Hydroxychloroquine, chloroquine, and all-*trans* retinoic acid regulate growth, survival, and histone acetylation in breast cancer cells

Rayhana Rahim[a] and Jeannine S. Strobl[b]

The antimalarial drugs chloroquine (CQ) and hydroxychloroquine (HCQ) have potential applications in cancer treatment. The growth of MCF-7 and MDA-MB-231 human breast cancer cells *in vitro* was inhibited by CQ and HCQ and these cells were more sensitive than nontumorigenic MCF-10A breast epithelial cells. Furthermore, all-*trans* retinoic acid (ATRA) augmented the anticancer effects of CQ and HCQ as evidenced by significant reductions in Ki67-positive cancer cells and clonogenicity compared with cells treated with CQ or HCQ in the absence of ATRA. As an earlier study suggested that CQ, HCQ, and ATRA are breast cancer cell differentiation agents, these agents were screened in cell-free histone deacetylase (HDAC) and histone acetyltransferase (HAT) assays. ATRA, but not CQ or HCQ, inhibited HDAC activity in HeLa nuclear extracts. Growth inhibitory concentrations of HCQ and ATRA stimulated purified p300/CBP-associated factor, where CBP is the cAMP-response element binding protein, HAT activity. To investigate whether growth inhibitory concentrations of these agents influenced protein acetylation in cells, gel-purified histone H3 and histone H4 were analyzed using mass spectrometry. HCQ alone and HCQ + ATRA treatments altered the acetylation status in the N-terminal lysines of histones H3 and H4 compared with dimethyl sulfoxide (DMSO) controls. The results indicated that HCQ and ATRA regulate protein acetylation events in MCF-7 breast cancer cells, and identify a potential mechanism for their effects on breast cancer cell growth and differentiation. *Anti-Cancer Drugs* 20: 736–745 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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[a]Department of Biochemistry, West Virginia University, Morgantown, West Virginia and [b]Division of Biomedical Sciences, Edward Via Virginia College of Osteopathic Medicine, Virginia Tech Corporate Research Center, Blacksburg, Virginia, USA

Correspondence to Jeannine Strobl, PhD, Division of Biomedical Sciences, Virginia College of Osteopathic Medicine, Research Building II, VT Corporate Research Center, 1861 Pratt Drive, Blacksburg, VA 24060, USA
Tel: +1 540 231 1463; fax: +1 540 231 5252; e-mail: jstrobl@vcom.vt.edu

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Introduction

The discovery of compounds that cause tumor-selective cell differentiation and apoptosis without toxicity in normal cells spurred development of conceptually novel anticancer therapies [1–4]. Cancer treatment with differentiation agents is largely experimental, but has the potential to reduce adverse drug toxicities, circumvent epigenetic-mediated drug-resistance mechanisms [4], and block tumor angiogenesis [5]. Although the potential value of tumor differentiation therapies is widely recognized, these drugs display complex and incompletely understood mechanisms of action. Tumor differentiation agents include chromatin-remodeling drugs that target histone deacetylases and DNA methyltransferases, as well as cell cycle regulatory proteins, transcription factors, regulatory RNA molecules, and cytoskeleton elements [6–9].

In breast cancer models, analogs of vitamin D and vitamin A (retinoids), histone deacetylase (HDAC) inhibitors, DNA methylation inhibitors, and chaperone modifiers are experimental tumor differentiation agents [3,10–14]. These compounds act upon cell cycle checkpoint deficiencies [15], c-*myc* and bcl-2 oncogenes, and expression of proteins with cell differentiation activities including retinoic acid receptors, estrogen receptors (ERs), and β-catenin [5,14,16–18]. In addition, reactivation of ER gene expression by HDAC inhibitors and DNA methyltransferase inhibitors can overcome resistance to hormonal therapies in experimental breast cancer models [13].

Our laboratory has characterized a distinct category of breast tumor cell differentiation drugs, namely, the quinoline antimalarial drugs that include quinidine, quinine, and chloroquine (CQ) [19–23]. In response to these drugs, MCF-7 breast cells exhibit a more differentiated phenotype characterized by accumulation in G_0 of the cell cycle and expression of the mammary cell-specific differentiation marker, milk fat globule membrane protein [22]. Levels of the cyclin-dependent kinase inhibitor, p21/WAF-1, are elevated five-fold to 15-fold [22]. The profile of cell cycle protein expression is consistent with nonproliferating cells including hypophosphorylated pRb, reduced c-*myc*, and E2F1

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protein levels [20,23]. In addition, these drugs stimulate apoptosis [22]. Collectively, the two quinoline antimalarial drugs we investigated most extensively, CQ and quinidine produced a pattern of cellular responses very similar to the pan-HDAC inhibitor, trichostatin A (TSA) in the MCF-7 human breast tumor cell model.

The quinoline antimalarial drugs are not HDAC enzyme inhibitors, but rather, as shown with quinidine, cause ubiquitin-mediated HDAC enzyme degradation accompanied by histone hyperacetylation, and a more differentiated cell phenotype [21]. Subsequently, we showed CQ was more active than quinidine against breast cancer cells *in vitro* [22], and others found CQ had anticancer activity in *myc*-induced lymphoma in mice [24,25] and chemopreventive activity against carcinogen-induced rodent mammary tumors [26]. The hydroxylated analog of CQ, hydroxychloroquine (HCQ) was also reported to induce apoptosis in leukemic cells [27]. Both CQ and HCQ exert anti-HIV effects [28,29]. CQ and HCQ are lysomotropic agents that inhibit autophagy, a protein degradation pathway implicated in both cell survival and cell death, and because there is evidence that agents capable of interrupting autophagy sensitize leukemic cells to apoptosis, this process might also be important to the actions of CQ and HCQ in breast cells [30].

Many women take HCQ or CQ as an immune suppressant to treat rheumatoid arthritis and serum lupus erythematosus [31–33], and it was observed that HCQ reduced osteoporosis in women [34,35]. Thus, the actions of CQ and HCQ in breast cancer are of particular interest. Whether therapeutic plasma levels of CQ or HCQ in patients are sufficient to exert anticancer or chemoprotective activity are uncertain. In rheumatoid arthritis patients, plasma CQ levels ranged between 0.1 and 1 μmol/l; however, 12 μmol/l is not unusual and levels as high as 30 μmol/l were found in patients who seemed to tolerate therapy [32]. Steady-state plasma HCQ levels of approximately 6 μmol/l were typical in patients taking a standard daily dose of 400 mg, but 1200 mg/day were safely administered and many patients displayed 15 μmol/l plasma concentrations at steady state [31,33]. The clinical pharmacokinetics suggest that as single agents, CQ and HCQ might not be sufficiently potent, however, because autophagy inhibitors, including CQ sensitized leukemic cells to HDAC inhibitor-mediated apoptosis [30], CQ and HCQ are potentially valuable as chemosensitizers in cancer treatment. In this investigation, we examined the anticancer effects of CQ and HCQ in combination with the retinoid, all-*trans* retinoic acid (ATRA).

Materials and methods

Materials

CQ [(7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline)], TSA, ATRA, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). HCQ [(7-chloro-4-[4]ethyl-(2-hydroxyethyl)amino]-1-methylbutylamino] quinoline)] was provided by Mylan Pharmaceuticals Inc. (Morgantown, West Virginia, USA). Ki67 antibodies were purchased from Dako Corporation (Glostrup, Denmark).

Cell culture and proliferation assays

MCF-7 (passage #36–60) and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, Maryland, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, Colorado, USA), and 0.04 mg/ml gentamicin (Abbott Laboratories, North Chicago, Illinois, USA) in a 93% air/7% CO_2, 37°C, humidified incubator. MCF-10A immortalized human mammary epithelial cells (passage #1–15) were maintained in Mammary Epithelial Cell Growth Medium (BioWhittaker).

For MTS assays, cells were seeded into 96-well plates in DMEM/5% FBS. After 48 h, 100 μl DMEM/5% FBS + 20 μl CellTiter 96 AQueous one solution (Promega, Madison, Wisconsin, USA) were added for 2 h at 37°C and then the formazan product was quantified by absorbance at 490 nm. Concentration–response data were fit to sigmoidal dose–response using Prism software (GraphPad Software Inc., San Diego, California, USA) and these curve fits were used to estimate the drug concentration at which the response is reduced by 50% (IC_{50}) and 25% (IC_{25}) levels.

Ki67-positive cells were detected using immunohistochemistry and a hematoxylin counterstain as described earlier [23]. At least 500 cells in each treatment group were evaluated. We computed the percentage of Ki67-negative (G_0) cells in drug-treated and in control cells. Then, the Ki67 index was calculated as the ratio (% Ki67-negative cells in drug-treated cells)/(% Ki67-negative cells in control cells). The Ki67 index is equal to one when the fraction of Ki67-negative (G_0) cells is identical in drug-treated and control cells, and increases as the fraction of cells in G_0 increases.

Apoptotic cells were identified by Hoechst 33342 (Invitrogen Corporation, Carlsbad, California) dye uptake and fluorescence microscopy [36]. Percentage of apoptotic cells were determined after counting 300–500 cells per treatment condition.

Cancer cell clonogenic potential was measured in DMEM/10% FBS after preexposing cells to the test agents. Cells were seeded at a density of 6×10^3 cells/60 mm^2 dish; MCF-7 cells were grown undisturbed for 7 days before counting colonies. Dishes were stained with 0.5% crystal violet, 5% formalin, 50% ethanol, 0.85% NaCl and rinsed

with tap water. The clonogenic fraction of untreated cells (defined as the number of colonies in control dishes/ the number of cells plated) was set to 1. Drug effects on clonogenicity were expressed relative to this control in each experiment. Colonies were defined at ≥ 20 cells. Either visual or automated colony counting was performed (Oxford Optronix Ltd., Oxford, UK). Drug combinations were tested for synergy using Berebaum's equation: $Ac/Ae + Bc/Be < 1$, where Ae and Be were the concentrations of the drugs alone and Ac and Bc were the concentrations used in combination [37].

Enzyme assays

Beta-galactosidase activity

Cells were assayed for senescence-associated β-galacto sidase activity (SA-β-gal) (Cell Signaling Technology Inc., Danvers, Massachusetts, USA).

HDAC activity

Drugs were screened using the HDAC Fluorescent Activity Assay (BIOMOL Research Laboratories Inc., Plymouth Meeting, Pennsylvania, USA) and the protocols provided.

HAT assay

Drugs were screened using an indirect ELISA HAT Assay Kit (Upstate Biotechnology, Lake Placid, New York, USA). Acetylated histone H3 or histone H4 peptides were used as positive controls. Reactions were initiated by the addition of 75 ng of recombinant purified p300/ CBP-associated factor, where CBP is the cAMP-response element binding protein (PCAF).

Histone analysis

Histones were extracted from treated cells as described earlier [21,22,38] and 20 µg of total histones were electrophoresed in 15% polyacrylamide gels. The Coomassie Blue stained histone H3 and H4 protein bands were excised from the gels and digested in-gel with 50 µl of 100 ng of the endoprotease trypsin in 25 mmol/l NH_4HCO_3 overnight at 37°C. Histone digests were separated by reverse-phase high-performance liquid chromatography on a C18 column (Phenomenex, Torrance, California, USA) and an acetonitrile gradient, and then the peptides were cocrystallized with equal volumes of matrix (α-cyano-4 hydroxycinnamic acid in 49.5% ethanol, 49.5% acetonitrile, and 0.1% trifluoroacetic acid) (Sigma-Aldrich, Milwaukee, Wisconsin, USA). The resulting tryptic peptides were analyzed by mass spectrometry (ThermoFinnegan LCQ Deca XP Plus ion trap; ThermoElectron, Waltham, Massachusetts, USA) to determine their individual mass values. Collision energy was set at 35% to perform a full-scan MS followed by MS/MS of the three most abundant ions. Mass values for the specific peptide sequences of the N-terminal portion of histone H3/H4 were attained by matching the measured masses with expected calculated mass values acquired from the International Protein Index human database using Sequest Software (Scripps Research Institute, LaJolla, California, USA). Acetylation sites were identified using a differential modification of 42 Da added to lysine (K) residues.

Statistical analysis

Statistically significant differences ($P < 0.05$) were determined using one-way analysis of variance and Dunnett's test for comparison of multiple groups with control, the Tukey–Kramer test for comparison between the different groups, or the Bonferoni's *t*-test for comparing selected pairwise groups.

Results

CQ and HCQ inhibit breast cancer cell growth in cell culture

We compared the effects of CQ and HCQ on growth of breast tumor-derived MCF-7 and MDA-MB-231 cells and immortalized, nontumorigenic MCF-10A cells after 48 h using the MTS assay (Table 1). The IC_{50} were derived independently from the curve fit of three separate experiments and IC_{25} were estimated from a composite concentration–response curve using the data from all three experiments. Both ER-positive (ER +) MCF-7 cells and ER-negative (ER–) MDA-MB-231 cells were significantly more sensitive to CQ and HCQ than immortalized MCF-10A cells. The antiproliferative activity of CQ and HCQ in MCF-7 cells was shown in cell growth studies using their respective IC_{50} as determined in the MTS assays (Fig. 1), and compared with that of 1 µmol/l ATRA. Combined treatment of ATRA and either CQ or HCQ slightly reduced cell numbers in these growth studies.

Cells actively engaged in the cell cycle stain positively for the Ki67 nuclear antigen [39]. In control cultures of MCF-7 cells to which either a water (0.1%) or 0.1% DMSO solvent control was added for 48 h, 93.9% of all cells were Ki67 positive ($n = 6$) and 6.1 ± 2.5 ($n = 6$ and SEM) were Ki67 negative. The results in control cells indicated that a very high percentage of cells were cycling

Table 1 MTS assay

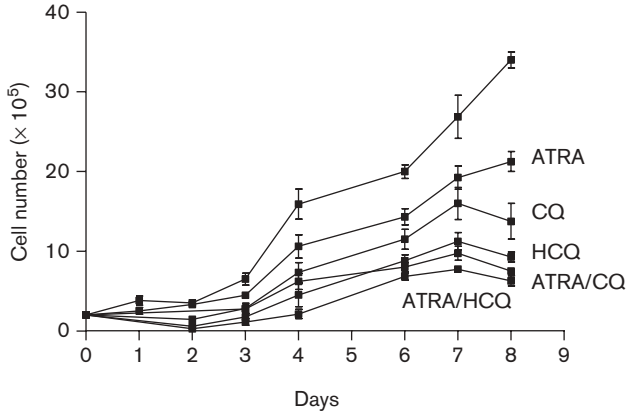
	MCF-7	MDA-MB-231	MCF-10A
	IC_{50}/IC_{25} (µmol/l)	IC_{50}/IC_{25} (µmol/l)	IC_{50}/IC_{25} (µmol/l)
CQ	33 ± 1.5*/14	11 ± 1.8*/7	365 ± 5.9/195
HCQ	57 ± 1.3*/30	28 ± 1.4*/12	218 ± 3.1/90

Cells were plated in quadruplicate, and then incubated in the presence of a range of CQ or HCQ concentrations for 48 h. MTS assays were performed. IC_{50} data represent the mean ± SE of three independent experiments. Reported IC_{25} values were estimated from concentration–response curve fits of all data obtained in all three experiments.

CQ, chloroquine; HCQ, hydroxychloroquine; IC_{50}, the drug concentration at which the response is reduced by 50%; IC_{25}, the drug concentration at which the response is reduced by 25%.

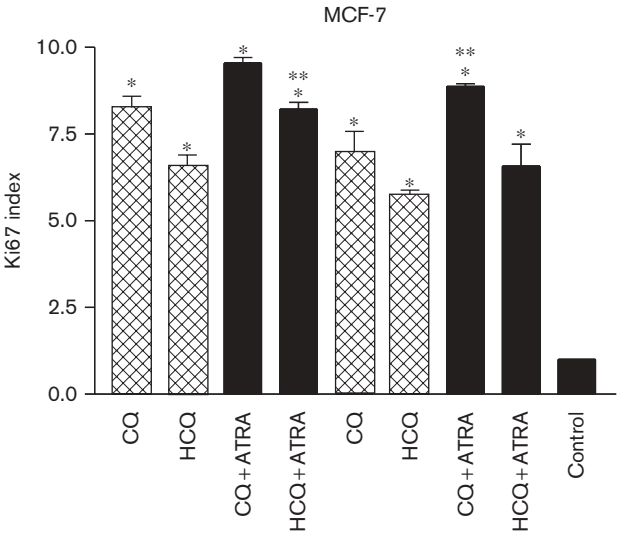
*Statistically significant differences ($P < 0.05$) in the IC_{50} values between MCF-7 or MDA-MB-231 cells and MCF-10A cells are indicated.

Fig. 1



MCF-7 growth curve. Cells (2×10^5) were replica plated in 35 mm^2 dishes on day 0, and 3 h later, dimethyl sulfoxide (DMSO) (0.1% solvent control) or 1 μmol/l all-*trans* retinoic acid (ATRA) in 0.1% DMSO were added to the culture medium. On day 1, 24 h after seeded, 33 μmol/l chloroquine (CQ) or 57 μmol/l hydroxychloroquine (HCQ) were added to the culture medium. Viable cells excluded trypan blue (0.04%) and were counted using a hemacytometer on the days indicated. Data shown are the mean ± SEM of duplicates in three independent experiments.

Fig. 2



Ki67 index. MCF-7 cells grown on glass coverslips (2×10^5 cells/35 mm^2 dish) were stained for Ki67 antigen expression after 48 h treatment times. The first four bars show data from the higher concentrations of chloroquine (CQ) and hydroxychloroquine (HCQ); the last four bars show data from the lower concentrations of CQ and HCQ. Controls were treated with solvent alone (0.1% dimethyl sulfoxide) for the final 24 h; CQ-treated cells were exposed to 33 or 14 μmol/l (IC_{25}) CQ for the final 24 h; HCQ-treated cells were exposed to 57 or 30 μmol/l (IC_{25}) HCQ for the final 24 h. In the all-*trans* retinoic acid (ATRA)-treatment groups, cells received 1 μmol/l ATRA 24 h before addition of CQ or HCQ for the final 24 h. Thus, the total ATRA treatment time was 48 h and that of CQ or HCQ was 24 h. The Ki67 index was determined as described in Methods. Data shown are the mean of three independent experiments (± SEM). Statistically significant differences from the control are indicated (*$P < 0.05$). Significant effects of 1 μmol/l ATRA are indicated (**$P < 0.05$).

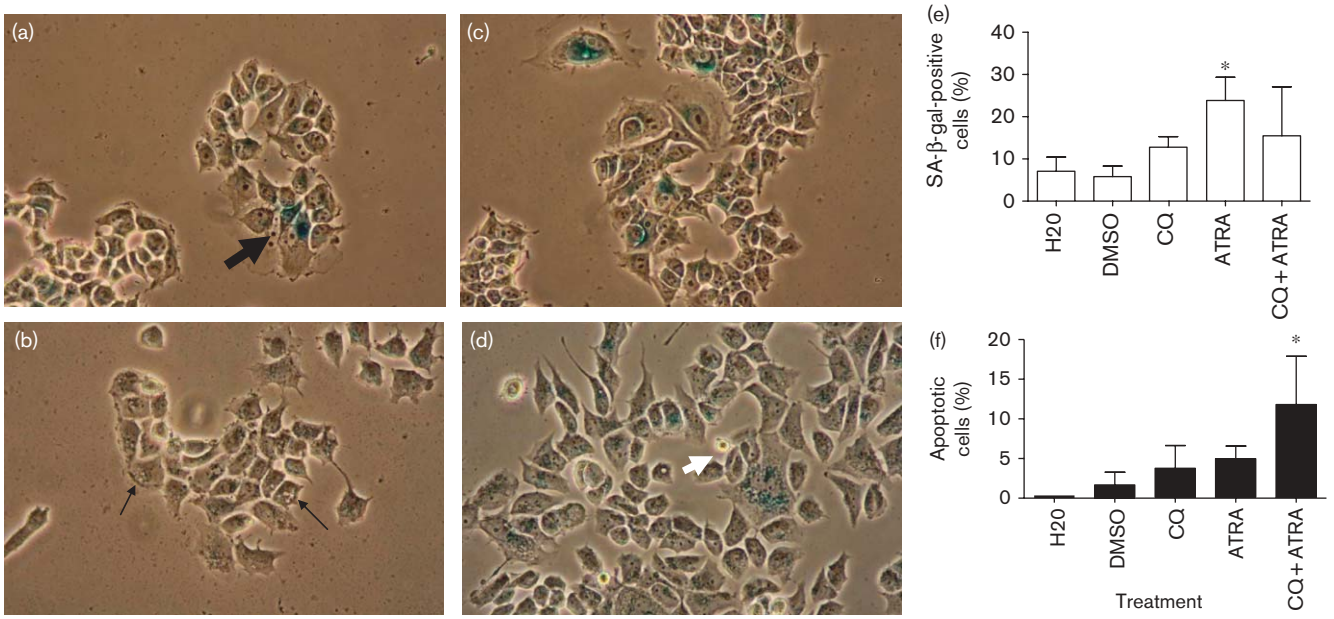
and only 6.1% of cells were in G_0 phase of the cell cycle. We used Ki67 staining to assess the effects of CQ and HCQ on cycling cells. These drugs significantly increased the percentage of MCF-7 cells in G_0 in MCF-7 as measured by a six-fold to eight-fold increase in the Ki67 index (Fig. 2). The magnitude of change in Ki67 index indicated that within 24 h, CQ and HCQ significantly reduced the fraction of cycling MCF-7 cells and raised the percentage of cells in G_0 to 36.6–48.8%. The addition of 1 μmol/l ATRA significantly raised the Ki67 index in cells treated with IC_{50} HCQ and IC_{25} CQ. Reductions in the proportion of cycling cells are likely to contribute to the inhibition of cell numbers by CQ, HCQ, and ATRA as shown in Fig. 1.

We compared the actions of CQ, HCQ, and ATRA on the induction of cellular senescence by assaying SA-β-gal in treated cells [40]. The solid arrows in Fig. 3 identify the typical blue stain produced by this enzyme. Less than 10% of MCF-7 cells in 0.1% DMSO control cultures were SA-β-gal positive, and neither CQ (33 μmol/l) or HCQ (57 μmol/l) treatments significantly enhanced SA-β-gal activity. However, SA-β-gal-positive cells doubled after exposure to ATRA indicating that ATRA significantly stimulated senescence in these breast cancer cells.

We examined the treated MCF-7 cells for the presence of cytoplasmic vacuoles which appear in cells when autophagy is inhibited; the thin arrows in Fig. 3b, c and d point to the cytoplasmic vacuoles in cells exposed to CQ, HCQ, or in combination with ATRA. ATRA treatment alone did not result in cytoplasmic vacuole formation. The effects of these drugs on SA-β-gal and cytoplasmic vacuolization suggest that in the presence of CQ + ATRA or HCQ + ATRA, MCF-7 cells undergo events resulting in the promotion of cellular senescence and inhibition of autophagy.

Figure 4 shows the effect of CQ, HCQ, and ATRA on the clonogenicity of MCF-7 cells. In these experiments, cells were pretreated with ATRA for 24 h, then treated for an additional 48 h in the presence of CQ or HCQ bringing the total time of exposure to drugs to 72 h. Afterwards, the cells were harvested and counted and viable cells were seeded at a low density to test their clonogenic potential. In Fig. 4a, significant reductions in clonogenicity in response to CQ and HCQ were noted, and the addition of ATRA to the treatments further reduced clonogenicity ($P < 0.05$) compared with CQ or HCQ treatment alone. Figure 4b illustrates typical crystal violet stained colonies in which the staining intensity is an estimate of the total number of cells present, 7 days post-plating. Fig. 4c depicts the results of an isobologram analysis performed using automated colony counting. The drug combination is additive or slightly synergistic.

Fig. 3



Senescence-associated β-galactosidase (SA-β-gal) assay. Replica plated MCF-7 cells were pretreated for 24 h with 0.1% dimethyl sulfoxide (DMSO) (control) or 1 μmol/l all-*trans* retinoic acid (ATRA) (final 0.1% DMSO), and then CQ (33 μmol/l) or HCQ (57 μmol/l) were added to the culture medium for an additional 48 h. Cells were fixed and incubated overnight at 37°C with the β-galactosidase substrate at pH 6. (a) 0.1% DMSO; (b) 57 μmol/l HCQ; (c) 33 μmol/l CQ; (d) 1 μmol/l ATRA + 33 μmol/l CQ. The thick black arrow indicates the typical blue color of β-gal positive cells; the thin black arrow indicates the cytoplasmic vacuoles; the thick white arrow identifies an apoptotic cell. (e, f) Quantify the SA-β-gal-positive and apoptotic cells. Data shown are results from counting 300–500 cells/condition (mean ± SD) and are representative of two independent experiments.

HDAC and HAT enzymes are in-vitro targets of CQ, HCQ, and ATRA

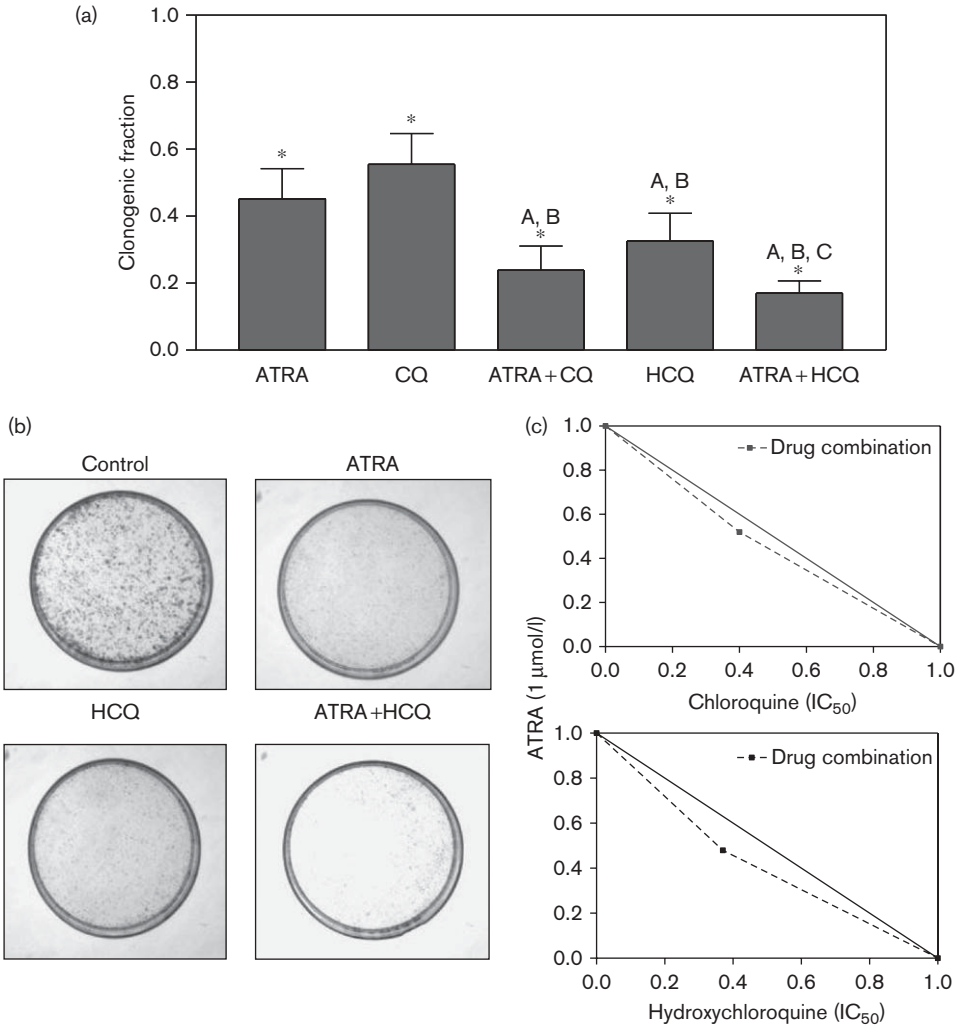
HCQ and CQ do not directly inhibit HDAC enzyme activity *in vitro* in HeLa cell nuclear extracts (Fig. 5a). ATRA (1 μmol/l) added directly to HeLa cell extracts reduced HDAC activity by 40% ($P < 0.05$) compared with the solvent control. ATRA inhibition of HDAC activity was unaffected in the presence of CQ or HCQ. The inhibitory effect of ATRA was not because of interference with the production of the fluorophore by the developer reagent. We also investigated the effect of HCQ and ATRA on HAT activity in an in-vitro system that used PCAF as the HAT activity source. ATRA and HCQ significantly stimulated in-vitro HAT activity as measured by increased acetylation of histone H3 and histone H4 substrate peptides (Fig. 5b). These results identify HCQ and ATRA as new HAT regulatory agents.

Global histone acetylation patterns assessed by mass spectrometry

Mass spectrometry was used to detect qualitative changes in the lysine acetylation profiles in the N-terminal peptides produced from the total pool of H3 and H4 histones isolated from MCF-7 cells. For these experiments, cells were treated for 8 h with 57 μmol/l HCQ ± pretreatment with 1 μmol/l ATRA for 24 h. There are 13 potential lysine acetylation sites in the N-terminal peptide of histone H3 (Fig. 6a), and 11 potential lysine acetylation sites in the N-terminal peptide of histone H4 (Fig. 6b). Acetylated lysine residues were detected by mass spectrometry at 11/13 possible sites in histone H3 prepared from control (DMSO treated) MCF-7 cells; only the most N-terminal sites, K_4 and K_9 lacked acetylation (Fig. 6a). Similarly, histone H4 in control cells displayed acetylated lysine residues at all possible sites except the most N-terminal sites K_5, K_8, and K_{12} (Fig. 6b). The results indicate N-terminal lysines in histone H3 and H4 are hypoacetylated compared with lysines residing in the central histone core in control MCF-7 cells.

HCQ-treated MCF-7 cells showed an increase in acetylation of histone H3 marked by the appearance of acetylated K_9; after HCQ treatment, only the K_4 of histone H3 remained hypoacetylated. In HCQ-treated cells, histone H4 contained acetylated K_{12}, whereas K_5 and K_8 of histone H4 remained hypoacetylated (Fig. 6). HCQ treatment specifically increased acetylation of lysine, H3-K_9 and H4-K_{12}, leaving the most N-terminal lysines in histones H3 and H4 hypoacetylated. In comparison, TSA-treated cells had acetylated residues at all three lysines in the N-terminal tails of histones H3 and H4, including H3-K_9K_4 and H4-$K_{12}K_8K_5$ (Fig. 6a

Fig. 4



Clonogenicity assay. MCF-7 were plated (2×10^5 cells/35 mm^2 dish) and allowed to adhere for 3 h, then solvent only (0.1% dimethyl sulfoxide, DMSO) or 1 μmol/l all-*trans* retinoic acid (ATRA) in 0.1% DMSO was added. Twenty-four hours later, chloroquine (CQ) (33 μmol/l) or hydroxychloroquine (HCQ) (57 μmol/l) were added. Cells were harvested on day 4 after exposure to CQ or HCQ for 48 h in the presence of either 0.1% DMSO or ATRA for a total of 72 h; cells were counted in the presence of trypan blue, then 6×10^3 viable cells were reseeded into 60 mm^2 dishes in DMEM + 10% FBS and grown undisturbed for 7 days. Cells were fixed and stained and colonies ($\geq$ 20 cells) were counted visually. (a) Data are expressed as the fractional clonogenic survival compared with cells treated with the solvent alone, and are the mean of three independent experiments (± SEM) carried out in duplicate. *Significant differences from control cells (0.1% DMSO) are indicated; 'A' indicates a difference from ATRA alone; 'B' indicates a difference from CQ alone; 'C' indicates a difference from HCQ alone. (b) Representative crystal violet-stained colonies of MCF-7 cells. (c) Isobologram analysis performed using automated colony counting; results are the mean of three independent experiments. *$P < 0.05$.

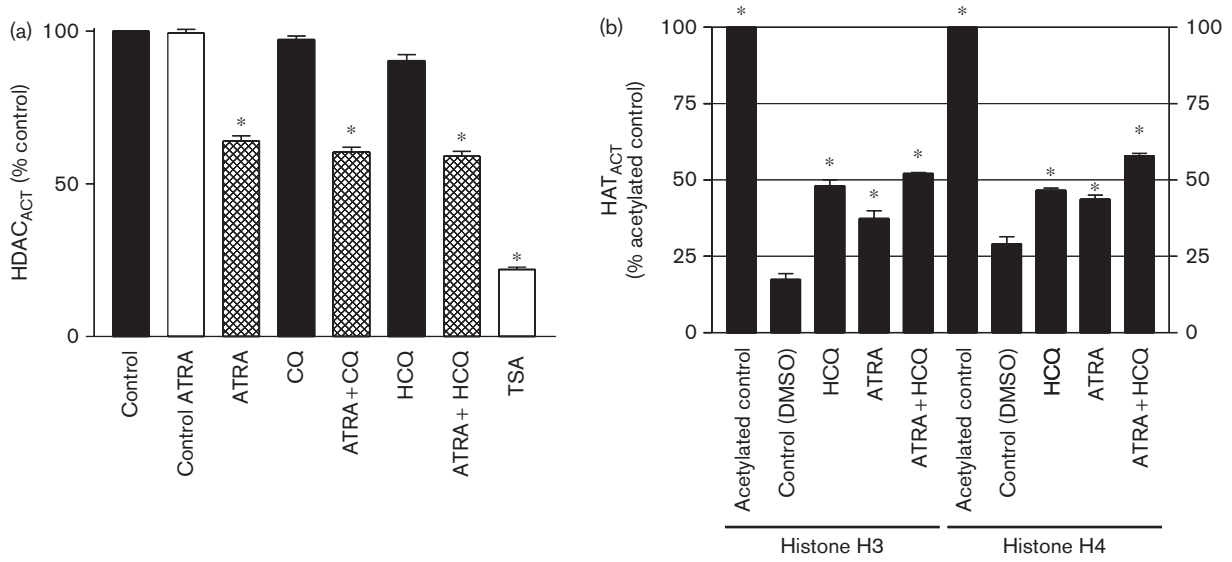
and b). The results of these mass spectrometry analyses are the first report of global distinctions in N-terminal lysine acetylation in histones H3 and H4 by two different cell differentiation agents, TSA and HCQ.

Cells treated with ATRA + HCQ exhibited the same mass spectrometry profile of histone acetylation induced by TSA to include acetylation of H3-K_9, H3-K_4 and H4-K_{12}, H4-K_8, and H4-K_5 (Fig. 6a and b). HCQ-treated cells displayed acetylated N-terminal lysines in histone H3 (K_9) and histone H4 (K_{12}), and in the presence of HCQ + ATRA, acetylation of additional N-terminal lysines in histone H3 (K_4 and K_9) and histone H4 (K_5, K_8, and K_{12}) were detected.

Discussion

The use of CQ in cancer treatment has attracted interest as a result of demonstrated preclinical [24–26,41–42] and clinical [43] activity in breast, lung, lymphoma, and glioblastoma tumors. Here, we showed that HCQ as well as CQ inhibited the growth and clonogenicity of human

Fig. 5



(a) Histone deacetylase enzyme activity ($HDAC_{ACT}$). Drugs or solvent controls were added directly to HeLa cell nuclear extracts, and HDAC activity was measured using the Fluor-de-Lys Fluorescence Assay. The HDAC inhibitor, trichostatin A (TSA) (35 nmol/l), was used as a positive control. Chloroquine (CQ) (33 μmol/l) and hydroxychloroquine (HCQ) (57 μmol/l) were tested for effects on HDAC ± 1 μmol/l ATRA. Data are the mean of three experiments ± SEM carried out in triplicate. (b) HAT enzyme activity (HAT_{ACT}). Drugs or solvent controls were added to an indirect ELISA to measure the formation of acetylated biotin-tagged histone H3 and biotin-tagged histone H4 peptides. Reactions contained 75 ng of recombinant PCAF HAT enzyme, 100 μmol/l acetyl-CoA and assay buffer provided by the supplier. Acetylated histone H3 or histone H4 peptides were used as positive controls. HCQ (57 μmol/l) ± 1 μmol/l all-*trans* retinoic acid (ATRA) were added to the reactions before initiating reactions by the addition of the PCAF enzyme. Data are the mean of three experiments ± SEM carried out in triplicate. Statistically significant differences from the control are indicated (*$P < 0.05$).

breast cancer cells. Furthermore, the anticancer effects of HCQ and CQ were amplified in the presence of the retinoid, ATRA. The actions of these drugs on enzymes that regulate protein acetylation in cells, including histone acetylation, provide insight into a possible mechanism of action in cancer cells.

We found agreement between HCQ concentrations that inhibited growth and clonogenicity in breast cancer cells and concentrations of HCQ and ATRA, which stimulated HAT activity of PCAF *in vitro*. HCQ (57 μmol/l) significantly stimulated HAT activity, reduced MCF-7 cell growth by 50%, and reduced MCF-7 clonogenicity by 70%. Under these conditions, mass spectrometry indicated that acetylation of H3-K_9 and H4-K_{12} was increased relative to control MCF-7 cells. In the presence of HCQ + ATRA, significant reductions in cancer cell growth and clonogenicity were observed compared with HCQ alone, and under these conditions, increased acetylation at additional lysines in histones H3 (K_4, K_9) and H4 (K_5, K_8, K_{12}) were observed. The histone analyses established that regulation of protein acetylation occurred in breast cancer cells in response to HCQ and ATRA. However, the determination of how acetylation of histones or possibly other regulatory proteins leads to inhibition of breast cancer growth and survival will require additional study [3,9,16].

Our observations on histone acetylation in MCF-7 cells in response to HCQ + ATRA suggest that the use of drug combination to stimulate HAT activity and inhibit HDAC enzymes is a rationale paradigm for arresting growth of breast cancer. The HAT activator, HCQ, stimulated acetylation of the internal lysines of histone H3 (K_9) and H4 (K_{12}); these are sites predicted by the 'zip' model of histone acetylation [44] to be preferential targets of HAT activity. Measurable hyperacetylation of the ultimate N-terminal tails of histone H3 and H4 in MCF-7 cells occurred only after exposure to HDAC inhibitors (TSA or ATRA). In other words, the observations suggest a model in which HAT activators will typically elevate acetylation of lysines closer to the nucleosome core where histone–histone interactions might undergo modulation, while HDAC inhibitors evoke acetylation most distally from the nucleosome core and influence interactions with DNA to modulate the extent of chromatin condensation.

HAT has been identified as a potential target for antiproliferatives in yeast, nonbreast human tumor cell lines, and a protozoan parasite *Toxoplasma gondii* [45–49]. In addition to HCQ, three other experimental quinoline compounds are known to regulate HAT [46,47]. These quinolines, represented by the prototype MC1626, inhibited HAT activity [48]. HAT inhibition in human

Fig. 6

(a) Histone H3 lysine (K) acetylation sites

ART**K$_4$**QTQR**K$_9$**S TGG**K$_{14}$**APR**K$_{18}$**QL AT**K$_{23}$**AAR**K$_{27}$**SAP ATGGV**K$_{36}$K$_{37}$**PHR
YRPGTVALRE IRRYQ**K$_{56}$**STEL LIR**K$_{64}$**LPFQRL VREIAQDF**K$_{79}$**T
DLRFQSSAVM ALQEACEAYL VGLFEDTNLC AIHA**K$_{115}$**RVTIM
P**K$_{122}$**DIQLARRI RGERA

Treatment	K$_4$	K$_9$	K$_{14}$	K$_{18}$	K$_{23}$	K$_{27}$	K$_{36}$	K$_{37}$	K$_{56}$	K$_{64}$	K$_{79}$	K$_{115}$	K$_{122}$
DMSO	0	0	+	+	+	+	+	+	+	+	+	+	+
HCQ	0	+	+	+	+	+	+	+	+	+	+	+	+
ATRA/HCQ	+	+	+	+	+	+	+	+	+	+	+	+	+
TSA	+	+	+	+	+	+	+	+	+	+	+	+	+

(b) Histone H4 lysine (K) acetylation sites

SGRG**K$_5$**GG**K$_8$**GL G**K$_{12}$**GGA**K$_{16}$**RHR**K$_{20}$** VLRDNIQGIT **K$_{31}$**PAIRRLARR
GGV**K$_{44}$**RISGLI YEETRGVL**K$_{59}$**V FLENVIRDAV TYTEHA**K$_{77}$**R**K$_{79}$**T
VTAMDVVYAL **K$_{91}$**RQGRTLYGF GG

Treatment	K$_5$	K$_8$	K$_{12}$	K$_{16}$	K$_{20}$	K$_{31}$	K$_{44}$	K$_{59}$	K$_{77}$	K$_{79}$	K$_{91}$
DMSO	0	0	0	+	+	+	+	+	+	+	+
HCQ	0	0	+	+	+	+	+	+	+	+	+
ATRA/HCQ	+	+	+	+	+	+	+	+	+	+	+
TSA	+	+	+	+	+	+	+	+	+	+	+

Mass spectrometry analysis of histone acetylation. 1 $\times$ 10^7 MCF-7 cells were pretreated with (0.1% dimethyl sulfoxide, DMSO) or 1 μmol/l all-*trans* retinoic acid (ATRA) for 24 h and incubated for another 8 h with trichostatin A (TSA) (300 nmol/l), or hydroxychloroquine (HCQ) (57 μmol/l). Purified histone proteins (20 μg) from each treatment group were resolved on 15% polyacrylamide gels. The Coomassie Blue-stained bands corresponding to histone H3 and histone H4 were excised, trypsin-digested, and separated on a C18 column using reverse-phase high-performance liquid chromatography. Samples were analyzed on a ThermoFinnegan LCQ Deca XP Plus ion trap. (a) Histone H3. The upper panel shows the amino acid sequence of N-terminal histone H3; all lysine (K) residues are numbered and appear in bold. Acetylated K in histone H3 were detected by mass spectrometry at sites indicated by '+' in the table, and nonacetylated sites are indicated by '0'. (b) Histone H4. The upper panel displays the amino acid sequence of N-terminal histone H4 where all of the lysine (K) residues are numbered and appear in bold. The acetylated lysine sites identified by mass spectrometry in each treatment group are indicated in the table as '+' and nonacetylated sites are indicated by '0'. Data shown are representative of two experiments with identical results.

colon cell lines was correlated with histone hypoacetylation and growth inhibition. This study on colon cancer cells contrasted directly with the findings of this study in which HAT activation and histone hyperacetylation were correlated with growth inhibition in MCF-7 breast tumor cells [47]. Furthermore, we showed that CQ and HCQ were more toxic to breast tumor cells than normal cells, a property shared by HDAC inhibitors that also stimulate histone hyperacetylation. We and others [49] suggest that the complex assortment of mechanisms involved in the regulation of histone acetylation status explains why disruption of histone acetylation either by hyperacetylation or hypoacetylation impairs cell growth.

The direct actions of ATRA on acetylation enzymes *in vitro* constitute new findings. HeLa cell extracts lack retinoic acid receptors [50] implicating HDAC as a direct target of ATRA. Similarly, ATRA stimulated HAT activity *in vitro* independently of the retinoic acid receptor. These experimental results raise the possibility that ATRA is capable of regulating protein acetylation in cells independently of retinoic acid receptor function. In support of this idea, anacardic acid, a natural product HAT regulator derived from cashew nut oil, bears structural similarities with ATRA [51] and establishes a precedent for simple compounds comprised of a 6-membered ring and a long alkyl side chain extension to regulate HAT. Although anacardic acid inhibits HAT, an amide analog of anacardic acid is a HAT activator [51]. The identification of HCQ and ATRA as new HAT activators is additional support for the role of HAT enzymes as anticancer drug targets.

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