A 28-Amino-Acid Peptide Fragment of the Cupredoxin Azurin Prevents Carcinogen-Induced Mouse Mammary Lesions

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Abstract

Azurin, a member of the cupredoxin family of redox proteins, preferentially penetrates human cancer cells and exerts cytostatic and apoptotic effects. Azurin and amino acids 50-77 (p28) of azurin also produce a dose-dependent reduction in the proliferation of human mammary cancer by increasing the level of the tumor suppressor protein p53 in the cancer cell nucleus. We show that the development of 7,12-dimethylbenz[a]anthracene–induced hormone-dependent premalignant mammary ductal lesions and hormone-independent mammary alveolar lesions in mouse mammary gland organ culture is also significantly reduced by azurin and p28. The dose-dependent reduction in carcinogen-induced mammary cell proliferation by p28 was associated with an increase in the expression of p53. p28 also enhanced the inhibitory effect of a low dose of the antiestrogen tamoxifen on the development of hormone-dependent mammary ductal lesions, but did not enhance the inhibitory activity of fenretinide (N-4-hydroxyphenyl retinamide) on hormone-independent mammary alveolar lesions. These observations suggest that cupredoxins and fragments derived from them can exert a chemopreventive effect on carcinogen-induced mammary gland transformation, irrespective of hormonal environment, and enhance the inhibitory effects of tamoxifen in this model of preneoplastic mammary development.

Introduction

Azurin is a 128-amino-acid (aa) copper-containing redox protein (cupredoxin) secreted by the opportunistic pathogen Pseudomonas aeruginosa. The role of azurin as an electron transfer agent in the denitrification process (1–4) has recently been expanded to include the preferential penetration of cancer cells (5) where azurin exerts both cytostatic and cytotoxic effects (6–8).

Azurin and p28 bind tightly to and stabilize the tumor suppressor protein p53 (9–11) and significantly reduce the proliferation of breast cancer xenografts (6, 11). This suggests that azurin and p28 could also act as chemopreventive agents in breast cancer, as 50% to 60% of human breast cancers are positive for wild-type or mutant p53 (12–14). The mouse mammary gland organ culture (MMOC) model offered an opportunity to extend these observations to carcinogen-induced premalignant lesions that can develop into adenocarcinoma when serially transplanted into syngeneic mice (15), as p53 is rarely mutated or altered (16) by chemical transformation. The MMOC model has been used repeatedly to determine the efficacy of putative chemopreventive agents (17) and proven a reliable predictor of preclinical efficacy in vivo (18). The MMOC model also offers the opportunity to analyze the effect of chemopreventive agents on the induction of estrogen-independent mammary alveolar lesions (MAL) and estrogen- and progesterone-dependent mammary ductal lesions (MDL; refs. 18–20), alone or as an adjuvant to proven chemopreventive agents (i.e., tamoxifen and fenretinide). We used the MMOC model to establish that azurin and p28 were effective chemopreventive agents against the induction of MAL and MDL, whereas p28 also enhanced the activity of a known chemotherapeutic adjuvant, tamoxifen, on carcinogen-induced murine MDL.

Materials and Methods

Azurin and p28

Azurin was purified to >99% from the periplasmic fraction of recombinant E. coli strain JM109 as previously described (5). p28, LSTAADMQGVVTDGMASGLDKDYLKPDD, was chemically synthesized to >95% purity and mass balance by CS Bio, Inc. Azurin and p28 were aliquoted, dried, and stored desiccated in sterile vials at –20°C. All compounds were dissolved in culture medium and filter (0.22 μm) sterilized immediately before use.
Mouse mammary gland organ culture

The MMOC model and assay were designed to induce ovarian hormone-dependent and hormone-independent precancerous mammary lesions (19). The effect of azurin and p28 were determined in a standard MMOC assay of 15 glands per treatment group. Briefly, thoracic mammary glands were aseptically dissected from estrogen- and progesterone-pretreated 4-week-old female BALB/c mice (Charles River Laboratories) and explanted into chemically defined serum-free Weymouth 752/MB culture medium (Life Technologies) containing growth-promoting hormones for 10 days with or without the test agent. The carcinogen 7,12-dimethylbenz[a]anthracene (DMBA; 2 μg/mL; Sigma Chemicals) was added for 24 hours on day 3 and azurin or p28 was added to the medium during the initial 10-day growth phase of mammary gland development.

Hormone-independent MAL were developed in culture media containing insulin (5 μg/mL), prolactin (5 μg/mL), aldosterone (1 μg/mL), and hydrocortisone (1 μg/mL; Sigma; IPAF medium) alone or in the presence of azurin at 10, 20, and 50 μg/mL; p28 at 12.5, 25, 50, and 100 μg/mL; fenretinide at 1 μmol/L; a combination of fenretinide (1 μmol/L) and p28 (12.5, 25, 50, or 100 μg/mL); or an equal volume of media (vehicle control) for the first 10 days of the growth-promoting phase. After 10 days, glands were allowed to regress in the presence of insulin alone for an additional 14 days (24 days total). The glands were then stained with alum carmine, the number of lesions was counted, and incidence relative to control was calculated (19, 21).

Ovarian steroid–dependent MDL were developed by incubating glands with insulin, prolactin, 17β-estradiol (0.001 μg/mL), and progesterone (1 μg/mL) during the 10-day growth phase of the culture (IPEPg media). Azurin, p28, and tamoxifen (0.37-3.7 μg/mL, 1-10 μmol/L) alone or in combination were included in the medium during the growth-promoting phase. After 10 days of incubation in the presence of growth-promoting hormones, the glands were allowed to regress in the presence of insulin alone for an additional 14 days (24 days total).

Morphometric analysis

The incidence and severity (number) of MAL were determined from whole-mount preparations of each mammary gland as previously described (22). Mammary glands were scored as positive or negative for MAL, and the incidence was recorded as the ratio of the number of glands exhibiting lesions to the total number of glands in that group. Duct dilation or a disintegration of mammary structure was considered as a toxic effect of the chemopreventive agent.

The incidence of MDL was analyzed in 4-μm longitudinal sections of glands fixed in 10% formalin for 24 hours, embedded in paraffin, and stained with H&E to identify MDL. Longitudinal sections of individual mammary glands from each experiment were divided into four to five fields depending on the length of the entire gland (~12-18 mm; ref. 21), with each field representing the area covered within the lens objective (40×). The number of ductal sections with either hyperproliferation or dysplastic lesions was recorded, and the number of ducts containing lesions compared with the total number of ductal fields was counted to determine the number of MDL (23). Percent MDL was calculated as the ratio of MDL between treatment and control glands. Although the entire gland was evaluated, smaller glands have fewer total fields than larger glands, leaving each gland with a variable number of fields. The number of sections through each duct is also variable from gland to gland, resulting in group-to-group variation. Fields containing ductal hyperplasia or atypia were identified and expressed as a percentage of the total number of fields evaluated for each gland. No discrimination was made between hyperplasia or atypia and severely occluded glands. Any field containing any of these histologic patterns was considered positive for the lesion. A 60% or greater inhibition when using 15 glands per treatment group ensures that the results are significant (P < 0.05; ref. 23). All treatment groups were compared with DMBA alone, and the severity and percent inhibition calculated.

Immunocytochemistry

Formalin-fixed (10%, buffered), paraaffin-embedded tissue sections (4 μm) were deparaffinized, hydrated, and fixed onto glass slides. Additional sections adjacent to those stained with H&E were processed for Ki-67 and p53. Each section was covered in p53 antigen retrieved by microwave for 3 minutes. Slides were then placed in an oven at 95°C to 100°C for 1 hour in citrate buffer. The expression of Ki-67 was determined by heating deparaffinized, hydrated sections in pressure chambers in 10 mmol/L citrate buffer for 6 minutes. After a PBS rinse, nonspecific staining was blocked by incubating the slides in 5% gelatin, followed by avidin and biotin (Zymed Laboratories). Sections were incubated with a primary p53 antibody (Vector/Novacastra) or Ki-67 (Santa Cruz Biotechnology), rinsed in PBS, and then incubated with the appropriate biotinylated goat anti-rabbit/anti-mouse antibody (ABC kit, Vector Laboratories). Diaminobenzidine (Sigma) was used to visualize immunoreactivity. All sections were counterstained with Gill’s hematoxylin. Sections of intact glands were photographed (10×) and nuclear staining in epithelial cells was quantified. The numbers of cells positive for p53 and Ki-67 and cells stained for hematoxylin were counted. Data represent the mean percent (±SE) of a minimum of three determinations.

Induction of p53 by azurin and p28

Mammary glands exposed to DMBA for 24 hours on day 3 developed MAL and MDL over a 24-day culture period, which can be altered by exposure to chemoprotective or chemopreventive agents over the first 10 days of culture. The significant decreases in human breast cancer xenograft growth induced by azurin (6) and p28 (11) are clearly mediated by posttranslational stabilization of the p53
protein (11), suggesting that p53 may also be involved in the chemoprotective or chemopreventive effects of azurin and p28. We analyzed any potential changes in p53 transcription and the level of mature protein in glands exposed to azurin, p28, and tamoxifen against changes in proliferation in glands exposed to estrogen and progesterone for 4 days. The expression of p53 was also analyzed after 10 days of treatment with hormones, azurin, p28 or tamoxifen, and DMBA (24-hour exposure on day 3). In all cases, mammary glands were divided into two sets, with half of the glands fixed and processed for histologic and immunochemical analyses and the rest snap frozen in liquid nitrogen and stored at −80°C before RNA extraction.

**RNA extraction and quantitative reverse transcription-PCR**

Total RNA was isolated from four individual mammary glands incubated in the presence (IPEPg) and absence (IPAF) of estrogen and progesterone and snap frozen in TRIzol reagent (Invitrogen; ref. 24). Total RNA was extracted from individual mammary glands, collected, and snap frozen in TRIzol reagent (Invitrogen; ref. 24). Quantitative reverse transcription-PCR incorporated 500 ng of total RNA for each reverse transcription reaction (20 μL), with 2 μL of reverse transcription product subjected to PCR analysis as previously described (23) using IQ SYBR Green PCR Supermix (Bio-Rad) according to the manufacturer's guidelines and the MyiQ real-time PCR detection system (Bio-Rad). Mouse hypoxanthine ribosyltransferase (mHPRT) was used to normalize all reactions (CC). The primers were mHPRT (forward: 5′-AGTCCCAGCGTGAGGAG-3′ reverse: 5′-TTTCCAAATCCTCGGCTATA-3′) and p53 (forward: 5′-GATGACTGGCATGGAGGAG-3′ reverse: 5′-GTCCATGCGAGTGATG-3′). The data represent the mean ± SE of three independent observations.

**Statistical analyses**

Chi-square analyses and Fisher’s exact test were used to evaluate the incidence of lesions (15 mammary glands per experimental group) between treated and untreated MAL and MDL exposed to DMBA. All quantitative reverse transcription-PCR results were analyzed with the ANOVA and Tukey-Kramer multiple comparison tests. The data in Figs. 2B and 4A represent the mean ± SE of three independent observations. The level of significance of immunostaining of p53 and Ki-67 in Figs. 3 and 4 was determined by ANOVA followed by Dunnett’s multiple comparison test (control versus treatment). Groups were compared using Student’s t test.

**Results**

**Effects of azurin, p28, and fenretinide on the induction of MAL**

Azurin produced a dose-related inhibition in hormone-independent MAL (Table 1). Whole mounts of mammary glands incubated in the presence of azurin or p28 (Fig. 1A) had fewer alveolar structures, with mammary ducts devoid of lobulo-alveolar structures (Fig. 1B). At 50 μg/mL (~4 μmol/L), azurin reduced the incidence of MAL from ~63% (average) to 20% (or ~43%; IC₅₀ 40 μg/mL ~3 μmol/L) compared with DMBA exposure alone (Table 1).

p28 (12.5 μg/mL, 4 μmol/L) also inhibited the incidence of MAL by 56%, increasing to ~80% at 100 μg/mL or ~34 μmol/L (IC₅₀ 12.5 μg/mL, ~4 μmol/L; Table 1). These results suggest that p28 makes up the active fragment of azurin. Previous studies have shown that fenretinide (N-4-hydroxyphenyl retinamide) inhibits MAL development by >60% (25) and shows clinical efficacy against breast cancer development in high-risk premenopausal women (26).

The activity of fenretinide and fenretinide plus p28 on the development of MAL in MMMC is shown in Table 1. Fenretinide alone (1 μmol/L) inhibited MAL development by 67%. This was not enhanced by increasing the concentration of p28 (Table 1; Fig. 2A) or lowering the concentration of fenretinide (data not shown). Statistical analyses indicated that p28 and fenretinide alone as well as in combination significantly inhibited the development of MAL as compared with glands exposed to DMBA alone (P < 0.01, Fisher’s exact test; Table 1). There was also no apparent toxicity associated with any concentration of azurin or p28, as no dilation of mammary ducts or disintegration of gland structure was observed in any of the treatment groups.

**Effects of azurin, p28, and tamoxifen on MDL**

MDL that develop in response to DMBA in the presence of estrogen and progesterone are structurally comparable to human ductal carcinoma in situ (27). Hyperplastic or dysplastic lesions were present in 69 of 84 (~82%) areas evaluated in DMBA-treated glands (Table 2). The azurin fragment p28 produced a dose-related inhibition (IC₅₀ 18 μg/mL, ~6 μmol/L) of MDL development (Table 2), but inhibition essentially plateaued above 25 μg/mL (~8.5 μmol/L). Azurin (50 μg/mL, ~4 μmol/L) also inhibited (P < 0.05) the development of MDL, reducing the number of lesion areas by 79%. The antiestrogen tamoxifen selectively inhibits MDL development but does not inhibit the development of MAL in the MMOC model (27). Tamoxifen (0.3 μg/mL, 1 μmol/L) and p28 (12.5 μg/mL, ~4 μmol/L) each inhibited MDL formation by ~52 and 32%, respectively. Combined exposure of mammary glands in MMOC to the same concentrations of tamoxifen and p28 inhibited MDL development by ~70% (P < 0.05), suggesting a complementary, if not additive, effect of these two agents (Fig. 2B), perhaps influenced through a similar mechanism.

**Antiproliferative activity of azurin and p28 in MAL and MDL**

p28 and azurin inhibited the staining for Ki-67 in mammary epithelial cells exposed to DMBA (Fig. 3A). The decrease in incidence and number of MAL and MDL correlating (P < 0.01) with the loss of Ki-67 expression (Fig. 3A) suggests that the azurin- and p28-induced
reduction in the incidence of MDL and MAL is associated with a decrease in cell proliferation.

**Induction of p53 by azurin and p28**

We determined whether the level of p53 transcription or the translated protein was associated with the azurin- and p28-mediated inhibition of MAL and MDL development by incubating glands in the presence (IPAF) and absence (IPEPg) of estrogen and progesterone and either azurin or p28 for 10 days during the growth-promoting phase of MAL development. Glands were stained after 24 d of culture with alum carmine. a, control; b, azurin (50 μg/mL); c, p28 (12.5 μg/mL); d, p28 (25 μg/mL); e, p28 (50 μg/mL); f, p28 (100 μg/mL). A dose-related reduction in the number of MAL is observed in glands exposed to p28. B, effect of p28 on MDL in MMOC. Occluded mammary ducts (a) and normal histology of mammary ducts (b) in DMBA-treated glands exposed to p28.

Fig. 1. Effect of p28 on the development of mammary lesions in MMOC. A, effect of p28 on the development of MAL. Mammary glands were incubated either with DMBA alone or with DMBA and p28 or azurin for 10 d during the growth-promoting phase of MAL development. Glands were stained after 24 d of culture with alum carmine. a, control; b, azurin (50 μg/mL); c, p28 (12.5 μg/mL); d, p28 (25 μg/mL); e, p28 (50 μg/mL); f, p28 (100 μg/mL). A dose-related reduction in the number of MAL is observed in glands exposed to p28. B, effect of p28 on MDL in MMOC. Occluded mammary ducts (a) and normal histology of mammary ducts (b) in DMBA-treated glands exposed to p28.

nor p28 significantly altered p53 transcription in mammary cells cultured in IPEPg (Fig. 3B, right). However, nuclear staining for p53 was significantly greater following exposure to either azurin or p28 under both culture conditions (Fig. 3C). These results suggest that estradiol and progesterone inhibit the transcription of p53 in mouse mammary cells (28) but may not alter the stabilizing effect of either azurin or p28 on the mature protein (10, 11).

Estrogen receptor α (ERα) reportedly binds to the COOH terminus of the p53 tumor suppressor protein directly and represses its function (28), whereas p53 transcriptionally upregulates ERα (29), suggesting that p53 and ERα are reciprocally regulated at least in human breast cancer cells. In mice, the absence of p53 function abrogates the protective effect of hormones against chemical carcinogen–induced mammary cancer (30, 31).

The antiestrogen tamoxifen exerts a chemopreventive effect in the MMOC model (27), suggesting that the expression of p53 should be reduced in mouse mammary cells cultured in the presence of estradiol and progesterone and increased when exposed to tamoxifen. We tested the
hypothesis that any increase in p53 should reduce the overall incidence of preneoplastic lesions (e.g., MAL and MDL) and enhance the activity of an antiestrogen by exposing mammary cells cultured in IPEPg to p28 and tamoxifen alone or in combination in the absence of DBMA; then, we measured the changes in the levels of Ki-67 staining, p53 transcription, and p53. This would suggest whether tamoxifen inhibited MDL development solely through its antiestrogenic properties or if it also inhibited the estrogen-mediated suppression of p53 transcription. A decrease (P < 0.001) in Ki-67 staining in mammary epithelial cells was observed in the presence of both tamoxifen and p28 at 25 μmol/L compared with either agent alone (Fig. 4A). p53 transcription in mammary glands cultured in the presence of IPEPg is shown in Fig. 4B. Mammary glands cultured in IPEPg alone did not express a significant amount of p53. However, p53 transcription in mammary glands exposed to either p28 or tamoxifen alone increased in a dose-related manner relative to untreated glands. Tamoxifen at the highest dose tested (10 μmol/L) induced an ~7-fold increase over control (Fig. 4B), whereas p28 (12.5 μg/mL, 4.3 μmol/L, and 50 μg/mL, 17.2 μmol/L) increased transcription by ~2- to 5-fold, respectively. Interestingly, a combination of p28 (12.5 μg/mL, ~4.3 μmol/L, and 50 μg/mL, ~17 μmol/L) and tamoxifen (1 μmol/L) induced an ~25-fold increase in p53 expression relative to untreated mammary cells. A 4-fold increase in the concentration of p28 (50 μg/mL, 17 μmol/L) did not increase the transcription of p53, whereas an increase in the concentration of tamoxifen to 10 μmol/L in combination with 12.5 μg/mL p28 induced a 125-fold increase in transcription. The results suggest that p28 increases the level of p53 in estrogen- and progesterone-treated mouse mammary glands, which is significantly enhanced when estrogen levels are minimal or estrogen function is sufficiently blocked (Fig. 3A, right). The increase in transcription was reflected in the increased level (P < 0.001) of mature p53 protein in the nucleus of mammary cells exposed to p28 and tamoxifen (Fig. 4C).

Discussion

The drugs currently in use for adjuvant chemotherapy generally have significant side effects, which makes them unsuitable as chemopreventive agents against the initiation of breast cancer in normal healthy but high-risk postmenopausal individuals and limits their overall dose in an adjuvant setting to prevent recurrence. This has generated considerable interest in the identification of novel nontoxic chemopreventive agents (17). The current preclinical focus on chemopreventive agents is on small molecules, including nonsteroidal anti-inflammatory drugs and natural products and their derivatives including stilbenoids and flavans (32, 33) and fat-soluble vitamin A derivatives (retinoids), but has not included proteins or peptides. Azurin and aa 50–77 (p28) of azurin inhibited the development of hormone-dependent (MDL) and hormone-independent (MAL) precancerous lesions in MMOC. Although azurin inhibited the development of MAL as well as MDL, p28 seemed to be responsible for most, if not all, of the cytostatic and cytotoxic properties of its 128-aa parent protein (11). p28 inhibition of MAL and MDL development was associated with a decrease in cellular proliferation evident from the reduction in the expression of Ki-67. Although p28 inhibited the development of MAL to a similar extent as did fenretinide, a known inhibitor of this system (21), p28 activity did not seem to be dependent on either an estrogen- or a progesterone-mediated pathway. This is not the case for the antiestrogens tamoxifen and raloxifene, currently the most widely prescribed chemopreventive agents, which inhibit MDL, but not MAL development (27). Retinoids signal through multiple pathways, generally through cellular retinoic acid–binding protein sites. One such pathway is through activation of p53 independent of effects on p53 stability or DNA binding that may be through a general mechanism that contributes to G1 arrest (34). Although fenretinide does not bind to cellular retinoic acid–binding protein sites (35), it increases p53 and p21 protein expression in immortalized breast cancer cells (36). This suggests that p28 and fenretinide may act through possibly independent p53-mediated mechanism(s) that were maximally activated at the doses used, obviating any additive or synergistic activity on MAL development.
Fig. 3. Effects of azurin and p28 on cell proliferation and p53 expression in MMOC. A, effects of azurin and p28 on the expression of Ki-67 in mouse mammary glands incubated with IPAF (right) or IPEPg (left). a and d, media control; b and e, 50 μg/mL p28; c and f, 50 μg/mL azurin for 10 d. A decrease in Ki-67 is observed in glands exposed to p28 and azurin. Columns, mean; bars, SE. *, significant difference. B, effect of azurin and p28 on p53 transcription in mammary glands. p53 expression was normalized to control (DMBA alone). Columns, mean of three independent observations; bars, SE. C, effect of azurin and p28 on p53 levels in mouse mammary glands cultured in either IPAF (right) or IPEPg (left) in the presence of azurin or p28 for 10 d. a and d, media control; b and e, 50 μg/mL p28; c and f, 50 μg/mL azurin. An increase in nuclear p53 staining is observed in glands exposed to azurin and p28. Columns, mean; bars, SE. **, significant difference from respective control.
Fig. 4. Effects of p28 and tamoxifen on mammmary cell proliferation and p53 expression. A, effect of p28 and tamoxifen on mammmary cell proliferation. Paraffin-embedded sections of mouse mammary glands cultured in IPEPg for 4 d in the absence of DMBA. Groups of glands were treated with or without tamoxifen (1 μmol/L) in the presence of p28. Immunohistochemical staining of Ki-67 was done after antigen retrieval. Left, no tamoxifen; right, 1 μmol/L tamoxifen. a and d, control; b and e, 12.5 μg/mL p28; c and f, 25 μg/mL p28. A decrease in Ki-67 staining in mammmary epithelial cells was observed in the presence of tamoxifen and p28 (e and f). Columns, mean; bars, SE. *, significant difference from control. B, effect of p28 and tamoxifen on p53 transcription in MMOC. Mammary glands were treated with p28 alone (12.5 and 50 μg/mL), tamoxifen (0.1, 1, or 10 μmol/L) alone, a combination of p28 (12.5 or 50 μg/mL) and 1 μmol/L tamoxifen, or a combination of p28 (50 μg/mL) and 10 μmol/L tamoxifen. Columns, mean of three replicates; bars, SE. C, effect of p28 and tamoxifen on the level of p53 in mouse mammary gland. MMOC cultured in IPEPg for 4 d in the absence of DMBA. Groups of glands treated with or without tamoxifen (1 μmol/L) in the presence of p28. Left, no tamoxifen; right, 1 μmol/L tamoxifen, a and d, control; b and e, 12.5 μg/mL p28; c and f, 25 μg/mL p28. An increase in nuclear staining for p53 was observed in the presence of tamoxifen and p28 as compared with either agent alone (columns). Columns, mean of at least three determinations; bars, SE. *, significant difference.
The critical role that a loss of p53 function plays in DMBA-induced experimental mammary tumors (30, 37) suggests that molecules that stabilize wild-type or mutant p53 or induce p53 could significantly influence neoplastic development. Azurin and p28 exert their antiproliferative activity by stabilizing p53 expression (5, 11). We show here that p28 also induces the transcription of p53 in mouse mammary gland in the absence of estrogen and progesterone and increases the accumulation of nuclear p53 in mammary cells irrespective of the hormonal milieu. The increase is maximal in the absence of estrogen and progesterone, however. These results suggest that estrogen and progesterone may inhibit the transcription of p53 in mouse mammary gland in vitro. The clear difference in the level of expression of p53 between mammary glands exposed to estrogen and progesterone (IPAF) and those that are not (IPEPg) and the increased nuclear accumulation of p53 following exposure to p28 suggest that stabilization of p53 may be responsible for the reduction in MDL development in these glands.

Tamoxifen, an ER antagonist, has been successfully used in the treatment of ER-positive breast cancer patients and

| Table 1. Effect of p28 and fenretinide on DMBA-induced mammary alveolar lesions in MMOC |
|---------------------------------|-----------------|---------------------|---------------------|---------------------|
| Experiment no. | Treatment | No. glands per group | No. glands with lesions | Incidence (%) | Percent inhibition |
|----------------|-----------|----------------------|------------------------|--------------|--------------------|
| 1              | None      | 15                   | 9                      | 60.0         |                   |
| 2              | 4-HPR (1 μmol/L) | 15                   | 3                      | 20.0         | 67                |
| 3              | p28 (12.5 μg/mL) | 15                   | 4                      | 26.7         | 56*               |
| 4              | p28 (12.5 μg/mL) + 4-HPR (1 μmol/L) | 15                   | 3                      | 20.0         | 67                |
| 5              | p28 (25 μg/mL) | 15                   | 3                      | 20.0         | 67                |
| 6              | p28 (25 μg/mL) + 4-HPR (1 μmol/L) | 15                   | 2                      | 13.3         | 78                |
| 7              | None      | 15                   | 10                     | 66.7         |                   |
| 8              | p28 (50 μg/mL) | 15                   | 3                      | 20           | 70                |
| 9              | p28 (50 μg/mL) + 4-HPR (1 μmol/L) | 15                   | 2                      | 13.3         | 80                |
| 10             | p28 (100 μg/mL) | 15                   | 2                      | 13.3         | 80                |
| 11             | p28 (100 μg/mL) + 4-HPR (1 μmol/L) | 15                   | 3                      | 20           | 70                |
| 12             | Azurin (10 μg/mL) | 15                   | 8                      | 53.3         | 15                |
| 13             | Azurin (20 μg/mL) | 15                   | 7                      | 46.6         | 26                |
| 14             | Azurin (50 μg/mL) | 15                   | 3                      | 20           | 68*               |

Abbreviation: 4-HPR, 4-hydroxyphenyl retinamide (fenretinide).
*Experiment 1 versus experiment 3: not significant (P = 0.1394); all others versus experiment 1: P < 0.05 (Fisher’s exact test).

| Table 2. Effect of p28 and tamoxifen on DMBA-induced MDL in MMOC |
|---------------------------------|---------------------|---------------------|---------------------|---------------------|
| Experiment no. | Treatment | No. fields examined | No. fields with ductal lesions | Incidence (%) | Percent inhibition |
|----------------|-----------|----------------------|------------------------|--------------|--------------------|
| 1              | DMBA      | 84                   | 69                     | 82.1         |                   |
| 2              | Tamoxifen (1 μmol/L) | 71                   | 30                     | 41.3         | 49                |
| 3              | p28 (12.5 μg/mL) | 79                   | 44                     | 55.7         | 32                |
| 4              | p28 (12.5 μg/mL) + tamoxifen (1 μmol/L) | 88                   | 21                     | 23.9         | 71                |
| 5              | p28 (25 μg/mL) | 73                   | 18                     | 24.7         | 70                |
| 6              | p28 (25 μg/mL) + tamoxifen (1 μmol/L) | 68                   | 12                     | 17.6         | 79                |
| 7              | DMBA      | 87                   | 62                     | 71.3         |                   |
| 8              | p28 (50 μg/mL) | 63                   | 15                     | 23.8         | 67                |
| 9              | p28 (50 μg/mL) + tamoxifen (1 μmol/L) | 76                   | 20                     | 20.0         | 72                |
| 10             | p28 (100 μg/mL) | 82                   | 12                     | 14.6         | 80                |
| 11             | p28 (100 μg/mL) + tamoxifen (1 μmol/L) | 73                   | 14                     | 19.2         | 73                |
| 12             | Azurin (50 μg/mL) | 61                   | 8                      | 13.1         | 79                |

NOTE: Fisher’s exact test: P < 0.0001, DMBA alone versus tamoxifen (1 μmol/L); P < 0.02, tamoxifen (1 μmol/L) or p28 (12.5 μg/mL) versus p28 (12.5 μg/mL) + tamoxifen (1 μmol/L).
is approved for use in breast cancer prevention trials (38, 39). We previously reported that tamoxifen inhibits the development of MDL (27). Because ERα reportedly binds directly to p53 and represses its function (28), it suggests that tamoxifen treatment should indirectly reduce ER binding to p53 by blocking the binding of estrogen to its receptor, potentially increasing p53 levels (Fig. 3B) and decreasing mammary cell proliferation. Exposure of mammary glands to p28 in the absence of estrogen and progesterone (IPAF) increases the transcription of p53 (Fig. 3B), suggesting that the increase in p53 transcription in the presence of p28 may be inhibited by estrogen. As p28 binds to the DNA binding region of p53 (11) and ERα to the COOH-terminal regulatory domain of p53, it is not likely that estrogen competes directly with p28 for binding to p53, but rather inhibits p53 transcription through a non-competitive effect that does not alter the p28-induced increase in the level of p53 (ref. 11; Fig. 4C). The significant dose-related increase in p53 transcription observed in the presence of p28 and tamoxifen (Fig. 4B) suggests that the apparent synergistic effect between p28 and tamoxifen (Fig. 2B) may arise from their ability to increase p53 through direct binding at a site that decreases proteolysis (11) and indirectly by blocking the inhibitory activity of estrogen on p53. Although p28 and azurin inhibited MAL and MDL development and exhibited enhanced nuclear expression of the p53 protein, only p28 induced p53 transcription. This difference may reside in the relative penetration of azurin and p28 into preneoplastic as well as malignant mammary epithelial cells (11, 40) because both bind with high affinity to p53 (1, 9–11).

In conclusion, this is the first report showing the chemopreventive effects of a redox protein, azurin, and its 28-aa peptide fragment, p28, against mammary gland transformation through a p53-mediated mechanism.

Disclosure of Potential Conflicts of Interest

All terms of the Sponsored Research Arrangement are managed by the University of Illinois at Chicago in accordance with its conflict of interest management policies. CDG Therapeutics, Inc., has an exclusive licensing agreement for the development and commercialization of cupredoxin-derived peptides. C.W. Beattie is Chief Scientific Officer, CDG Therapeutics, Inc. T.K. Das Gupta is a founder of CDG Therapeutics, Inc., and a shareholder in it.

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