In estrogen-independent breast adenocarcinoma cell line MDA-MB-231, NF-κB is constitutively activated. The addition of DHMEQ (10 μg/mL) completely inhibited the activated NF-κB for at least 8 hours. On the other hand, NF-κB is not activated in estrogen-dependent MCF-7 cells. In this cell line, DHMEQ completely inhibited the tumor necrosis factor-α-induced activation of NF-κB. DHMEQ did not inhibit the degradation of IκB but inhibited the nuclear translocation of NF-κB by both p65/p50 and RelB/p52 pathways. MDA-MB-231 cells secrete interleukin (IL)-6 and IL-8 without stimulation, and DHMEQ decreased the secretion levels of both cytokines. When MDA-MB-231 or MCF-7 cells were stimulated by tumor necrosis factor-α, the inhibitory effects of DHMEQ were still maintained. I.p. administration of DHMEQ (thrice a week) significantly inhibited the tumor growth of MDA-MB-231 (12 mg/kg) or MCF-7 (4 mg/kg) in severe combined immunodeficiency mice. No toxicity was observed during the experiment,

including the loss of body weight. An immunohistological study on resected MCF-7 tumors showed that DHMEQ inhibited angiogenesis and promoted apoptosis. Furthermore, in Adriamycin-resistant MCF-7 cells highly expressing multidrug resistance gene-1, DHMEQ also exhibited the above capability, including down-regulation of IL-8. Thus, DHMEQ might be a potent drug for the treatment of various breast carcinomas by inhibiting the NF-κB activity.

INTRODUCTION

Over the past few years, extensive studies conducted into breast cancer have led to the recognition that overexpression of HER2, estrogen receptor (ER), and mutation of genes including p53, BRCA1, and BRCA2 play important roles in the development and progression of breast cancer via induction of multiple angiogenic, proapoptotic regulators including nuclear factor κB (NF-κB). NF-κB was initially discovered as a heterodimeric protein consisting of p65 and p50. The members of Rel and NF-κB family include p50 (p105), p65 (RelA), p52 (p100), RelB, and c-Rel. Members p105 and p100 are processed into p50 and p52, respectively. Mammary NF-κB mainly consists of RelA/p50. NF-κB is the transcription factor that promotes the transcription of inflammatory cytokines, cell adhesion molecules, and inhibitor of apoptosis-associated proteins (1–4). Ligand-stimulated tumor necrosis factor-α (TNF-α) receptor 1 activates NF-κB-inducing kinase through TNF-α receptor-associated death domain protein, receptor interacting protein, and TNF-α receptor–associated factor-2 (5). NF-κB-inducing kinase then activates IκB kinase (IKK), which is composed of catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO). IKK induces the phosphorylation and degradation of IκB-α, and consequently, the liberated NF-κB molecules enter the nucleus. For the TNF-α-induced activation of NF-κB, IKKβ/γ is essential but IKKα is not. In contrast, it has recently been suggested that IKKα is essential for an alternative NF-κB-inducing kinase-dependent pathway (6–8). The lymphotoxin-β (LT-β) receptor signaling pathway utilizes both IKKβ/γ and IKKα for the activation of NF-κB. The RelB/p100 complex, which is usually inactive in the cytoplasm, is activated by IKKα after the stimulation with LT-β (9). Phosphorylated p100 is processed to p52, and this RelB/p52 complex is then translocated to the nucleus (10). Thus, translocation of activated NF-κB is mainly regulated by two distinct pathways, one triggered by TNF-α receptor 1 and the other by the LT-β receptor pathways.

NF-κB is often constitutively activated in breast carcinomas (11, 12), bladder carcinomas (13), prostate carcinomas (14), and melanomas (15). A previous study showed that constitutive activation of NF-κB contributed to the progression of breast cancer to hormone-independent growth (12), the inhibition of NF-κB may enhance the therapeutic efficacy for malignant

phenotypes such as those with less sensitivity to apoptosis or those that show hypersecretion of angiogenetic cytokines.

Therefore, low–molecular weight inhibitors of NF-κB should be useful as anticancer agents for breast carcinomas. In the course of our search for inhibitors of NF-κB function, we have designed new NF-κB inhibitors of low molecular weight by reference to the structure of the antibiotic epoxyquinomicin C (16). One of the designed compounds, dehydroxymethylepoxyquinomicin (DHMEQ; Fig. 1), inhibited TNF-α-induced activation of NF-κB, and was effective in suppressing rheumatoid arthritis in an *in vivo* model (16). Subsequently, DHMEQ was found to inhibit nuclear translocation of NF-κB in TNF-α-treated human T cell leukemia Jurkat and monkey kidney COS-1 cells (17). DHMEQ has been already shown to provide a potential for apoptosis induction in prostate cancer (18).

In the present study, we looked into the effect of DHMEQ on the tumorigenic activity of human breast carcinomas with respect to the cellular phenotypes related to malignancy and on tumor growth *in vivo*.

MATERIALS AND METHODS

Materials. DHMEQ was synthesized in our laboratory as described before (19). Recombinant human TNF-α, recombinant human LT-α $_1$/β$_2$, and Adriamycin were purchased from Sigma Chemical Company (St. Louis, MO). Rabbit polyclonal anti-IκB-α, mouse monoclonal anti-p65, mouse monoclonal anti-p50, and rabbit polyclonal anti-RelB antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. MDA-MB-231, MCF-7, and MCF-7/Adr cells were grown in DMEM supplemented with 10% fetal bovine serum, 200 μg/mL kanamycin, 100 units/mL penicillin G, 600 μg/mL L-glutamine, and 2.25 g/L NaHCO$_3$. For trypan blue exclusion analysis, cells were seeded into 24-well plates at 5 × 10^5 cells/well and treated with various concentrations of DHMEQ for 24 hours. Then the number of cells stained with trypan blue dye was counted.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared according to the method of Andrews and Faller (20). In brief, MCF-7 cells treated or not with DHMEQ were incubated for 2 hours at 37°C and then further incubated for 30 minutes in the absence or presence of TNF-α (20 ng/mL). The cells were harvested, washed with PBS, suspended in 400 μL of buffer A [10 mmol HEPES (pH 7.8), 10 mmol KCl, 2 mmol MgCl$_2$, 0.1 mmol EDTA, 1 mmol DTT, 0.1 mmol phenylmethylsulfonyl fluoride], and incubated on ice for 15 minutes. Nuclei were pelleted by centrifugation for 5 minutes
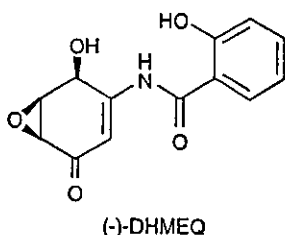

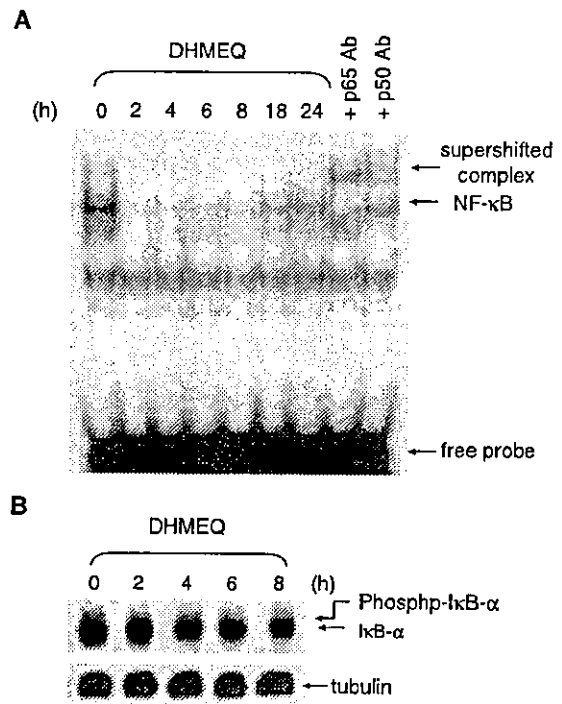*Fig. 1* Structure of (−)-DHMEQ. Racemic DHMEQ was used in all experiments.

Fig. 2 Inhibition of NF-κB activation by DHMEQ in breast carcinoma MDA-MB-231 cells. *A*, inhibition of NF-κB activation. MDA-MB-231 cells were treated without or with 10 μg/mL DHMEQ for the indicated time periods. Then the nuclear extract was assayed by EMSA. *B*, effect on IκB-α degradation. The cells were treated without or with 10 μg/mL DHMEQ for the indicated time periods. The lysates were fractionated on a 12.5% polyacrylamide gel.

at 14,000 rpm, resuspended in 40 μL of buffer C [50 mmol HEPES (pH 7.8), 50 mmol KCl, 300 mmol NaCl, 0.1 mmol EDTA, 1 mmol DTT, 0.1 mmol phenylmethylsulfonyl fluoride, 25% glycerol (v/v)], incubated on ice for 20 minutes, and centrifuged for 5 minutes at 14,000 rpm at 4°C. The supernatant was used as a nuclear extract. Binding reaction mixture containing 5 μg protein of nuclear extract, 2 μg poly dI-dC and ^{32}P-labeled probe were incubated for 20 minutes at room temperature. DNA-protein complexes were separated from free DNA on a 4% native polyacrylamide gel in 0.25 mmol Tris-borate EDTA buffer. The DNA probe used for NF-κB binding was the double-stranded oligonucleotide containing the κB site from the mouse κ light chain enhancer (5'-ATGTGAGGGGACTTTCCCAGGC-3').

Western Blotting Analysis. Cells were lysed with lysis buffer [20 mmol Tris (pH 8.0), 150 mmol NaCl, 2 mmol EDTA, 100 mmol NaF, 400 μmol/L Na$_3$VO$_4$, 1% NP40, 1 μg/mL leupeptin, 1 μg/mL antipain, 1 mmol phenylmethylsulfonyl fluoride]. Each extract (100 μg protein) was fractionated on a polyacrylamide/SDS gel, and then transferred to a polyvinylidene difluoride membrane (Amersham). The membrane was incubated overnight at 4°C for blocking in TBS buffer [20 mmol Tris-HCl (pH 7.6), 137 mmol NaCl] containing 5% skim milk. After having been washed thrice with 0.1% Tween 20 in TBS, the membrane was incubated for 1 hour at room temperature with the anti-IκB or anti-RelB antibody in TBS buffer. After
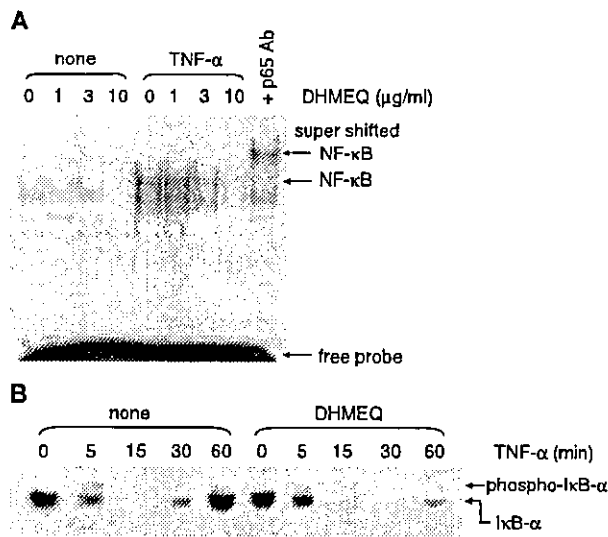
A

none   TNF-α   + p65 Ab

0 1 3 10 0 1 3 10 +   DHMEQ (μg/ml)

super shifted
← NF-κB
← NF-κB

← free probe

B

none   DHMEQ

0 5 15 30 60   0 5 15 30 60   TNF-α (min)

←phospho-IκB-α
IκB-α

Fig. 3 Inhibition of NF-κB activation by DHMEQ in breast carcinoma MCF-7 cells. *A*, inhibition of NF-κB activation. MCF-7 cells were treated with the indicated concentrations of DHMEQ in the presence or absence of 20 ng/mL TNF-α for 30 minutes. *B*, effect on IκB-α degradation. The cells were cultured with 20 ng/mL TNF-α in the presence or absence of DHMEQ for the indicated time periods.

three more washes with the TBS-Tween buffer, the membrane was incubated for 1 hour at room temperature with anti-rabbit immunoglobulin sheep antibody linked to horseradish peroxidase (Amersham). Immunoreactive proteins were visualized by use of the ECL detection system (Amersham).

Detection of Cytokines. MDA-MB-231, MCF-7, and MCF-7/Adr cells (1 × 10⁵ per well) were seeded into 24-well plates. After 24 hours, the culture medium was removed, and cells were washed twice with PBS (−), after which 1 mL of serum-free medium was added to each well, along with various concentrations of DHMEQ. For nonstimulated MDA-MB-231 and MCF-7 cells, the cells were treated with DHMEQ for 6 hours. In the case of stimulation with TNF-α, the cells were pretreated with DHMEQ for 2 hours, and then TNF-α (20 ng/mL) was added and the incubation continued for 4 hours. For MCF-7/Adr cells, the duration of stimulation of TNF-α combined with DHMEQ was 3 hours. Collected culture media were centrifuged at 15,000 rpm for 2 minutes, and the supernatants were used as samples for an (ELISA). Concentrations of the interleukin (IL)-6 and IL-8 in the collected samples were determined by using commercially available ELISA kits (Techne, Minneapolis, USA) according to the manufacturer's instructions.

In vivo Antitumor Activity. All procedures involving animals and their care in this study were approved by the Animal Care Committee of Tokyo Metropolitan Komagome Hospital in accordance with institutional and Japanese government guidelines for animal experiments. Male BALB/c severe combined immunodeficiency (SCID) mice were obtained from Sankyo Laboratory Service, Co. (Tokyo, Japan). MDA-MB-231 (1 × 10⁶) or MCF-7 cells (5 × 10⁶) were implanted s.c. in the flank of each nude mouse. Once the animals had developed palpable

MDA-MB-231 tumors, they were randomly assigned into four groups. Then DHMEQ suspended in 0.5% chloromethyl cellulose was given i.p. for 8 weeks to three of the groups (12 mg/kg once a week, 4 mg/kg thrice a week, and 12 mg/kg thrice a week), and the vehicle only medium was injected into the fourth group (control). In each mouse inoculated with MCF-7 cells, an estrogen pellet was implanted s.c. 7 days before the cell inoculation. Once the animals had developed palpable MCF-7 tumors, they were randomly assigned into two groups. Then
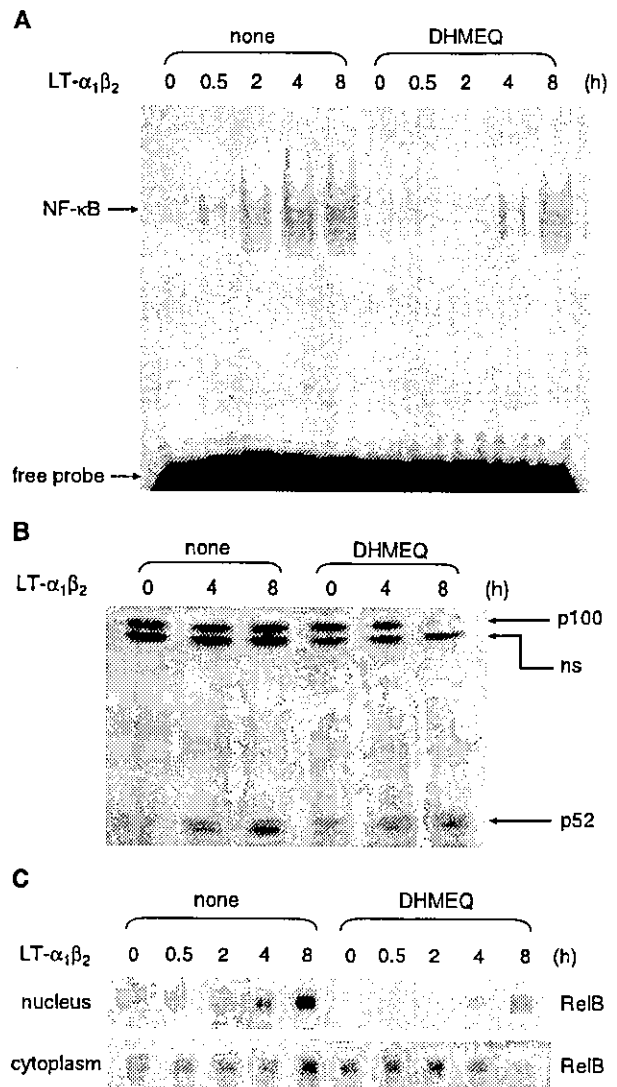
A

none   DHMEQ

LT-α₁β₂ 0 0.5 2 4 8   0 0.5 2 4 8 (h)

NF-κB →

free probe →

B

none   DHMEQ

LT-α₁β₂ 0 4 8   0 4 8 (h)

← p100
← ns
← p52

C

none   DHMEQ

LT-α₁β₂ 0 0.5 2 4 8   0 0.5 2 4 8 (h)

nucleus   RelB

cytoplasm   RelB

Fig. 4 Effect of DHMEQ on LT-α₁β₂-induced NF-κB activation in breast cancer cells. *A*, inhibition of NF-κB activation in EMSA. MCF-7 cells were pretreated or not with 10 μg/mL DHMEQ for 30 minutes, and stimulated with 20 ng/mL LT-α₁β₂ for the indicated time periods. The nuclear extract was assayed by EMSA. *B*, effect on cellular level of p52 (p100). The cells were treated with chemicals as in *A*. The lysates were fractionated on a 10% polyacrylamide gel. *C*, inhibition of nuclear translocation of RelB. The cells were treated with chemicals as in *A*. The nuclear and cytosolic fractions were prepared and each preparation was fractionated on a 9% polyacrylamide gel. Then the blotted membrane was stained with anti-RelB antibody.
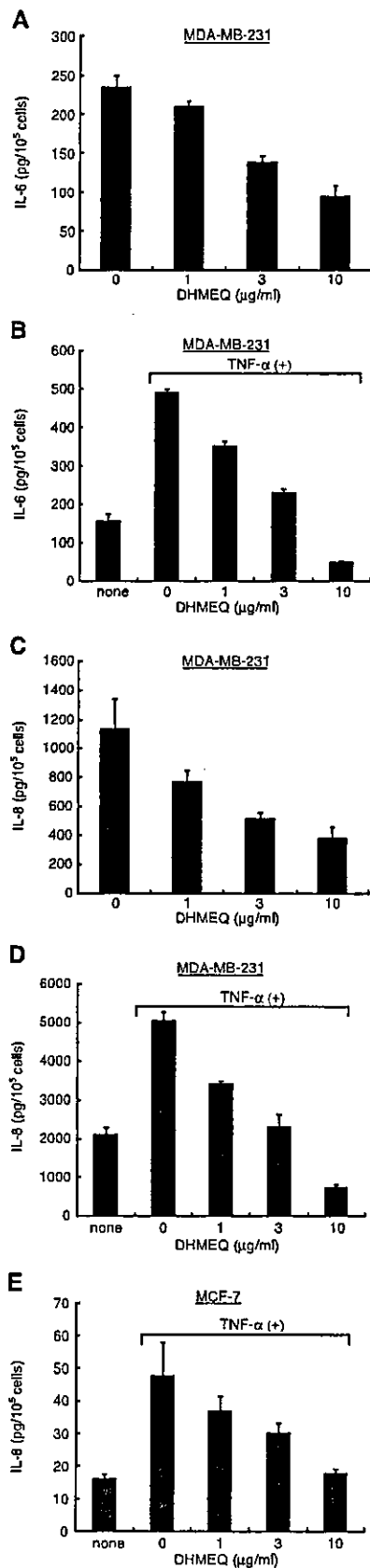
DHMEQ was given for 8 weeks to one group (4 mg/kg thrice a week), and the vehicle only medium was injected into the other group (control). Each experimental group consisted of five mice. Animals were carefully monitored, and tumor size as well as body weight was measured weekly. Tumor volume was calculated according to the formula $a^2 \times b \times 0.5$, where "a" and "b" are the smallest and largest diameters, respectively.

Immunostaining. Resected tumors from SCID mice were immediately frozen in liquid nitrogen and stored at $-80°C$. Frozen samples in OCT compound were sectioned with a cryostat at a 7-μm thickness and mounted on silane-coated glass slides. Tissue sections were incubated at room temperature for 30 minutes with anti-CD31 antibody (1:200 dilution; ref. 21) for the detection of tumor vasculature or with M30 (1:200 dilution) for the detection of apoptosis or mouse monoclonal antibody M30 (Roche, Basel, Switzerland) against caspase-cleaved neo-epitope of cytokeratin 18 was used to recognize apoptotic cancer cells (22). Slides were processed by using a commercial Elite ABC kit (Vectastain, Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as the final chromogen, and Meyer's hematoxylin was used as a counterstain.

Statistical Evaluation. The potential of DHMEQ for inhibition of *in vivo* tumor growth was analyzed by using Scheffe F test. Statistical tests were done with a StatView software package (Abacus Concepts, Inc.), and findings were considered significant when the P value was < 0.05.

RESULTS

Inhibition of Constitutively Activated NF-κB by DHMEQ in MDA-MB-231 Cells. NF-κB was constitutively activated in MDA-MB-231 in the EMSA assay, as shown in Fig. 2A. DHMEQ completely inhibited the activation at 10 μg/mL for about 8 hours. DHMEQ was previously shown to inhibit nuclear translocation of p65 in COS-1 cells (17). Therefore, as expected, DHMEQ did not change the phosphorylation or the amount of IκB in MDA-MB-231 cells (Fig. 2B).

Inhibition of TNF-α-Induced NF-κB Activation by DHMEQ in MCF-7 Cells. NF-κB was not activated in MCF-7 cells without stimulation. TNF-α was shown to activate NF-κB in MCF-7 in the EMSA assay, and DHMEQ inhibited the

Fig. 5 Inhibition of inflammatory cytokine secretions by DHMEQ in breast carcinoma cells. *A* and *B*, effects of DHMEQ on IL-6 secretion from MDA-MB-231 cells with and without stimulation by 20 ng/mL TNF-α were assessed by ELISA. *A*, cells were treated with the indicated concentrations of DHMEQ for 6 hours. *B*, cells were treated with the indicated concentrations of DHMEQ for 2 hours, and then TNF-α was added and incubation continued for 4 hours. *C* and *D*, effects of DHMEQ on IL-8 secretion from MDA-MB-231 cells with and without stimulation by 20 ng/mL TNF-α were assessed by ELISA. *C*, nonstimulated cells were treated with the indicated concentrations of DHMEQ for 6 hours. *D*, cells were treated with the indicated concentrations of DHMEQ for 2 hours, and then TNF-α was added and the incubation continued for 4 hours. *E*, effect of DHMEQ on TNF-α-induced IL-8 secretion from MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of DHMEQ for 2 hours, and then TNF-α was added and the incubation continued for 3 hours. The values are mean ± SD of quadruplicate determinations.
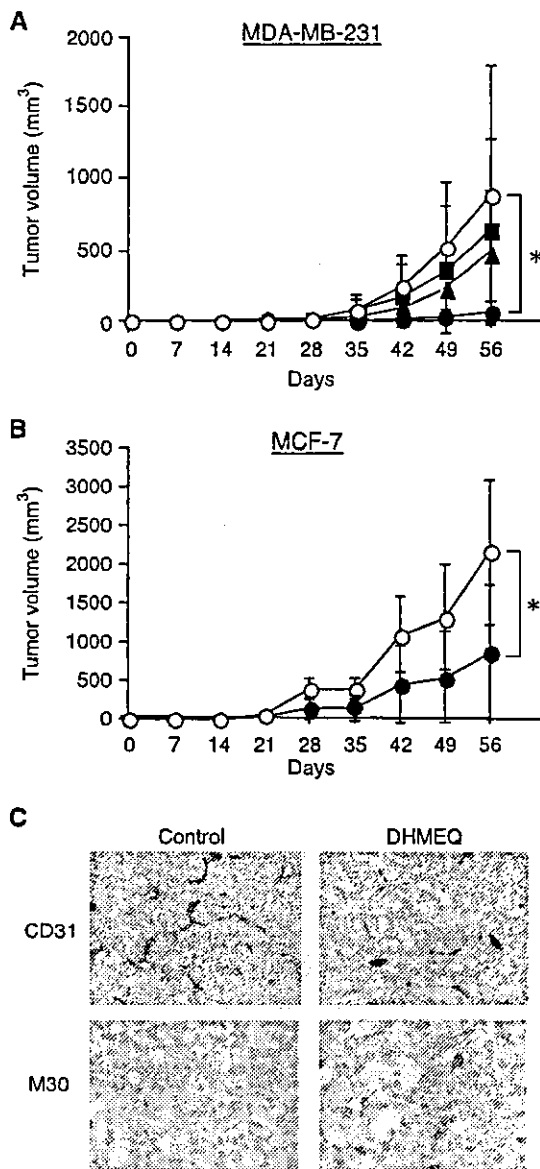
Fig. 6 Antitumor effect of DHMEQ in mice. A, inhibition of MDA-MB-231 tumor growth in SCID mice. MDA-MB-231 cells (5 × 10⁶) were implanted s.c. in the flank of each of five SCID mice. Once the animals had developed palpable tumors, they were randomly assigned into four groups. Then DHMEQ was given i.p. 12 mg/kg once a week (■), 4 mg/kg thrice a week (▲), or 12 mg/kg thrice a week (●). DHMEQ was dissolved or suspended in 0.5% chloromethyl cellulose. Control animals only received the vehicle medium injection (○). Animals were carefully monitored, and the tumor size as well as the body weight was measured weekly. Tumor volume was calculated according to the formula $a^2 \times b \times 0.5$ where "a" and and "b" are the smallest and largest diameters, respectively. Statistical significance was observed (*, ● versus ○, P = 0.0407). B, inhibition of MCF-7 tumor growth in SCID mice. MCF-7 cells (5 × 10⁶) were implanted s.c. in the flank of each of five SCID mice. The mice were i.p. injected with 4 mg/kg DHMEQ thrice a week (●). Control animals received the vehicle only medium injection (○). Statistical significance was observed (*P = 0.0003). C, histological examination of MCF-7 tumor. After the treatment with DHMEQ in "B", the tumor was removed and the tissue preparation was stained with anti-CD31 (top, ×200) and M30 (bottom, ×100) antibodies for the observation of angiogenesis and apoptosis in the tissue, respectively.

activation in a dose-dependent manner at 3 and 10 µg/mL (Fig. 3A). DHMEQ did not inhibit TNF-α-induced phosphorylation and degradation of IκB-α as shown in Fig. 3B. However, the synthesis of IκB-α at 60 minutes was strongly inhibited by DHMEQ treatment, indicating that liberated NF-κB could not induce transcription of the IκB-α gene due to failed nuclear translocation.

Inhibition of LT-α₁β₂-Induced NF-κB Activation by DHMEQ in MCF-7 Cells. We also determined whether DHMEQ inhibited the nuclear translocation of RelB/p52, which is a major form of NF-κB in the LT-β receptor pathway. LT-α₁β₂ (20 ng/mL) induced the activation of NF-κB in MCF-7 cells, as shown in Fig. 4A. This activation was apparently inhibited by DHMEQ at 10 µg/mL. DHMEQ inhibited the activation of NF-κB in the stimulated MCF-7 cells without influencing the processing of p100 (Fig. 4B); i.e., the production of p52 by the stimulation with LT-α₁β₂ was not influenced by DHMEQ. However, synthesis of p100 was inhibited at 8 hours after the addition. Fig. 4C clearly shows that DHMEQ inhibited the activation of NF-κB in stimulated MCF-7 cells, possibly by inhibiting the nuclear translocation of RelB up to 4 hours. Thus, DHMEQ inhibited the activation of NF-κB by inhibiting the nuclear translocation of both RelA/p50 and RelB/p52.

Inhibition of IL-6 and IL-8 Secretion by DHMEQ. MDA-MB-231 cells were found to secrete IL-6 constitutively, and the addition of TNF-α further accelerated the secretion (Fig. 5A and B). DHMEQ inhibited the constitutive IL-6 secretion of MDA-MB-231 in a dose-dependent manner (Fig. 5A). Even when cells were stimulated with TNF-α (20 ng/mL), 10 µg/mL of DHMEQ completely inhibited the IL-6 secretion (Fig. 5B). MCF-7 cells failed to produce detectable IL-6 protein even after stimulation (data not shown). MDA-MB-231 cells constitutively expressed a high level of IL-8 (Fig. 5C), whereas ER-positive MCF-7 cells, expressed a low level of IL-8 (Fig. 5E). DHMEQ inhibited the constitutive expression of IL-8 in MDA-MB-231 cells (Fig. 5C). TNF-α treatment enhanced the levels in both cell lines. DHMEQ inhibited these elevated IL-8 secretions in both kinds of cells (Fig. 5D and E).

Antitumor Effects of DHMEQ in Mice. We then tested the antitumor effects of DHMEQ on tumors arising from MDA-MB-231 and MCF-7 cells inoculated into SCID mice. I.p. administration of DHMEQ at 12 mg/kg, thrice a week, resulted in a significant decrease in the MDA-MB-231 tumor volume found for the controls (Fig. 6A). In the MCF-7 xenograft model, the mice receiving DHMEQ at 4 mg/kg, thrice a week, also showed a significant decrease in tumor growth when compared with the controls (Fig. 6B). DHMEQ treatment at the dosage used was well tolerated, leading to no gross or histopathological changes in liver, kidney, stomach, intestine, or lung attributable to toxicity, and to no body weight loss in the treated animals when compared with that of the controls (data not shown).

Induction of Apoptosis and Antiangiogenesis in Xenografted Tumors Caused by DHMEQ. Tumor vessels were identified by staining frozen sections of tumor tissue with antibody against CD31, a specific marker of angiogenesis. There was a large reduction in the number of CD31-positive vessels in the tumors of DHMEQ-treated mice as compared with that of tumors in the controls (Fig. 6C, top). Apoptosis was then