Ataxia Telangiectasia-Mutated and p53 Are Potential Mediators of Chloroquine-Induced Resistance to Mammary Carcinogenesis

Christian R. Loehberg,1,3 Tiia Thompson,1 Michael B. Kastan,2 Kirsteen H. Maclean,2 Dean G. Edwards,1 Frances S. Kittrell,1 Daniel Medina,1 Orla M. Conneely,1 and Bert W. O’Malley1

1Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas; 2Department of Oncology, St. Jude Children’s Research Hospital, Memphis, Tennessee; and 3Department of Obstetrics and Gynecology, University of Erlangen, Erlangen, Germany

Abstract

The use of agents to prevent the onset of and/or the progression to breast cancer has the potential to lower breast cancer risk. We have previously shown that the tumor-suppressor gene p53 is a potential mediator of hormone (estrogen/progesterone)-induced protection against chemical carcinogen–induced mammary carcinogenesis in animal models. Here, we show for the first time a breast cancer–protective effect of chloroquine in an animal model. Chloroquine significantly reduced the incidence of N-methyl-N-nitrosourea–induced mammary tumors in our animal model similar to estrogen/progesterone treatment. No protection was seen in our BALB/c p53-null mammary epithelium model, indicating a p53 dependency for the chloroquine effect. Using a human nontumorigenic mammary gland epithelial cell line, MCF10A, we confirm that in the absence of detectable DNA damage, chloroquine activates the tumor-suppressor p53 and the p53 downstream target gene p21, resulting in G1 cell cycle arrest. p53 activation occurs at a posttranslational level via chloroquine-dependent phosphorylation of the checkpoint protein kinase, ataxia telangiectasia-mutated (ATM), leading to ATM-dependent phosphorylation of p53. In primary mammary gland epithelial cells isolated from p53-null mice, chloroquine does not induce G1 cell cycle arrest compared with cells isolated from wild-type mice, also indicating a p53 dependency. Our results indicate that a short prior exposure to chloroquine may have a preventative application for mammary carcinogenesis.

[Int. Cancer Res. 2007;67(24):12026–12033]

Introduction

Breast cancer remains the major cancer among women in the United States with >200,000 new cases annually (1). Breast cancer incidence rates are influenced by age, genetics, radiation, socioeconomic status, diet, ethnicity, and reproductive history. However, the strongest protective factor is related to reproductive history, that is, early age at first pregnancy (≤20 years of age), which confers a 50% reduction in lifetime risk compared with the lifetime risk of breast cancer in nulliparous women (2, 3).

The protective effect of early pregnancy against chemical carcinogen–induced mammary carcinogenesis has been shown in both rat and mouse models (4–6). The effect of pregnancy on the protection against carcinogen-induced mammary cancer is mimicked by treatment with estrogen and progesterone (4, 5, 7). However, the molecular mechanisms that underlie the protective effect are poorly understood. In studies using the Wistar-Furth rat and BALB/c mouse models, we showed that hormone-induced protection is manifested at the cellular level as a block in carcinogen-induced proliferation shortly after carcinogen treatment (4, 5, 7). The availability of the BALB/c p53-null mammary epithelium model provided a means to test the functional role of p53 in hormone-induced protection (8–11). We showed that the absence of p53 abrogates the protective effect of hormones against chemical carcinogen–induced mammary cancer (10–12). These results suggested the general hypothesis that p53 is up-regulated in a persistent fashion by hormonal stimulation of the virgin gland and that p53 functions in part to retard cell cycle progression in response to carcinogen-induced DNA damage.

p53 can be activated by several phosphatidylinositol 3-kinase (PI3K)–related kinases (PIKK), including ataxia telangiectasia-mutated (ATM), ATM- and Rad3-related (ATR); and the ATM downstream effector checkpoint kinases Chk1 or Chk2 that phosphorylate the NH2-terminal transactivation domain of p53 at multiple sites (e.g., serines 6, 9, 15, 20, 37, and 46; refs. 13–17). Recent studies showed that not only DNA damage but also chromatin modifications are sufficient to activate the ATM protein kinase, which, in turn, is able to activate p53 (18). Chromatin modifiers such as the anti-malarial agent chloroquine activate ATM via autophosphorylation at serine 1981, which results in the phosphorylation of p53 at serine 15 in fibroblasts (18, 19). Furthermore, members of the quinoline sulfonamide family, like chloroquine, induce cell cycle arrest, apoptosis, and differentiation in breast cancer cell lines (20–23).

Importantly, it was recently shown that chloroquine not only reduces proliferation in cellular models, but impairs spontaneous lymphoma development in a mouse model (24). Here, we further show a cancer protective effect of chloroquine and show that a short prior exposure to chloroquine is preventative against N-methyl-N-nitrosourea (NMU)–induced mammary carcinogenesis in our rat model similar to estrogen/progesterone treatment. No protection was seen in our BALB/c p53-null mammary epithelium model, suggesting a p53 dependency of the chloroquine effect. We show that in a human nontumorigenic mammary gland epithelial cell line, MCF10A, chloroquine up-regulates the tumor-suppressor p53, its downstream target gene p21 leading to G1 cell cycle arrest. These findings indicate that the tumor protective effect of chloroquine may be mediated in part by a p53 pathway.

Materials and Methods

Cell Culture

Culture conditions for cell lines used in this study have been described previously (25). MCF10A is a human breast epithelial cell line that arose by
spontaneous immortalization from cell cultures established from the s.c. mastectomy of a woman with fibrocystic disease (25). Briefly, MCF10A cells were maintained in DMEM/F-12 medium supplemented with 5% horse serum, 0.02 μg/mL epidermal growth factor (EGF), 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 0.1 μg/mL cholera toxin, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Cells were treated with 10 or 50 μmol/L chloroquine (Sigma) for 4, 24, 48, or 72 h. For Western blot analyses, cells were pretreated with medium containing reduced horse serum (0.1%) for 24 h. As positive controls for several antibodies, cells were either γ-irradiated with 10 Gy or treated with 10 μmol/L trichostatin A (Sigma).

The primary mammary gland epithelial cells were derived from 8-week-old p53-null or p53-WT BALB/c mice 9. The left and right thoracic and inguinal mammary glands were aseptically removed, pooled, and the epithelial cells were isolated according to ref. 9. The cells were plated on collagen-coated flasks and grown in DMEM/F-12 medium supplemented with 5% FCS, 5 ng/mL EGF, 1 mg/mL bovine serum albumin, 10 μg/mL insulin, 50 μg/mL gentamicin, and 20 units/mL nystatin.

**Short Interfering RNA Transfection**

Validated small interfering RNAs (siRNA) for p53 and ATM were purchased from Dharmaco (siGENOME SMART pool reagent) and transiently transfected into MCF10A cells using TransIT siQuest from Mirus according to the manufacturer's protocol. At 24 h posttransfection, MCF10A cells were treated with chloroquine, as described above and harvested 24 h later for protein and flow cytometry analyses.

**Western Blot Analysis**

Nuclear and cytoplasmic extracts were prepared with NEPER lysis buffer kit (Pierce). For Western blotting, the lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane according to standard protocols. The blots were stained with the following primary antibodies: p53 (FL-393), p53 (DO-1), Chk1 (G-4), Chk2 (A-11) from Santa Cruz Biotechnology; phospho–p53-S6,9,15,20,37,46, phospho–Chk1-S345, phospho–Chk2-p53-K373/382, phospho–histone-H2AX-S139 (JBW301), ATM from Upstate; phospho–p53-S36,S46,phospho–ATM-S1981 antibody was kindly provided by Dr. M.B. Kastan (18). Protein bands were visualized with anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase (Santa Cruz) and transferred with a scanner densitometer (Molecular Dynamics). The phosphorylation status of each protein band was confirmed by an immunoblot with an appropriate loading control (from Sigma). The membranes were scanned and densities were quantified with a scanner densitometer (Molecular Dynamics).

**Flow Cytometry**

MCF10A cells were trypsinized into single-cell suspension, fixed in ice-cold 70% ethanold, and stored at 4 °C. Before analysis, cells were resuspended in 100 μg/mL RNase A (Promega), 40 μg/mL propidium iodide in PBS. To determine the percentage of apoptotic and necrotic cells, trypsinized cells were collected and stained with Annexin V FITC and 7- aminoactinomycin D (7-AAD), according to standard protocols. Analysis was done on a Becton Dickinson fluorescence activated cell sorter (FACScan).

Differences of MCF10A cells after chloroquine treatment were examined by Student's t test with P < 0.05 considered as statistically significant.

**Animals**

**Rats.** Female Wistar-Furth rats were purchased from National Cancer Institute. The Wistar-Furth female rat is an inbred strain of rats sensitive to both NMU- and 7,12-dimethylbenz[a]anthracene–induced mammary adenocarcinomas. Rats were acclimatized to our animal facility before experimental manipulation.

**Mice.** All donor and recipient BALB/c mice were bred and maintained at Baylor College of Medicine. The donor mice were p53 homozygous null, and the recipient mice were p53 wild-type (WT; ref. 8).

All animals were housed by using approved American Association of Laboratory Animal Care guidelines under conditions of a 12-h light/dark cycle, and permitted ad libitum access to food and water. All experiments were performed in accordance with NIH guidelines for experimental animals.

**Tumorigenesis Studies in Chloroquine-Treated Wistar-Furth Rats and BALB/c Mice**

**Wistar-Furth rats.** For the studies on chloroquine-induced resistance to the carcinogen NMU (Sigma), 48-day-old Wistar-Furth virgin female rats, were given three i.p. injections of chloroquine, 3.5 mg/kg of body weight, 1 week apart. At 63 days old, 1 day after the final chloroquine-injection (at day 62), Wistar-Furth Rats were given an i.p. injection of NMU, 50 mg/kg of body weight. An age-matched control group of rats also received NMU injections. All mammary tumors appearing in the ensuing 35 weeks were collected, and processed for histology and pathologic evaluation. Tumors were evaluated by routine H&E staining. The vast majority of the mammary tumors of both groups were adenocarcinomas of the solid or papillary variety. Tumor incidences and latencies were statistically evaluated and differences between groups were tested for statistical significance by using the two-sided Student's t test. Results were considered significantly different at P < 0.05.

**BALB/c mice.** The mammary ducts were isolated from 7- to 8-week-old p53-null BALB/c mice and transplanted into the cleared mammary fat pads of 3-week-old WT BALB/c mice. The transplanted duct samples grew and filled the fat pad in 6 to 8 weeks. For each group, two mammary fat pads were processed as whole mounts at 8 weeks to examine the growth rate and morphology of the outgrowth. Those mice were treated weekly for 8 weeks with chloroquine (3.5 mg/kg of body weight) starting at 8 weeks after transplantation so the mice were 11 to 12 weeks old when the treatment started. The mice (n = 20 per group) were palpated weekly for mammary tumors for 60 weeks posttransplantation. Tumors and nontumoral tissue were evaluated by routine H&E staining. Tumor incidences and latencies were statistically evaluated, and differences between groups were tested for statistical significance by using the Fisher's exact test. Results were considered significantly different at P < 0.05.

**Results**

Chloroquine shows breast cancer protective effects in animal models in a p53-dependent manner. To investigate whether chloroquine has an effect in mammary gland tissue in vivo, we designed an experiment in rats based on our earlier work showing that estrogen/progesterone treatment is protective against NMU-induced mammary tumors in female Wistar-Furth rats (7). Rats were treated thrice (once a week) over a period of three weeks with chloroquine (3.5 mg/kg of body weight) before treatment with NMU (50 mg/kg of body weight) to induce mammary gland adenocarcinomas (Fig. 1A). In the untreated rats, mammary tumor incidence was 67% (16 of 24) with the first tumor onset at 8 weeks after NMU. In comparison, rats that received chloroquine treatment exhibited a reduced tumor incidence of only 41% (10 of 24; P < 0.05) with a delayed tumor onset at 12 weeks (P < 0.05) post-NMU (Fig. 1A). In addition, the growth rate of tumors was significantly slower in the chloroquine-treated rats compared with the untreated controls (data not shown).

A mouse model that mimics essential biological and genetic attributes of a subset of human breast cancer is the BALB/c p53-null mammary epithelium, in which deletion of the tumor-suppressor gene p53 results in enhanced tumorigenic risk (8–11). The p53-null mammary epithelium progresses through ductal hyperplasia and ductal carcinoma in situ before becoming invasive breast cancer (8–11). Because the p53-null mammary epithelial cell mimics the expression patterns found in subsets of human invasive breast cancers, we investigated the possible p53-dependency of the chloroquine-induced breast cancer protective effects in this model. Using this model, we have previously shown that the p53 null...
mammary epithelium develops spontaneous breast cancer when transplanted into WT BALB/c mice, and showed that the absence of p53 tumor-suppressor gene function abrogates the protective effect of estrogen/progesterone against carcinogen-induced tumorigenesis in those BALB/c mice (10–12).

Accordingly, the mammary ducts were isolated from p53-null BALB/c mice and transplanted into WT BALB/c mice. The mice were treated weekly for 8 weeks with chloroquine (3.5 mg/kg of body weight) starting at 8 weeks after transplantation (at 11–12 weeks of age). No protection was seen in our BALB/c p53-null mammary epithelium model, suggesting a p53 dependency (Fig. 1B). Importantly, this is the first indication that chloroquine can prevent breast cancer in an animal model similar to estrogen/progesterone treatment and shows a p53 dependency supporting the cellular results.

**Chloroquine inhibits MCF10A cell proliferation via G1 cell cycle arrest.** To address the molecular mechanisms by which chloroquine prevents mammary tumorigenesis in vivo, we performed flow cytometry to determine whether chloroquine affects the cell cycle in MCF10A cells at concentrations that do not induce significant cytotoxicity. The DNA content of propidium iodide–stained nuclei from untreated and chloroquine-treated cells were analyzed as shown in Fig. 2A. At 24 h incubation, the percentage of cells in the G1 phase was 52% in the control, 64% with 10 μmol/L, and 76% with 50 μmol/L chloroquine treatment (P < 0.05; similar numbers occurred at 48 and 72 h, data not shown). To determine whether chloroquine induces apoptosis in addition to G1 cell cycle arrest, we stained the MCF10A cells with Annexin V–FITC (apoptosis marker) and 7-AAD (necrosis marker). As shown in Fig. 2B, in the untreated MCF10A cell population only 5% of the cells undergo apoptosis, and after treatment with 10 μmol/L chloroquine no significant change in apoptosis is detectable. Although the 50 μmol/L chloroquine treatment results in an increase in apoptotic cells, the level of apoptosis (10% of cells) is still very low, indicating that chloroquine does not cause significant cytotoxicity.

In summary, chloroquine induces cell cycle arrest in MCF10A cells at a low 10 μmol/L concentration that does not induce cytotoxicity.

**Chloroquine up-regulates p53 and its downstream target gene p21.** Because loss of p53 abrogates the tumor protective effect of chloroquine, we next examined whether chloroquine also induces up-regulation of p53 in the MCF10A cells as shown previously in fibroblasts (18) and MCF7 breast cancer cells (22). Indeed, p53 protein levels were increased in nuclear extracts of chloroquine-treated MCF10A cells at 4 h (1.5-fold at 10 μmol/L, and 2.7-fold at 50 μmol/L) and 24 h (3-fold at 10 and 50 μmol/L; Fig. 2C). Increase in nuclear p53 protein level after chloroquine treatment was confirmed by immunocytochemistry (data not shown). Increases in p53 protein levels generally reflect regulation at the posttranscriptional rather than at the transcriptional level (26–28). In agreement with this, we did not detect any increase in p53 transcripts after chloroquine treatment, measured by quantitative PCR from control and chloroquine-treated MCF10A cells (data not shown), suggesting that the increase in p53 protein levels reflects regulation at the posttranscriptional level.

To determine whether chloroquine-induced cell cycle arrest is associated with up-regulation of the p53-dependent cell cycle inhibitor p21, we examined the levels of the p21 protein after chloroquine treatment. As shown in Fig. 2C, significant up-regulation (3-fold at 10 μmol/L and 6-fold at 50 μmol/L) of p21 (Waf1/Cip1) was detected by Western blot analysis in our model. In addition to p21, p27 (Kip1), a member of the Cip/Kip family of cyclin-dependent kinase (cdk) inhibitors (29), was up-regulated (up to 4.7-fold at 10 μmol/L) after 24 h chloroquine treatment (Fig. 2C). Both p21 and p27 play a significant role in cell cycle arrest via enforcing G1 restriction point by inhibitory binding to cdk2/cyclin E or other cdk/cyclin complexes (29). We also examined the level of expression of bax, a proapoptotic mediator of p53-dependent apoptosis (30); no significant up-regulation was detected for bax (Fig. 2C).

Mdm2, an E3 ubiquitin ligase for p53, plays a central role through a p53-Mdm2 autoregulatory loop to regulate the stability of p53 levels, and both genes are targeted by Mdm2 (31). In these experiments, we examined the Mdm2 expression in chloroquine-treated MCF10A cells. We did not detect any increase in Mdm2 transcripts after chloroquine treatment, measured by quantitative PCR from control and chloroquine-treated MCF10A cells (data not shown), suggesting that the increase in Mdm2 protein levels reflects regulation at the posttranscriptional level.
of p53 via ubiquitination and degradation (31). Due to increased p53 levels, total mdm2 protein levels were also increased (up to 6.6-fold at 10 μmol/L for 4 h) with chloroquine treatment (Fig. 2C), consistent with the role of mdm2 as a p53 target gene. Taken together, we show chloroquine-induced up-regulation of p53 and p21, the key players for G1 cell cycle arrest in the MCF10A cells similar to our estrogen/progesterone protection model.

p53 is required for the chloroquine-induced G1 cell cycle arrest of MCF10A cells. To determine if there is a functional requirement for p53 in the chloroquine-induced G1 cell cycle arrest, flow cytometry propidium iodide staining was performed in p53-siRNA-transfected MCF10A cells, and p53 expression was monitored by Western blotting. As shown in Fig. 3A, no increased G1 cell cycle arrest was detected after 10 μmol/L chloroquine treatment in cells transfected with p53-siRNA. Moreover, treatment with p53-siRNA abolished the chloroquine stimulation of the cell cycle regulator p21 (Fig. 3B).

To further confirm the functional requirement of p53, we isolated primary mammary gland epithelial cells from our WT and p53-null BALB/c mice. Flow cytometry profiles of nuclear DNA content revealed that chloroquine promotes G1 cell cycle arrest only in the p53-WT cells but not in p53-null cells neither at 10 μmol/L (Fig. 3C), nor at 50 μmol/L (data not shown). Thus, p53 is required for the chloroquine-induced G1 cell cycle arrest in nontumorigenic mammary epithelial cells.

Chloroquine causes p53 phosphorylation. The level of p53 activity is regulated by a variety of posttranslational modifications, including phosphorylation and acetylation that are activated by distinct stimuli (13–15, 32–34). To determine whether exposure of mammary epithelial cells to chloroquine results in covalent modification of p53 in the MCF10A cells, we analyzed the phosphorylation and acetylation status of p53 in multiple sites (e.g., serines 6, 9, 15, 20, 37, and 46; refs. 13–15). Our analysis of the phosphorylation status of these residues using phosphorylation site–specific antibodies showed that chloroquine treatment of MCF10A cells increased p53 phosphorylation only at serine 15 (8.2-fold at 10 μmol/L, and 11-fold at 50 μmol/L for 24 h), a crucial p53 phosphorylation site that can be phosphorylated by ATM and that plays an important role in p53 functional activity (refs. 35, 36; Fig. 4). The time course of chloroquine-induced p53 phosphorylation at serine 15 correlated with the chloroquine stimulation of p21 production (Fig. 2C). In contrast, phosphorylation of serine 46, which has been shown to be linked to the transactivation of apoptotic target genes (37), was not increased with chloroquine treatment. This result is consistent with our flow cytometry data showing that chloroquine induces only slight apoptosis at high amounts (Fig. 2B).

The COOH terminus of p53 also plays a key role in the regulation of p53 function; it is rich in lysines, which are subjected to acetylation, ubiquitination, and sumoylation (32–34). Phosphorylation of serine 20 and 46 strengthens the association with the acetyltransferases p300 and CBP, leading to acetylation at lysines 373 and 382 in the COOH-terminal regulatory domain (13). This increases the DNA-binding activity of p53 and modulates its interaction with transcription factors (32–34). Consistent with our data showing no significant phosphorylation at serine 20 or 46.
we did not detect any increase in acetylated p53 after chloroquine treatment (Fig. 4). These results suggest a serine 15 selective modulation of p53 occurs following chloroquine.

**Chloroquine phosphorylates p53 through the ATM signaling pathway.** p53 phosphorylation at serine 15 is mainly transduced through the PIKKs ATM, ATR, and the DNA-activated protein kinase (DNA-PK; ref. 13), which predicts that inhibitors of the PI3K family of enzymes should suppress chloroquine-induced phosphorylation of p53. To test this concept, MCF10A cells were treated with chloroquine in the presence or absence of wortmannin, an inhibitor of PIKK ATM and DNA-PK (38). Wortmannin completely blocked ATM-mediated serine 15 phosphorylation of p53 in our model (data not shown), suggesting a PIKK requirement for serine 15 phosphorylation. Because previous studies have indicated that chloroquine can activate ATM by phosphorylation at serine 1981 in fibroblast cells (18, 19), we next asked whether specific inhibition of ATM using RNA interference abrogates p53 phosphorylation of serine 15. As shown in Fig. 5A, treatment with ATM-siRNA completely abolished the chloroquine induction of p53 protein phosphorylation at serine 15.

To determine whether chloroquine treatment regulates ATM phosphorylation in MCF10A cells, we performed Western blots with a phosphospecific antibody to the serine 1981 of ATM. As shown in Fig. 5B, ATM was specifically phosphorylated in chloroquine-treated nuclear extracts (6-fold at 10 μmol/L and up to 21-fold at 50 μmol/L for 4 h), under conditions in which p53 protein is phosphorylated at serine 15. Interestingly, ATM downstream effectors Chk1 (16) and Chk2 (17) only showed increased phosphorylation at the later time point in response to chloroquine (up to 5-fold at 10 and 50 μmol/L at 24 h; Fig. 5B), and ATM-siRNA still abolished chloroquine-induced phosphorylation of p53 at serine 15 at this later time point (24 h; Fig. 5A).

The ATM protein kinase may be activated by DNA damage or by chromatin modifications such as acetylation (18, 19). Therefore, we determined whether chloroquine activation of the ATM signaling pathway was due to formation of DNA double-strand breaks. Western blotting of isolated histone extracts showed that in MCF10A cells, chloroquine did not induce phosphorylation of H2AX at serine 139, a well-known DNA breakage marker (39), compared with irradiation, suggesting that chloroquine does not activate the ATM-mediated phosphorylation of p53 through induction of DNA double-strand breaks in MCF10A cells (Fig. 5C). Additionally, we did not detect any histone hyperacetylation after chloroquine treatment (Fig. 5C). Taken together, our data indicate that chloroquine can be used in nontumorigenic mammary epithelial cells, similar to fibroblasts (18), to activate the tumor-suppressor p53 via the ATM kinase pathway without detectable DNA damage.

**Figure 3.** p53 is required for the chloroquine-induced up-regulation of the cell cycle inhibitor p21 (Waf1/Cip1) and for G1 cell cycle arrest. A, MCF10A cells were transiently transfected with either control-siRNA or p53-siRNA, were treated with chloroquine (24 h), and flow cytometric profiles were plotted (representative example of experiments done in triplicate). *, P < 0.05. B, those cells were harvested and nuclear extracts were subjected to SDS-PAGE and Western blotting with indicated antibodies or actin as loading control (representative example of experiments done in triplicate). C, isolated primary mammary gland epithelial cells were treated with chloroquine (24 h) and flow cytometric profiles were plotted (representative examples of experiments were done in triplicate). *, P < 0.05.
Discussion

We report here that the preventative effects of early pregnancy or exposure to estrogen/progesterone (4, 7) against mammary carcinogenesis can be recapitulated by short-term pretreatment of rats with the drug chloroquine. The first indication that chloroquine may be useful for later cancer prevention, in addition to treatment of malaria and rheumatoid diseases, came from a recent study showing that chronic chloroquine treatment impairs spontaneous lymphomagenesis in mouse models (24). We and others have previously shown that mammary tissue in young rodents is very susceptible to the protective effects of pregnancy or pregnancy levels of estrogen/progesterone (4, 5, 7). Similarly, women who have undergone a full-term pregnancy early in life are ~50% less likely to develop breast cancer compared with nulliparous women (2, 3). Therefore, we took the approach to administer chloroquine for only a 2-week period in young rats to examine whether chloroquine may be used in prevention of mammary carcinogenesis. The prevention of mammary carcinogenesis by chloroquine was significant, and was similarly efficient to what we observed previously with lower doses of estrogen/progesterone treatment (4, 5, 7). However, we only gave the rats three injections of chloroquine, 3.5 mg/kg of body weight, during the 2-week treatment regimen. Therefore, by optimizing the chloroquine dosage, we would expect an even greater response. Previous studies have indicated that although chloroquine does not show many side effects during long-term use for treatment of malaria or rheumatoid diseases, retinal problems are a potential side effect (40). Based on the ophthalmologic safety guidelines for long-term use of chloroquine in humans (40), chloroquine can be administered with the low dose of 3.5 mg/kg of body weight daily for several years. Furthermore, chloroquine shows two interesting pharmacologic characteristics, extensive tissue accumulation and prolonged retention in the tissues after drug administration has ceased (41). Chloroquine has been shown to be retained in the body for several weeks after a single i.v. dose similar to the conditions in our animal model (42) and showed impaired spontaneous lymphomagenesis in mouse models with this low dose (24).

Therefore, our result of chloroquine-induced protection against mammary carcinogenesis at such low levels is highly significant and promising.

We have previously shown that estrogen/progesterone–induced prevention of carcinogen-induced mammary carcinogenesis in rodents is associated with up-regulation of the tumor-suppressor p53. This results in p53-dependent up-regulation of p21, leading to cell cycle arrest and a mammary phenotype that is refractory to carcinogenesis (4, 5, 7). The essential role of p53 was shown by the abrogation of the refractory phenotype in mammary epithelium derived from p53-null mice (10–12). As seen with estrogen/progesterone treatment, where abrogation of p53 diminished the protective effects, we showed that in the absence of p53, chloroquine-induced protection against mammary carcinogenesis in vivo and cell cycle arrest in vitro is lost. These results clearly confirm an essential role for p53 in the prevention of mammary carcinogenesis. However, the lack of an appropriate nontumorigenic mammary epithelial cell culture system that responds to estrogen/progesterone has hampered disclosure of the molecular mechanisms underlying estrogen/progesterone–dependent activation of p53. Therefore, our observation that chloroquine can mimic the protective effects of estrogen/progesterone in animals and induce p53 activation in cultured mammary epithelial cells suggests that chloroquine may be used in culture systems to elucidate the molecular mechanisms underlying prevention.

The tumor-suppressor p53 is induced in response to a wide array of signals that stress the cell, and these changes involve stabilization of the protein and increases in its rate of translation. The precise mechanism by which chloroquine induces p53 is not yet resolved. However, our analysis of the chloroquine-induced posttranslational modifications associated with p53 activation revealed site-specific phosphorylation at serine 15, whereas other known NH$_2$-terminal phosphorylation sites and the COOH-terminal acetylation status remained unaltered. Phosphorylation of p53 at serine 15 has been shown to increase in response to some, but not all, activating signals (35, 36, 38), with some evidence suggesting that the ATM kinase plays a crucial role in the phosphorylation of this site. Our results indicate that in the MCF10A cells, chloroquine indeed activates ATM, another known tumor suppressor. Specifically, we observed that treatment of cells with either wortmannin (inhibitor of ATM and DNA-PK; ref. 38), or ATM-siRNA, disrupts the chloroquine-induced phosphorylation of p53 at serine 15, indicating that ATM is a principal kinase that activates p53 in these cells in response to chloroquine. These results are in agreement with previous studies done in fibroblasts (18). In the above-mentioned murine lymphoma model, chloroquine still impaired spontaneous lymphoma development in ATM-deficient, but not in p53-deficient mice (24). However, an essential role for ATM was shown in chloroquine-induced insulin sensitivity and cardiovascular effects; chloroquine treatment improved blood pressure and glycemic control in ATM-WT but not ATM-null mice (43). This suggests that in select cells, chloroquine-induced signaling pathways require ATM to activate downstream targets whereas in other systems, p53 (or other targets) may be activated via ATM-independent pathways.

Previous studies have shown that hormonal (estrogen/progesterone) prevention of carcinogen (NMU)–induced mammary carcinogenesis in rats is associated with p53-dependent up-regulation of the cdk inhibitor p21. This leads to increased cell cycle arrest in the mammary gland and, importantly, to a mammary phenotype that is refractory to carcinogenesis (4, 5, 7).

**Figure 4.** Phosphorylation of p53 serine 15 by chloroquine. Chloroquine causes p53 serine 15 phosphorylation. MCF10A cells were treated for the indicated time points with chloroquine. Cells were harvested and nuclear extracts were subjected to SDS-PAGE and Western blotting with p53 or the indicated phosphorylated and acetyl antibodies, and quantitated by scanning densitometry. Results are shown as fold induction after normalization to loading control (representative example of experiments done in triplicate).
Our analysis of the cellular and molecular effects of chloroquine in cultured nontumorigenic mammary epithelial cells supports the hypothesis that low doses of chloroquine act at least in part also by inducing G1 cell cycle arrest through a p53-dependent up-regulation of p21. Specifically, we showed that chloroquine-induced G1 cell cycle arrest in the human breast epithelial cells was dependent on functional p53 and was abrogated in p53-siRNA–treated MCF10A cells and isolated primary mammary gland epithelial cells from p53-null BALB/c mice coinciding with loss of p21. We hypothesize that one mechanism of chloroquine-induced protection could be through the activation of p53, so that upon DNA damage (as induced by chemical carcinogen), a memory response occurs so that p53 is functionally activated to cause G1 cell cycle arrest. Similar to our estrogen/progesterone treatment model, we hypothesize that this heightened response to inhibit carcinogen-induced proliferation can protect normal proliferating cells from the initiation of carcinogenesis but not the promotion of p53-null preneoplastic cells as shown in our animal models.

Together with the excellent pharmacologic characteristics like extensive tissue accumulation, chloroquine offers the opportunity to develop an improved protective strategy for protection of normal proliferating tissues without affecting the sensitivity of tumors with mutant p53, for example chemotherapeutic antimitotic agents. Thus, chloroquine may offer a new modality for partial protection of normal proliferating tissues during antimitotic chemotherapy of p53-deficient tumors.

Currently, there are very few agents that have a proven ability to prevent breast cancer and their mechanism of action remains undetermined. We reasoned that possible preventative agents would include those that augment p53-dependent pathways. We focused our chemoprevention studies on the antimalarial drug chloroquine, which can activate the tumor-suppressors ATM and p53, and which is well-tolerated in humans. The activation of the tumor-suppressors ATM and p53 by chloroquine is highly interesting because ATM and p53 deficiencies increase susceptibility to cancer (44–48). Our studies provide further proof-of-principle for developing prevention therapies based on the modulation of ATM-p53 pathways, whether based on chloroquine itself or targeting of other steps in the pathway. Although the mechanism of chloroquine-induced activation of ATM remains undetermined and may be cell signaling and context specific, chloroquine is known to alter chromatin and chromosome structures, which, in turn, may activate ATM (49–51). Therefore, further studies are needed to provide insight into the role of chloroquine-regulated molecular pathways and on the consequences of these events to susceptibility to carcinogenesis. From a clinical standpoint, however, our results indicate that chloroquine may have important preventative applications in breast carcinogenesis.

**Acknowledgments**

Received 8/8/2007; revised 9/24/2007; accepted 10/2/2007.

Grant support: National Institute of Child Health and Human Resources, Nuclear Receptor Signaling Atlas, and Deutsche Krebshilfe (German Cancer Aid) grants.

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