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We have shown that the quinoline antimalarials chloroquine (CQ) and hydroxychloroquine (HCQ) inhibit proliferation and induce differentiation in breast cancer cell lines without toxicity to normal MCF-10A cells. The purpose of this project is to derive more efficacious antitumor agents that enhance the differentiation response by using CQ and HCQ in combination with the demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza-dC; Aza), or with the differentiating agent, all-trans-Retinoic acid (ATRA). Cell survival, cellular differentiation, histone H3 and/or histone H4 acetylation status, and HDAC protein and activity were measured to show that combination of Aza or ATRA with the quinolines augmented the antiproliferative effect, differentiation response, and acetylation status of either CQ or HCQ alone. A new and highly sensitive assay for histone acetylation by mass spectrometry was developed to illustrate the specific lysine sites that get modified (acetylated/deacetylated) by the most promising combination of chemotherapeutic agents. This approach will be pivotal in further developing more effective and less toxic therapeutic agents for breast cancer intervention.

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Introduction:

Preliminary studies showed that two of the quinoline antimalarials, chloroquine (CQ) and hydroxychloroquine (HCQ), displayed selective toxicity only to breast cancer cells. Hence the goal of these studies was to explore: (1) whether a drug combination modulating epigenetic events would sensitize breast cancer cells to the antitumor activity of CQ or HCQ, (2) and if so, which would be the most promising combination of agents for the generation of safer and less toxic chemotherapeutic agents for the prevention and treatment of advanced breast cancer. We hypothesized that the use of a drug combination modulating epigenetic events would lower the concentration of CQ or HCQ needed to produce the differentiation response. Hence, the proposed work seeks to use these two quinoline agents in combination with the demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza-dC or Aza), or with the differentiating agent, all-trans-Retinoic acid (ATRA) in order to lower the threshold for chemotherapy-induced cell death in breast cancer cells. MCF-7 and MDA-MB-231 human breast cancer cell lines will be treated with either CQ or HCQ at their IC_{25} and IC_{50} MTS values ± 5-Aza-dC or ATRA. Cell survival will be assessed using three endpoints: (1) MTS metabolism assay, (2) cell growth curve, and (3) tumor cell clonogenic survival. Cellular differentiation will be measured using three endpoints: (1) accumulation of cytoplasmic lipid droplets, (2) loss of Ki67 protein, and (3) expression of E2F-1 and c-myc cell cycle regulatory proteins. Histone acetylation status will be assessed by measuring acetylated histone H3 and/or histone H4 protein levels, as well as histone deacetylase, (HDAC1) protein degradation. Finally, the most promising combination of agents will be assessed by mass spectrometry in order to generate an overall profile of histone acetylation. The development of a new and highly sensitive assay for histone H3 and/or histone H4 acetylation by mass spectrometry will be pivotal in further developing HDAC inhibitors for the prevention and treatment of breast cancer. This new approach will illustrate the level and the specific lysine sites that get modified (acetylated/deacetylated) by the chemotherapeutic agents.

Body:

Research Accomplishments

Task #1 of this project was to determine optimal conditions for cellular differentiation using CQ or HCQ ± 5-Aza-dC (Aza) or ATRA (or a vitamin D-derivative as an alternative drug of choice if the other agents do not cause differentiation). Previous results attained from cell survival assays (MTS metabolism assay, cell growth curve, and tumor cell clonogenic survival) as well as differentiation assays (accumulation of cytoplasmic lipid droplets and loss of Ki67 protein) showed that the combination of low concentrations of Aza or ATRA with CQ or HCQ decreased breast cancer cell survival and augmented the differentiation response more than either antimalarial alone.

Additional experiments conducted for this task are outlined below. First, since tumor clonogenic survival seems to be one of the most sensitive tests for predicting the responsiveness of a tumor to clinical treatment (Kumala et al., 2003; West et al., 1997), this method was used to predict the effect of chloroquine and hydroxychloroquine upon pretreatment with ATRA or Aza on tumor cell survival (Figures 1 and 2, respectively).

Clonogenic survival of MDA-MB-231 cells exposed to combination of 1μM ATRA with CQ for seven days, was reduced by 83% compared to control cells exposed to solvent alone, whereas combination of 1μM ATRA with HCQ was reduced by 92% (Figure 1A). In MCF-7 cells, clonogenic survival was reduced by 76% upon combination treatment with ATRA and CQ and by 82% with ATRA and HCQ (Figure 1B). The interaction of ATRA with CQ or HCQ showed to be synergistic in both the MDA-MB-231 and MCF-7 breast cancer cell lines. These results support the hypothesis that combination of ATRA with CQ or HCQ would augment the antiproliferative effects of the antitumor quinolines. ATRA in combination with HCQ showed to have the most promise in decreasing tumor cell survival.

Clonogenic survival of MDA-MB-231 cells exposed to combination of 1μM Aza with CQ for seven days, was reduced by 82% compared to control cells exposed to solvent alone, whereas combination of 1μM Aza with HCQ was reduced by 91% (Figure 2A). In MCF-7 cells, clonogenic survival was reduced by 56% upon combination treatment with Aza and CQ and by 68% with Aza and HCQ compared to control cells exposed to solvent alone (Figure 2B). The antiproliferative response of Aza with CQ or HCQ showed to be synergistic in only the MDA-MB-231 breast cancer cell line. Aza in combination with HCQ showed to have the most promise in decreasing tumor cell survival in the MDA-MB-231 cell line.

Alterations in the regulation of a key cell cycle regulatory protein, E2F-1, was measured by Western blotting (Figure 3). E2F-1, which is tightly bound to the retinoblastoma (Rb) protein, interacts with HDAC to enhance cell cycle progression by stimulating the G1-S cell cycle transition (Martinez-Balbas et al., 2000). In order for cells to undergo differentiation, downregulation of E2F-1 is an essential precondition (Yamasaki et al., 1996); therefore, it was necessary to assess E2F-1 protein levels in order to confirm G1 cell cycle arrest as seen in the data above. E2F-1 protein was downregulated in MCF-7 breast cancer cells in response to combination treatment of CQ and ATRA. Chloroquine caused a 60%, 67%, and 72% decrease in E2F-1 protein levels when combined with 1,10, and 100μM ATRA, respectively. The response was statistically significant, but yet a modest effect. Nevertheless, it supports the hypothesis that

that combination of ATRA with CQ would augment the differentiation response as shown by downregulation of E2F-1 cell cycle regulatory protein.

c-myc, a protooncogene which codes for proteins that stimulate cell division, is a key regulator of cell cycle progression, cell differentiation, and apoptosis (Melkoumian et al., 2002; Spaventi et al., 1993). It is hypothesized that in order for cells to undergo differentiation, downregulation of c-myc protein may be an essential step in controlling the cell cycle regulatory profile. In order to confirm the cellular differentiation response seen in the above results, alterations in the level of c-myc protein was measured by Western blotting (Figure 4). c-myc protein was downregulated in MCF-7 breast cancer cells in response to combination treatment of CQ and ATRA. Chloroquine caused a 66%, 69%, and 79% decrease in c-myc protein levels when combined with 1,10, and 100μM ATRA, respectively; therefore, the cells experienced an enhanced downregulation of c-myc protein with increased concentration of ATRA. The effect was modest even though the response was statistically significant. These results support the hypothesis that that combination of ATRA with CQ CQ would augment the differentiation response as shown by downregulation of c-myc cell cycle regulatory protein.

In conclusion, both CQ and HCQ serve as selective breast tumor differentiating agents and the combination of low concentrations of 5-Aza-dC or ATRA with these antitumor antimalarials decreased breast cancer cell survival and augmented the differentiation response more than either CQ or HCQ alone. Task #1 is complete.

Task #2 of this project was to determine the optimum conditions for histone H4 hyperacetylation. To examine whether pretreatment with the demethylating agent, 5-Aza-dC (Aza), or the differentiating agent, ATRA, would alter the acetylation status of histones, western blot analyses were conducted to assess acetylated histone H3 and/or histone H4 and HDAC protein levels. The $RAR\beta_2$ gene is silenced in human carcinoma cells. Since growing evidence suggests that methylation may be responsible for the lack of $RAR\beta_2$ gene expression in cancer cell lines (Yang et al., 2002) and also that retinoids exert their chemopreventive effects by $RAR\beta_2$ induction (Lotan et al., 1995; Yang et al., 2002), $RAR\beta_2$ protein levels were measured as well. Whole cell lysates or histone proteins were extracted from MCF-7 cells pretreated with 1μM Aza or ATRA for 24 hours prior to chloroquine or hydroxychloroquine addition for different time points (Figures 5 and 6, respectively). Trichostatin A (TSA) was used as a positive control for HDAC inhibition and histone hyperacetylation.

Figure 5 shows that by 4 hours after chloroquine addition, there was a 56% reduction in HDAC protein levels in cells that were pretreated with Aza compared with a 40% reduction in cells with no pretreatment and only chloroquine addition. Also by 4 hours after chloroquine treatment, there was significant induction of histone hyperacetylation with a 6.6 fold increase in acetylated histone H4 protein in cells pretreated with Aza compared to cells with solvent alone. By 8 hours after chloroquine treatment, there was a significant 4.4 fold increase in $RAR\beta_2$ protein in cells pretreated with Aza and this increase was sustained even at 24 hours. By 4 hours after hydroxychloroquine addition, there was a 42% reduction in HDAC protein levels in cells that were pretreated with Aza compared with a 37% reduction in cells with no pretreatment and only hydroxychloroquine addition. Also by 4 hours after hydroxychloroquine treatment, there was significant induction of histone hyperacetylation with a 4.5 fold increase in acetylated histone H4 protein in cells pretreated with Aza compared to cells with solvent alone. There was

a maximum increase in RARβ_2 protein levels in cells pretreated with Aza by 12 hours after hydroxychloroquine treatment. Increased RARβ_2 protein levels were sustained even at 24 hours. The data show that combination of Aza with chloroquine or hydroxychloroquine increases histone H4 acetylation status, inhibits HDAC protein levels, as well as increases RARβ_2 tumor suppressor protein levels. These results combined with cell survival and differentiation assays, suggest that Aza in combination with either chloroquine or hydroxychloroquine are promising agents for the generation of safer and less toxic chemotherapeutic agents for the prevention and treatment of ER- breast cancer.

Figure 6 shows that by 4 hours after chloroquine addition, there was a 33%, 33%, and 36% reduction in HDAC protein levels in cells that were pretreated with 1, 10, or 100 μM ATRA, respectively, compared with a 27% reduction in cells with no pretreatment and only chloroquine addition. By 8 hours after chloroquine treatment, there was a significant induction of histone hyperacetylation with a 3.9 fold increase in acetylated histone H4 protein and a 3.4 fold increase in acetylated histone H3 protein in cells pretreated with 100μM ATRA compared to cells with solvent alone. There was a pronounced histone H3 hyperacetylation response even by 0.5hr in cells pretreated with 1μM ATRA and increased levels were sustained even at 12 hours. By 12 hours after chloroquine treatment, there was a significant induction of RARβ_2 protein levels in cells pretreated with Aza and this increase was sustained even at 24 hours. Cells pretreated with 1μM ATRA showed the greatest increase in RARβ_2 protein levels. By 4 hours after hydroxychloroquine addition, there was a 35%, 31%, and 41% reduction in HDAC protein levels in cells that were pretreated with 1, 10, or 100 μM ATRA, respectively, compared with a 30% reduction in cells with no pretreatment and only hydroxychloroquine addition. By 8 hours after hydroxychloroquine treatment, there was a significant induction of histone hyperacetylation with a 4.2 fold increase in acetylated histone H4 protein and a 5.6 fold increase in acetylated histone H3 protein in cells pretreated with 100μM ATRA compared to cells with solvent alone. By 12 hours after hydroxychloroquine treatment, there was a significant induction of RARβ_2 protein levels in cells pretreated with Aza and this increase was sustained even at 24 hours. Cells pretreated with 1μM ATRA showed the greatest increase in RARβ_2 protein levels. The data show that combination of ATRA with chloroquine or hydroxychloroquine increases histone H4 acetylation status, inhibits HDAC protein levels, as well as increases RARβ_2 tumor suppressor protein levels.

To examine whether these HDAC protein levels observed in the Western blotting experiments were directly or indirectly inhibited by chloroquine or hydroxchloroquine ± pretreatment with ATRA, HDAC fluorescent activity was measured (Figure 7). This assay system measures histone deacetylase activity present in a commercial HeLa (human cervical cancer cell line) cell nuclear extract, which is rich in HDAC activity. The HDAC substrate contains an acetylated Lysine side chain. Upon incubation of the substrate with the HeLa nuclear extract, HDAC-mediated deacetylation sensitizes the substrate so that it becomes a fluorophore with addition of a developer. The potent HDAC inhibitor, TSA, was used as a positive control. 1μM ATRA alone showed a significant inhibitory effect on HDAC. This effect on HDAC activity was sustained, but not decreased further upon combination treatment with either chloroquine or hydroxychloroquine. This data suggests that ATRA alone is responsible for the direct inhibition of HDAC. The results from the HDAC activity assay combined with cell survival and differentiation assays, suggest that ATRA in combination with either chloroquine or

hydroxychloroquine are promising agents for the generation of safer and less toxic chemotherapeutic agents for the prevention and treatment of both ER- and ER+ breast cancers. Task #2 is complete.

Task #3 of this project was to develop a qualitative and quantitative assay using mass spectrometry for assessment of the overall histone acetylation profile, while Task #4 is to utilize this new assay using the most promising combination of agents. Since ATRA in combination with HCQ had the most significant inhibitory effect on clonogenic survival, and since clonogenic survival is the most sensitive test for predicting the responsiveness of a tumor to clinical treatment, the combination of ATRA with HCQ was assessed subsequently by mass spectrometry to measure its effect on histone H3 and histone H4 acetylation sites.

Histone H3 and histone H4 samples were resolved by SDS-PAGE and visualized using Coomassie Blue staining. The histone bands were excised and digested with the endoprotease, trypsin. The resulting tryptic peptides were analyzed by mass spectrometry to determine their individual mass values. Mass values for the specific peptide sequences of the N-terminal portion of histone H3 or histone H4 were attained by matching the measured masses with expected calculated mass values acquired from the International Protein Index (IPI) human database using Sequest Software. Acetylation sites were identified using a differential modification of 42 Daltons added to Lysine residues. The acetylation sites for the N-terminal portion of histone H3 were established showing 13 possible acetylation sites at Lys(K)-4, 9, 14, 18, 23, 27, 36, 37, 56, 64, 79, 115, and 122 (Figure 8) and 11 possible acetylation sites for histone H4 at Lys(K)-5, 8, 12, 16, 20, 31, 44, 59, 77, 79, and 91 (Figure 9A).

In figure 8, control cells treated with solvent (DMSO) only displayed acetylated Lysine residues at all possible sites except for Lys-4, and 9. Cells treated with hydroxychloroquine only showed acetylated Lysine residues at all possible sites except for Lys-4; however, cells pretreated with 1μM ATRA before hydroxychloroquine addition displayed acetylated Lysine residues at all possible sites including Lys-4. Cells treated with only TSA (positive control) also were acetylated at all possible Lysine sites. These results were consistent with the hypothesis of a "zip" model, whereby acetylation of histone H3 proceeds in the direction of from the N-terminal tail Lysine residues to Lys-4, and deacetylation proceeds in the reverse direction. In summary, hydroxychloroquine alone showed acetylation at Lys-9 sites compared to control cells. The modification of acetylation sites proceeded in the direction of acetylation to cover the remaining Lys-4 sites when cells were pretreated with 1μM ATRA.

In figure 9, control cells treated with solvent (DMSO) only displayed acetylated Lysine residues at all possible sites except for Lys-5, 8, and 12. Cells treated with hydroxychloroquine only showed acetylated Lysine residues at all possible sites except for Lys-5, and 8. Interestingly, cells with only TSA treatment (positive control) and cells pretreated with 1μM ATRA before hydroxychloroquine addition displayed acetylated Lysine residues at all possible sites (Figure 9A,B). These results were again consistent with the hypothesis of a "zip" model, whereby acetylation of histone H4 proceeds in the direction of from the N-terminal tail lysine residues to Lys-5, and deacetylation proceeds in the reverse direction (Zhang et al., 2002). In summary, hydroxychloroquine alone showed modification at Lys-12 sites compared to control cells. The remaining acetylation sites, Lys-5, and 8 were modified when cells were pretreated with 1μM ATRA.

Task #3 is complete and Task #4 is partially complete. The combination of ATRA with HCQ will be reassessed using mass spectrometry in order to measure quantitively the level of acetylation. The overall data suggest that hydroxychloroquine may exert its effects by instead regulating acetylation via histone acetyltransferase (HAT) either directly or indirectly since both histone H3 and histone H4 lysine sites were modified in the direction of acetylation, but not all lysine sites were acetylated because histone deacetylase (HDAC) was not completely inhibited by hydroxychloroquine alone. Future experiments will also focus on measuring HAT activity to test the hypothesis that hydroxychloroquine will enhance histone acetylation via augmentation of HAT activity. On the other hand, ATRA displayed its chemotherapeutic effects via inhibition of HDAC activity; therefore, when both ATRA and hydroxychloroquine were combined all lysine sites of histones H3 and H4 were acetylated. Thus, the combination of ATRA with hydroxychloroquine lead to an increase in global acetylation levels.

In addition to the studies delineated, several other experiments were conducted in conjunction with other projects. The results of these additional studies have either been published with the projects that they had supported or have aided in the understanding of future directions.

Training Accomplishments

In addition to the above stated research accomplishments, the P.I. has made several training achievements during the July 1, 2003 through June 30, 2004 funded year. Per the requirements of the P.I.'s Pharmacology and Toxicology Ph.D. Program, an oral comprehensive exam was successfully completed in May of 2004. Results attained during the funded year were also presented in the departmental research forums each semester. The P.I. also completed two semesters of required courses as well as successfully presented and completed a progress report for her committee in June of 2004.

Figure 1A,B. Effect of Chloroquine or Hydroxychloroquine ± ATRA on tumor cell clonogenic survival. MDA-MB-231 or MCF-7 cells ($2x10^5$/35 mm^2 dish) were plated and allowed to attach for 3 hours before ATRA (1μM) addition (Day 0). After 24 hours of treatment with ATRA, the cells were then incubated in the presence of solvent (control, DMSO) or MCF-7 IC_{50} levels of chloroquine (CQ, 33μM) or hydroxychloroquine (HCQ, 57μM). MDA-MB-231 and MCF-7 cells were harvested on days 3 and 4, respectively, and replated at a cloning cell density of $6x10^3$ cells/60 mm^2 dishes in 5ml DMEM/10% FBS and subjected to an undisturbed incubation period (5 or 7 days, respectively). Colonies were visualized by staining the dishes with 3 ml of 0.5% crystal violet, 5% formalin, 50% ethanol, 0.85% NaCl for 3 minutes, then rinsing with tap water. Colonies were scored using a Nikon Eclipse TS100 microscope at 20X magnification with ≥20 MCF-7 cells and ≥50 MDA-MB-231 cells =1 colony. The data are represented as clonogenic fractions, where the clonogenic fraction of the control groups was set to equal 1. Data are the mean ±SEM of 3 independent experiments performed in duplicates.

* Statistically significant differences from the control ($p<0.05$).

a Statistically significant differences from ATRA alone ($p<0.05$).

b Statistically significant differences from CQ alone ($p<0.05$).

c Statistically significant differences from HCQ alone ($p<0.05$).

d Statistically significant differences from ATRA + CQ ($p<0.05$).

Figure 1A. MDA-MB-231

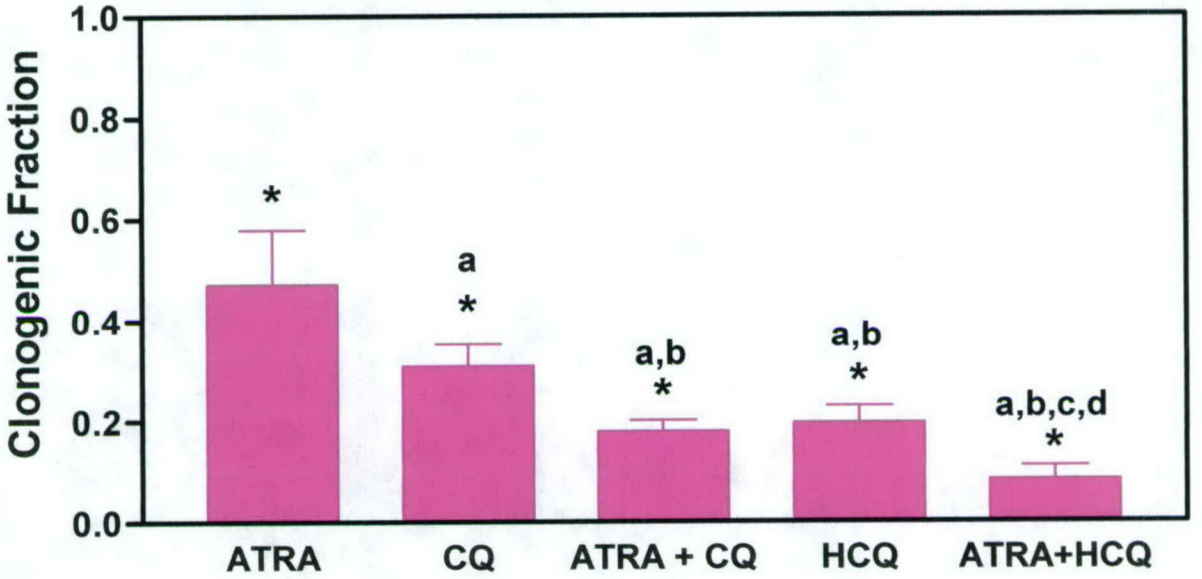


Figure 1B. MCF-7

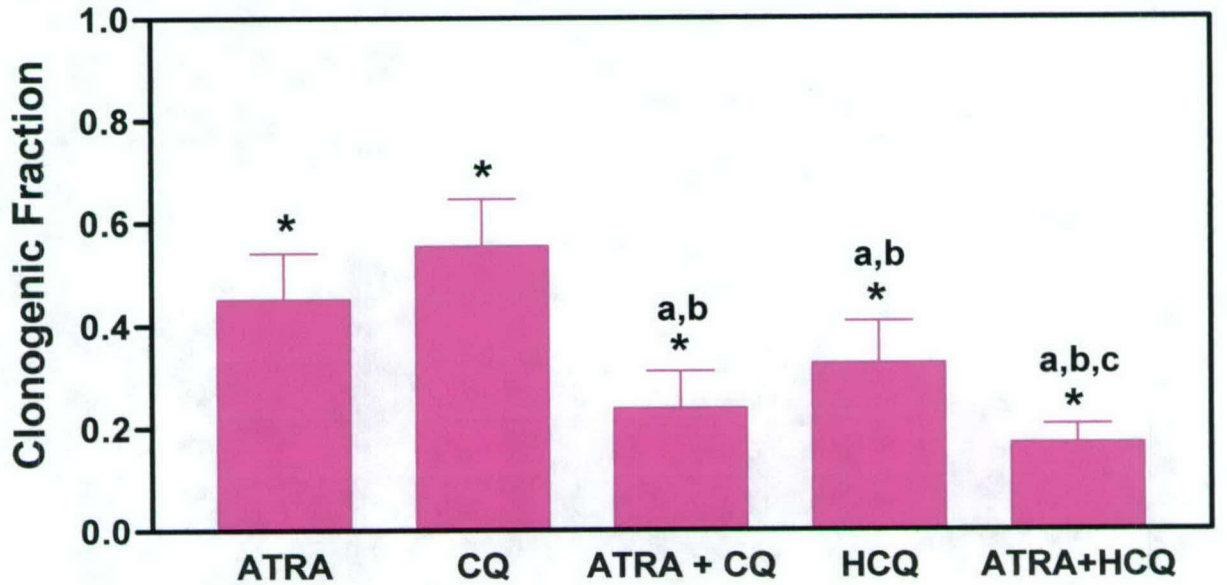


Figure 2A,B. Effect of Chloroquine or Hydroxychloroquine ± 5-Aza-dC on tumor clonogenic survival. MDA-MB-231 or MCF-7 cells ($2x10^5$/35 mm^2 dish) were plated and allowed to attach for 3 hours before 5-Aza-dC (Aza, 1μM) addition. Cells were incubated in the presence of solvent (control, DMSO) or MCF-7 IC_{50} levels of chloroquine (CQ, 33μM) or hydroxychloroquine (HCQ, 57μM) 24 hours later. MDA-MB-231 and MCF-7 cells were harvested on days 3 and 4, respectively, and replated at a cloning cell density of $6x10^3$ cells/60 mm^2 dishes in 5ml DMEM/10% FBS and subjected to an undisturbed incubation period (5 or 7 days, respectively). Colonies were visualized by staining the dishes with 3 ml of 0.5% crystal violet, 5% formalin, 50% ethanol, 0.85% NaCl for 3 minutes, then rinsing with tap water. Colonies were scored using a Nikon Eclipse TS100 microscope at 20X magnification with ≥20 MCF-7 cells and ≥50 MDA-MB-231 cells =1 colony. The data are represented as clonogenic fractions, where the clonogenic fraction of the control groups was set to equal 1. Data are the mean ±SEM of 3 independent experiments performed in duplicates.

* Statistically significant differences from the control ($p<0.05$).

a Statistically significant differences from Aza alone ($p<0.05$).

b Statistically significant differences from CQ alone ($p<0.05$).

c Statistically significant differences from HCQ alone ($p<0.05$).

d Statistically significant differences from Aza + CQ ($p<0.05$).

Figure 2A. MDA-MB-231

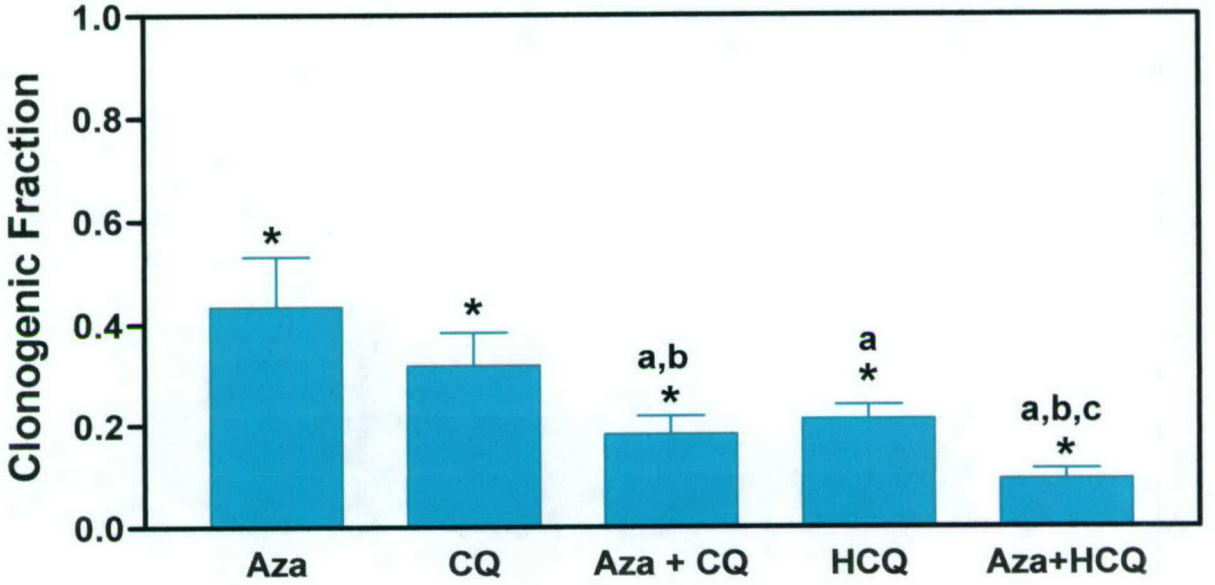


Figure 2B. MCF-7

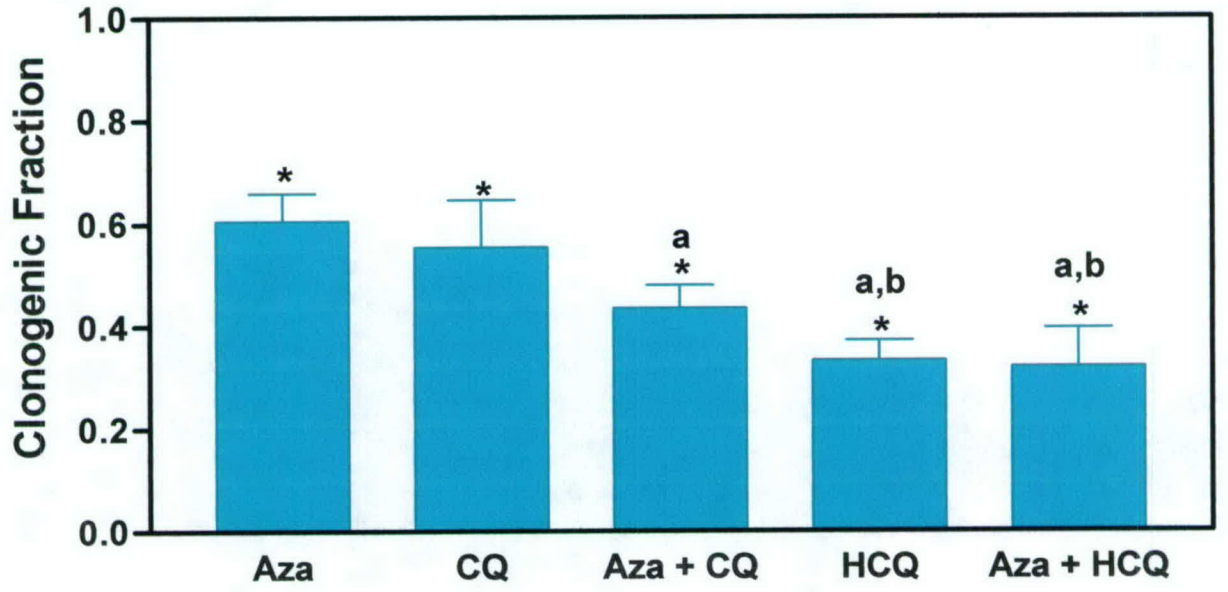


Figure 3. E2F-1 protein expression in MCF-7 cells following treatment with Chloroquine ± ATRA. MCF-7 ($2x10^6$ cells/60 mm^2 dish) were pretreated with 1, 10, or 100 μM ATRA for 24 hours before chloroquine (CQ, IC_{50}=33μM) addition for another 24 hours. Total cellular proteins were isolated and 80μg of whole cell extract proteins were resolved on a 10% acrylamide gel and analyzed by Western blot. Signals were quantitated by FluoroChem (Alpha Innotech, San Leandro, CA) spot densitometry using automatic background subtraction after normalization to β-catenin levels. Data shown represents the mean of three experiments ±SEM.

* Statistically significant differences from the control ($p<0.05$).

a Statistically significant differences from CQ alone ($p<0.05$).

b Statistically significant differences from 1μM ATRA + CQ ($p<0.05$).

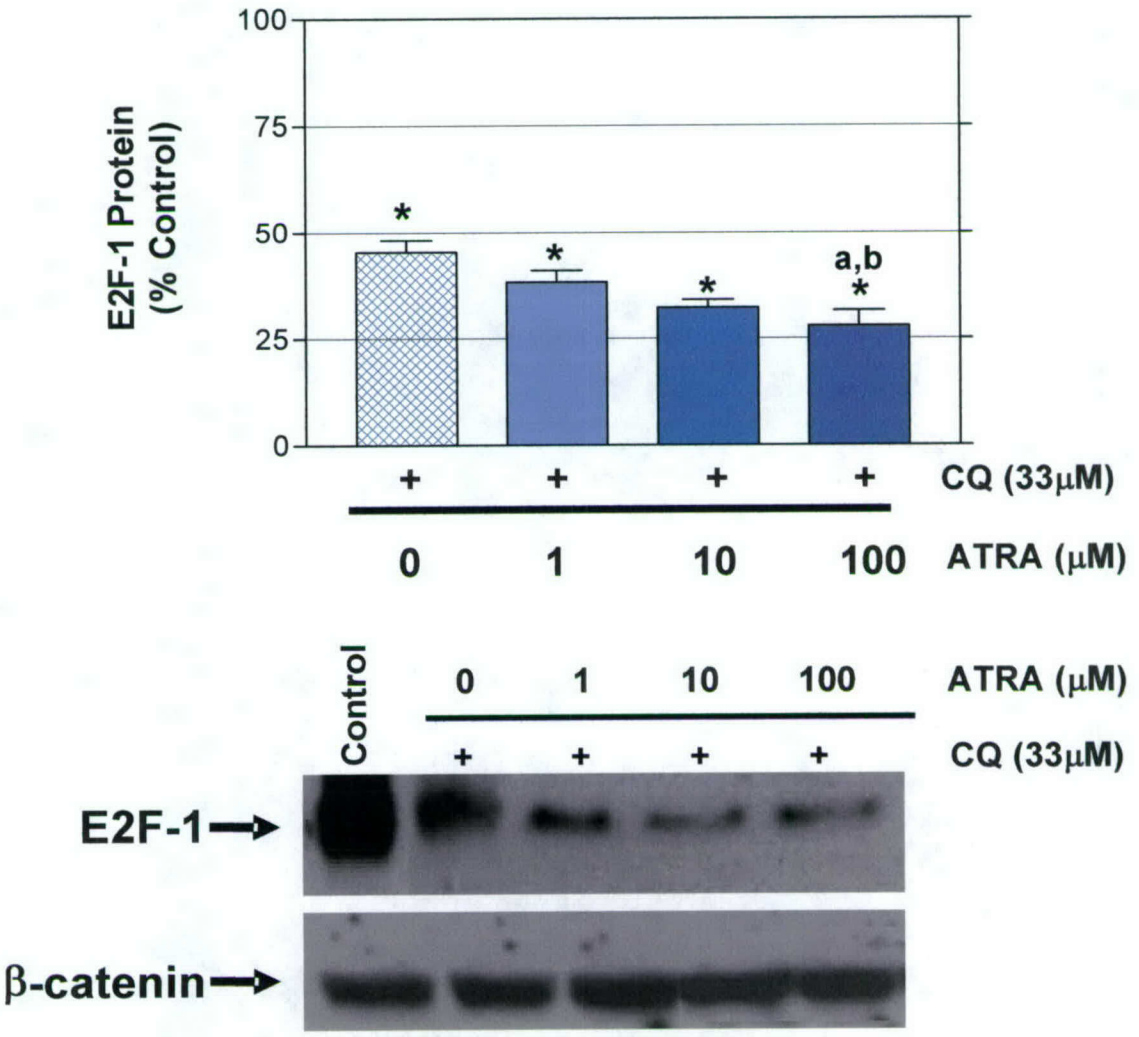

E2F-1 Protein
(% Control)
100
75
50
25
0
*
*
*
a,b
*
+ + + + CQ (33μM)
0 1 10 100 ATRA (μM)
Control
0 1 10 100 ATRA (μM)
+ + + + CQ (33μM)
E2F-1
β-catenin

Figure 4. c-myc protein expression in MCF-7 cells following treatment with Chloroquine ± ATRA. MCF-7 ($2x10^6$ cells/60 mm^2 dish) were pretreated with 1, 10, or 100 μM ATRA for 24 hours before chloroquine (CQ, IC_{50}=33μM) addition for another 24 hours. Total cellular proteins were isolated and 80μg of whole cell extract proteins were resolved on a 10% acrylamide gel and analyzed by Western blot. Signals were quantitated by FluoroChem (Alpha Innotech, San Leandro, CA) spot densitometry using automatic background subtraction after normalization to β-catenin levels. Data shown represents the mean of three experiments ±SEM.

* Statistically significant differences from the control ($p<0.05$).

a Statistically significant differences from CQ alone ($p<0.05$).

b Statistically significant differences from 1μM ATRA + CQ ($p<0.05$).

c Statistically significant differences from 10μM ATRA + CQ ($p<0.05$).

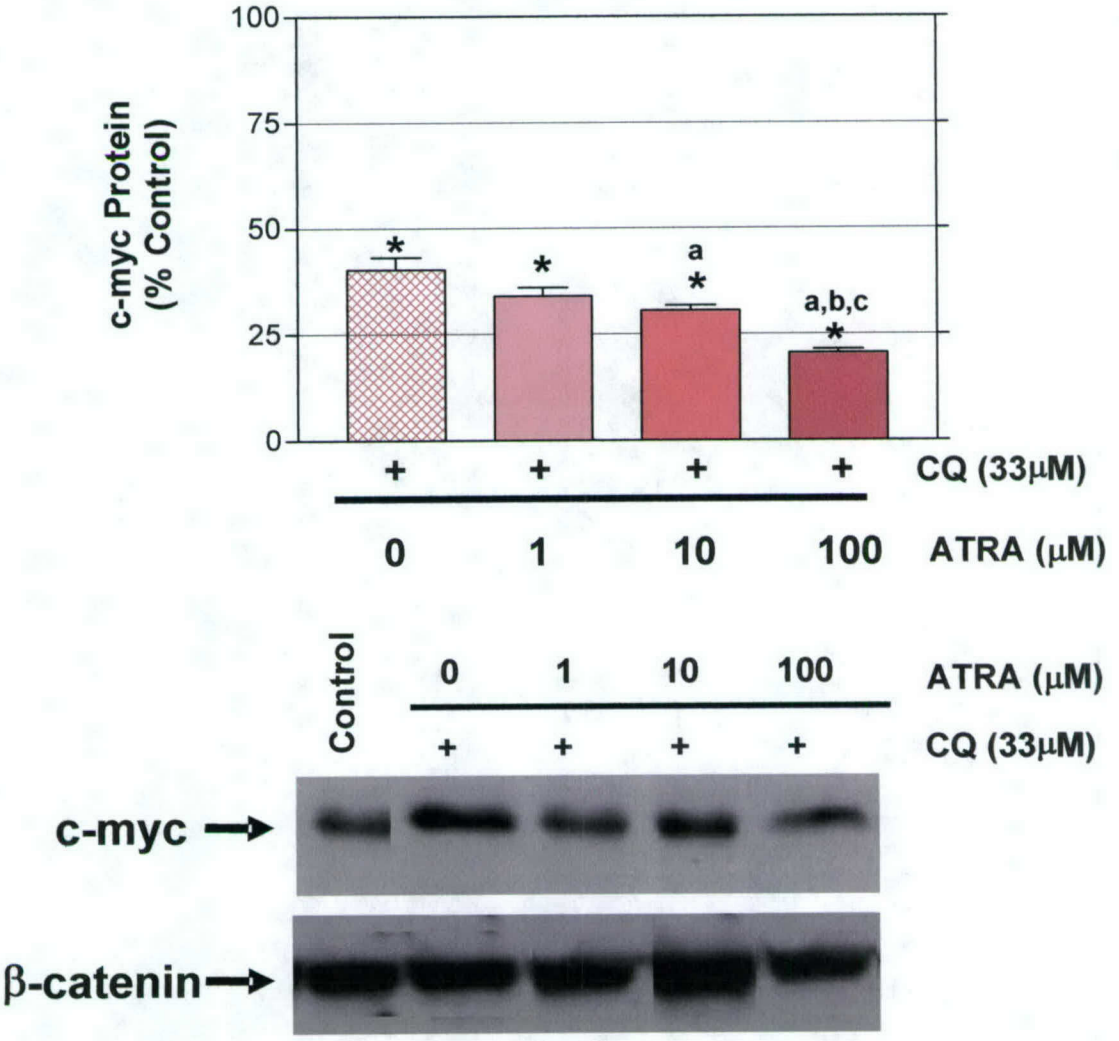

c-myc Protein
(% Control)
100
75
50
25
0
*
*
a
*
a,b,c
*
+
+
+
+
CQ (33μM)
0
1
10
100
ATRA (μM)
Control
0
1
10
100
ATRA (μM)
+
+
+
+
CQ (33μM)
c-myc
β-catenin

Figure 5A-E. HDAC, acetylated histone H4, and RARβ$_2$ protein levels in MDA-MB-231 cells following treatment with Chloroquine or Hydroxychloroquine ± Aza. $2x10^6$ or $6x10^8$ MDA-MB-231 cells were allowed to attach for 3 hours prior to treatment with 1μM Aza for whole cell or histone extraction, respectively. 24 hours after Aza addition, cells were treated with control (DMSO), trichostatin A (TSA, 300nM), chloroquine (CQ, IC_{50}=11μM), or hydroxychloroquine (HCQ, IC_{50}=28μM). At 0.5, 4, 8, 12, and 24 hours after TSA and quinoline treatment, either whole cell lysates were prepared or total histone proteins were isolated using acid-extraction. 80μg of proteins from whole cell lysates or 20μg of purified histone proteins were resolved on a 10% or 15% acrylamide gel, respectively, and analyzed by Western blot. Signals were quantitated by FluoroChem (Alpha Innotech, San Leandro, CA) spot densitometry using automatic background subtraction. Statistically significant differences from the control are indicated ($p<0.05$).

Figure 5A.

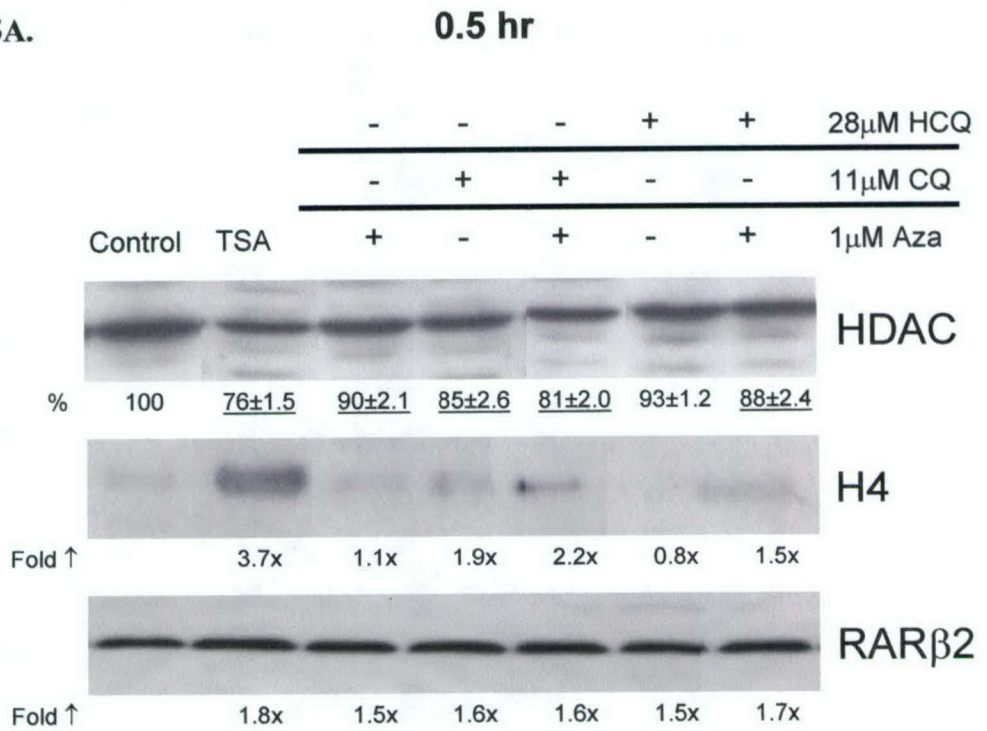


Figure 5B.

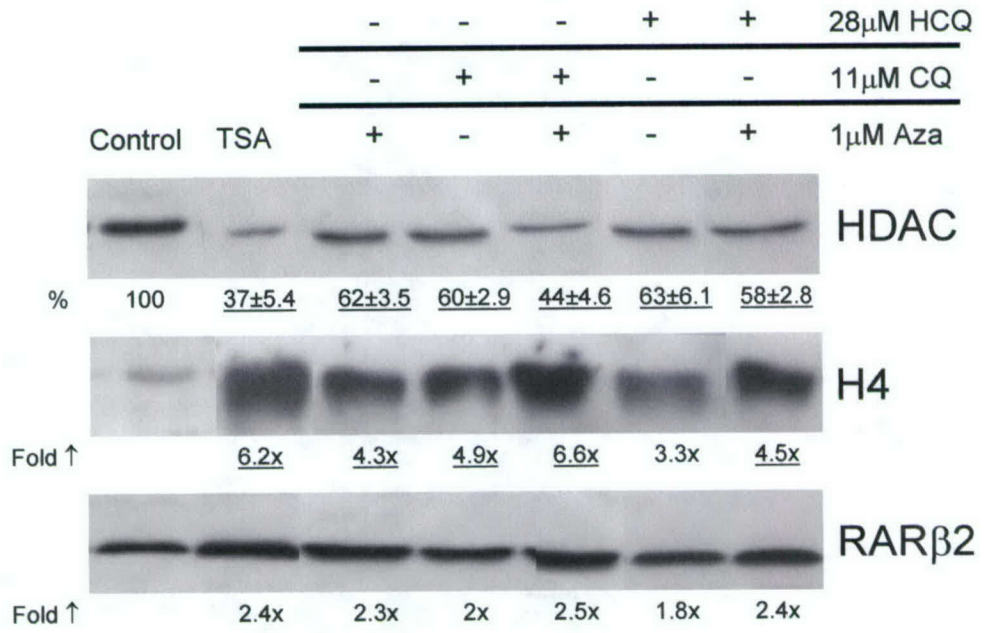


Figure 5C.

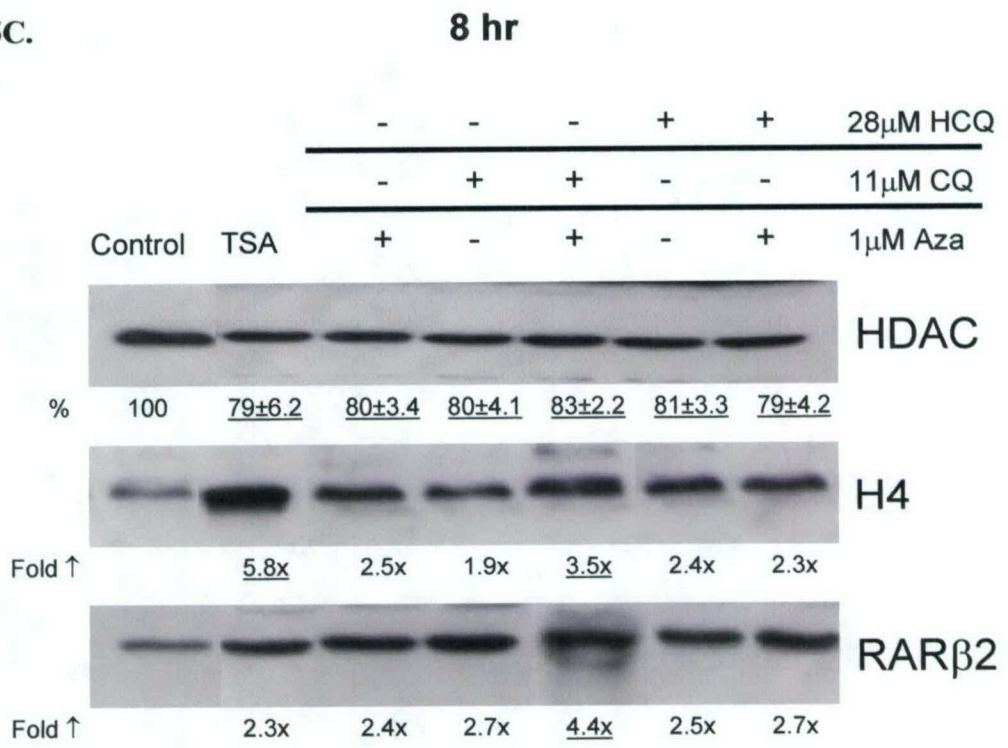


Figure 5D.

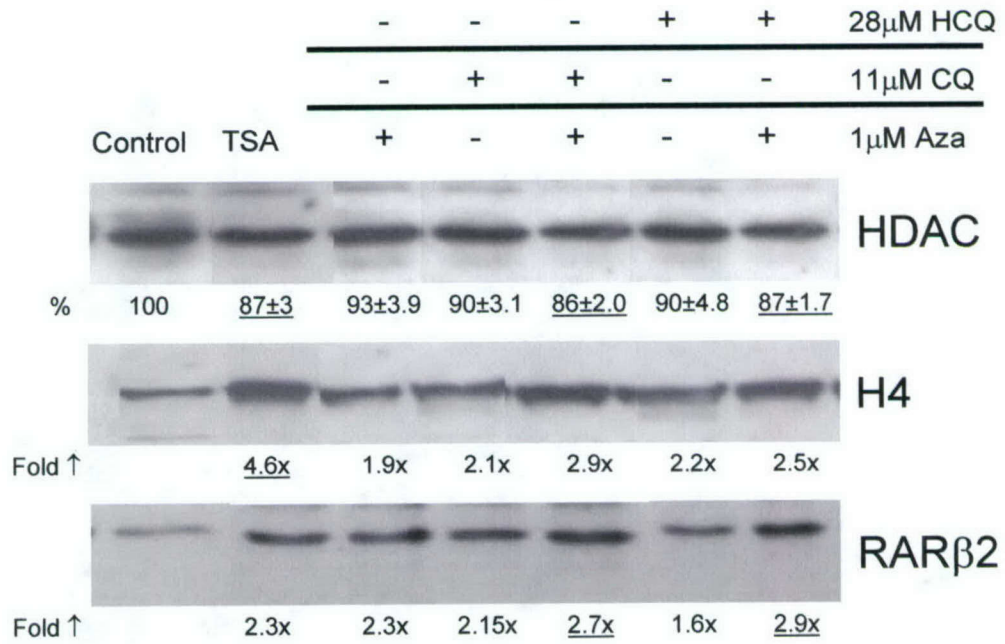


Figure 5E.

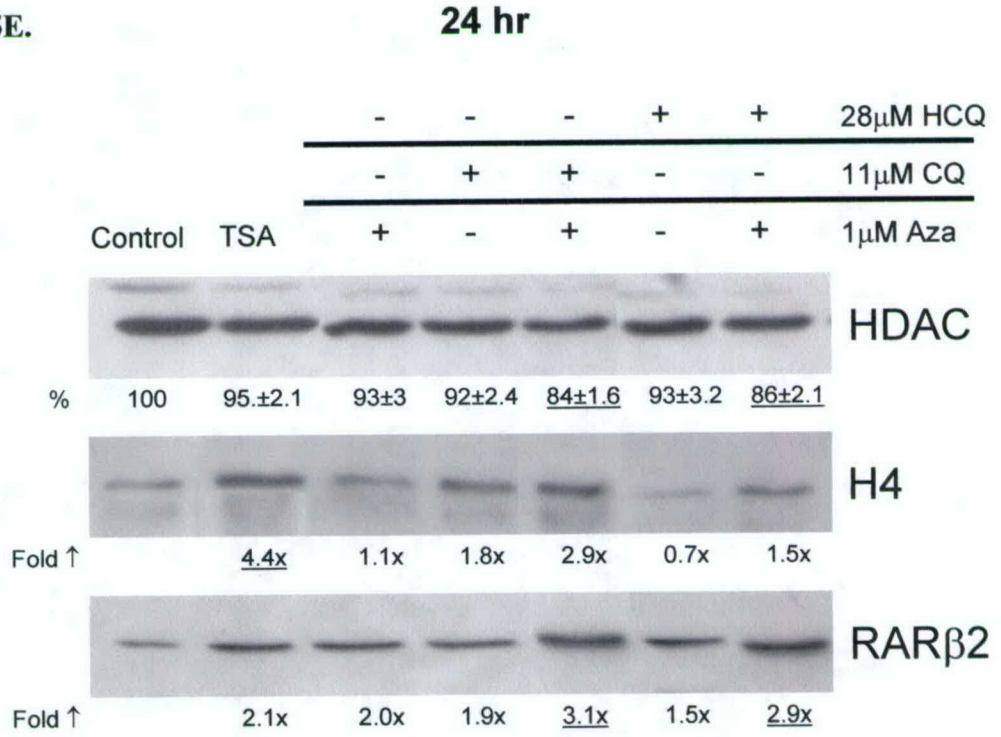

24 hr
- - - + + 28μM HCQ
- + + - - 11μM CQ
Control TSA + - + - + 1μM Aza
HDAC
% 100 95.±2.1 93±3 92±2.4 84±1.6 93±3.2 86±2.1
H4
Fold ↑ 4.4x 1.1x 1.8x 2.9x 0.7x 1.5x
RARβ2
Fold ↑ 2.1x 2.0x 1.9x 3.1x 1.5x 2.9x

Figure 6A-E. HDAC, acetylated histone H3/H4, and RARβ$_2$ protein levels in MCF-7 cells following treatment with Chloroquine or Hydroxychloroquine ± ATRA. $2x10^6$ or $1x10^7$ MCF-7 cells were allowed to attach for 3 hours prior to treatment with 1, 10, or 100μM ATRA for whole cell or histone extraction, respectively. 24 hours after ATRA addition, cells were treated with control (DMSO), trichostatin A (TSA, 300nM), chloroquine (CQ, IC_{50}=33μM), or hydroxychloroquine (HCQ, IC_{50}=57μM). At 0.5, 4, 8, 12, and 24 hours after TSA and antimalarial treatment, either whole cell lysates were prepared or total histone proteins were isolated using acid-extraction. 80μg of proteins from whole cell lysates or 20μg of purified histone proteins were resolved on a 10% or 15% acrylamide gel, respectively, and analyzed by Western blot. Signals were quantitated by FluoroChem (Alpha Innotech, San Leandro, CA) spot densitometry using automatic background subtraction. Statistically significant differences from the control are indicated ($p<0.05$).

Figure 6A.

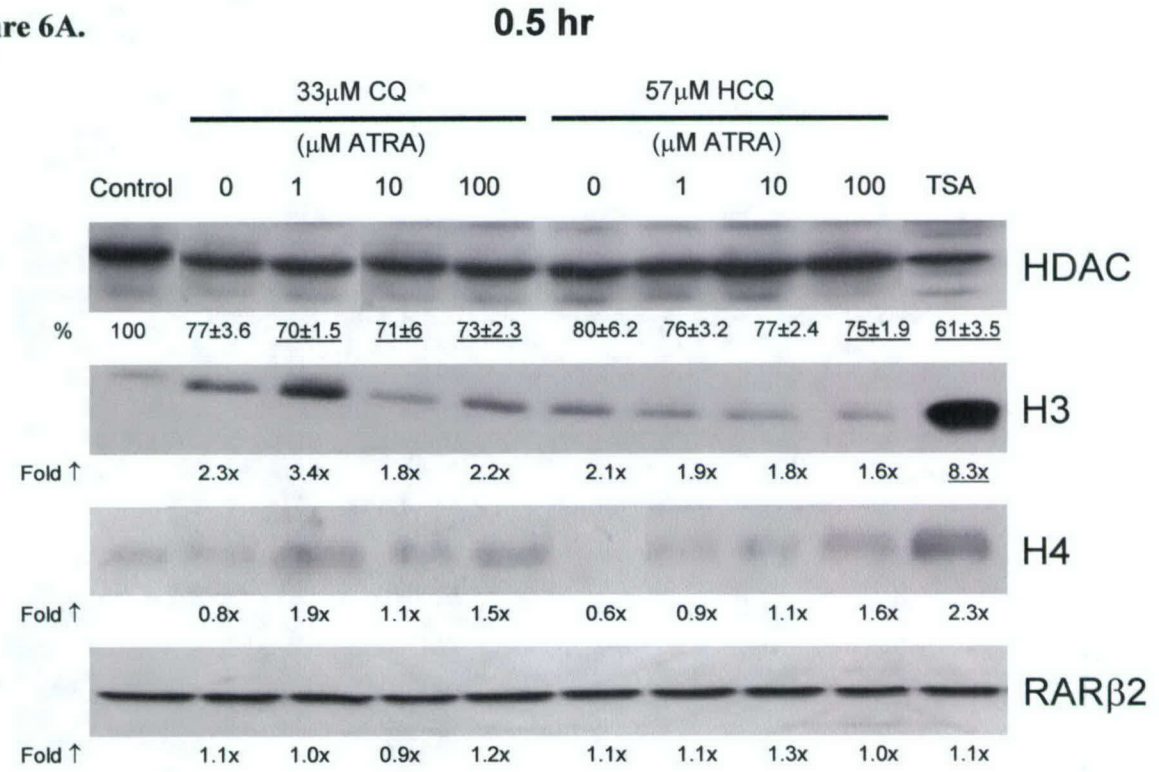


Figure 6B.

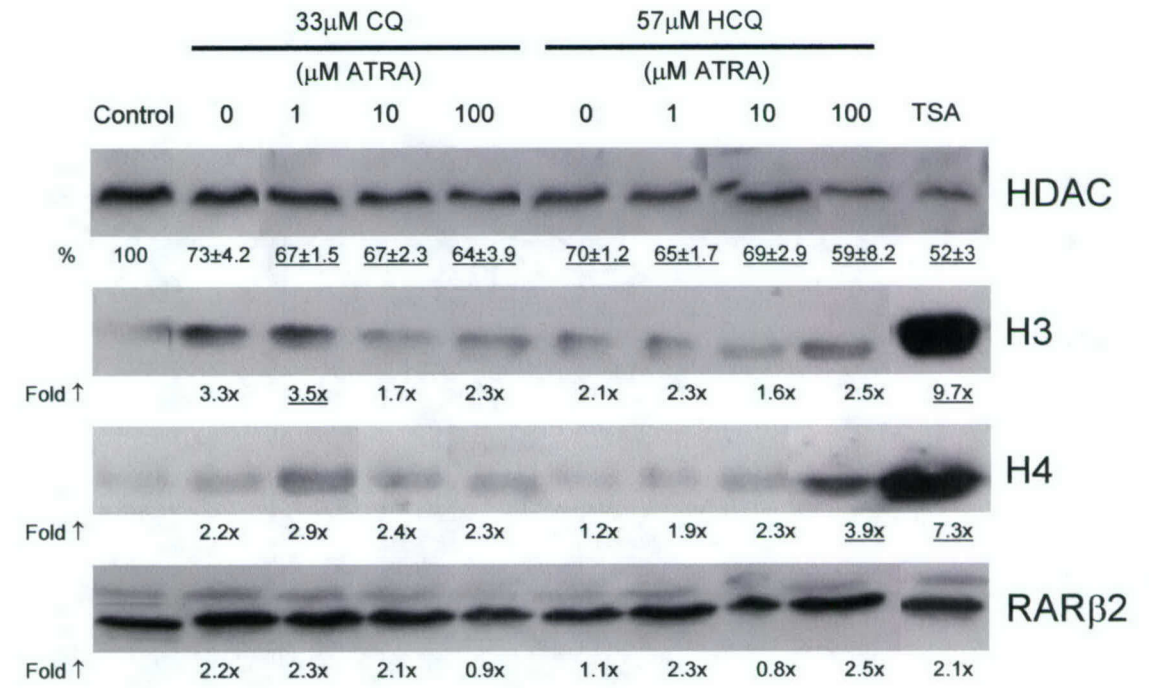


Figure 6C.

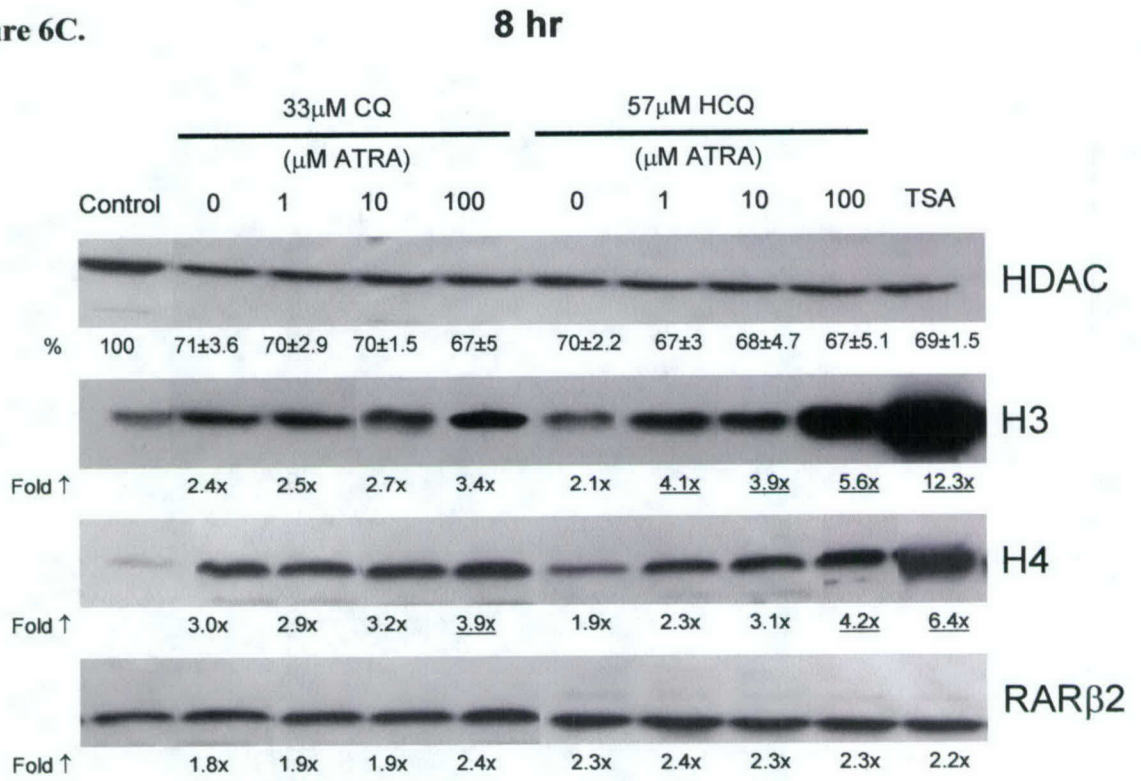


Figure 6D.

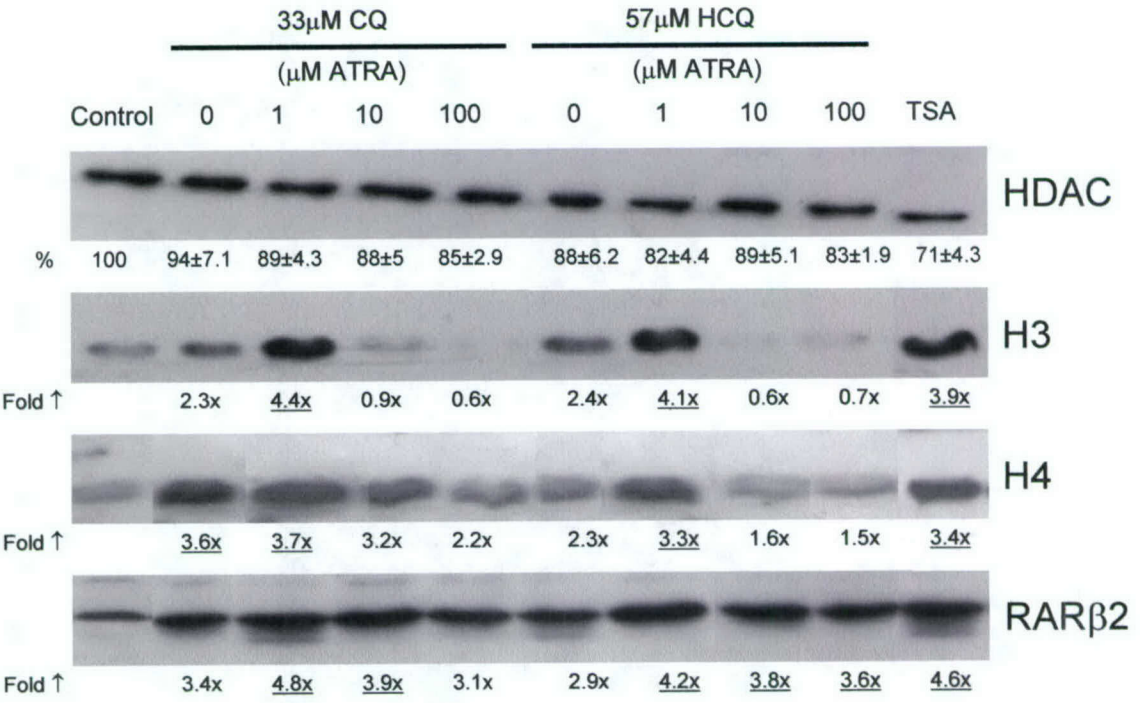
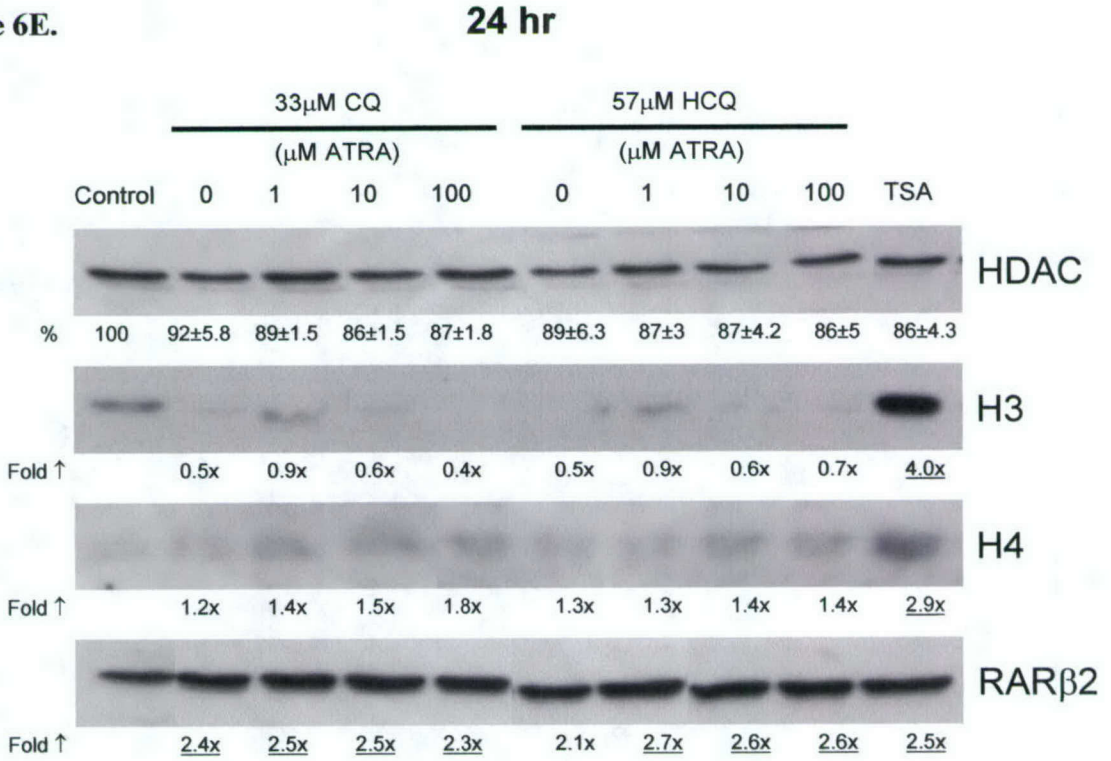


Figure 6E.


24 hr
33μM CQ
57μM HCQ
(μM ATRA)
(μM ATRA)
Control
0
1
10
100
0
1
10
100
TSA
HDAC
%
100
92±5.8
89±1.5
86±1.5
87±1.8
89±6.3
87±3
87±4.2
86±5
86±4.3
H3
Fold ↑
0.5x
0.9x
0.6x
0.4x
0.5x
0.9x
0.6x
0.7x
4.0x
H4
Fold ↑
1.2x
1.4x
1.5x
1.8x
1.3x
1.3x
1.4x
1.4x
2.9x
RARβ2
Fold ↑
2.4x
2.5x
2.5x
2.3x
2.1x
2.7x
2.6x
2.6x
2.5x

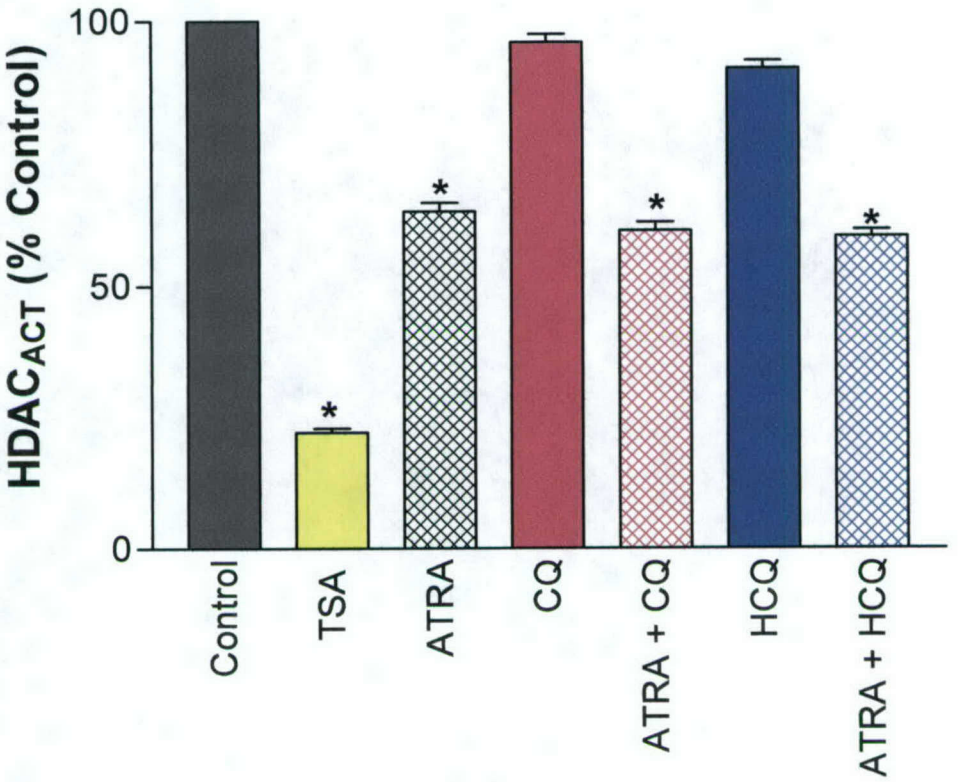


Figure 7. Histone Deacetylase (HDAC) Fluorescent Activity of Chloroquine or Hydroxychloroquine ± ATRA. Drugs were screened for the ability to directly inhibit histone deacetylase (HDAC) activity in HeLa cell nuclear extracts using the HDAC Fluorescent Activity Assay. TSA (35nM) was used as a positive control. Chloroquine (MCF-7 IC_{50}=33μM) or hydroxychloroquine (MCF-7 IC_{50}=57μM) ± 1μM ATRA were tested for HDAC activity. Data are the mean of n=3 experiments ± SEM performed in triplicates per treatment. Statistically significant differences from the control are indicated (*$p<0.05$).

Figure 8. Histone H3 acetylation status in MCF-7 cells following treatment with Hydroxychloroquine ± ATRA. $1x10^7$ MCF-7 cells were either pretreated with 1μM ATRA or not for 24 hours. Cells were then treated with solvent (DMSO; control), trichostatin A (TSA, 300nM), or hydroxychloroquine (CQ, IC_{50}=57μM) for another 12 hours. Total histone proteins were isolated using acid-extraction protocol from Upstate. 20μg of purified histone proteins were resolved on a 15% acrylamide gel and confirmed by Coomassie Blue staining. Histone H3 bands were excised, trypsin digested, and separated using reverse-phase HPLC with C18 column. Samples were analyzed on a ThermoFinnigan LCQ Deca XP Plus ion trap. MS/MS spectra were searched against International Protein Index (IPI) human database using Sequest Software. Acetylation sites were identified using a differential modification of 42 Daltons added to Lysine residues.

Acetylated Histone H3

ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE
IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSSAVM ALQEACEAYL
VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA

Control (DMSO)

ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE
IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSSAVM ALQEACEAYL
VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA

Hydroxychloroquine

ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE
IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSSAVM ALQEACEAYL
VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA

ATRA + Hydroxychloroquine

ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE
IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSSAVM ALQEACEAYL
VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA

TSA

ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE
IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSSAVM ALQEACEAYL
VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA

Figure 9A,B. Histone H4 acetylation status in MCF-7 cells following treatment with Hydroxychloroquine ± ATRA. A.) $1x10^7$ MCF-7 cells were either pretreated with 1μM ATRA or not for 24 hours. Cells were then treated with solvent (DMSO; control), trichostatin A (TSA, 300nM), or hydroxychloroquine (HCQ, IC_{50}=57μM) for another 24 hours. Total histone proteins were isolated using acid-extraction protocol from Upstate. 20μg of purified histone proteins were resolved on a 15% acrylamide gel and confirmed by Coomassie Blue staining. Histone H4 bands were excised, trypsin digested, and separated using reverse-phase HPLC with C18 column. Samples were analyzed on a ThermoFinnigan LCQ Deca XP Plus ion trap. MS/MS spectra were searched against International Protein Index (IPI) human database using Sequest Software. Acetylation sites were identified using a differential modification of 42 Daltons added to Lysine residues. **B.)** MS/MS spectra of a histone H4 peptide sequence in cells pretreated with 1μM ATRA before hydroxychloroquine (HCQ, IC_{50}=57μM) addition.

Figure 9A.

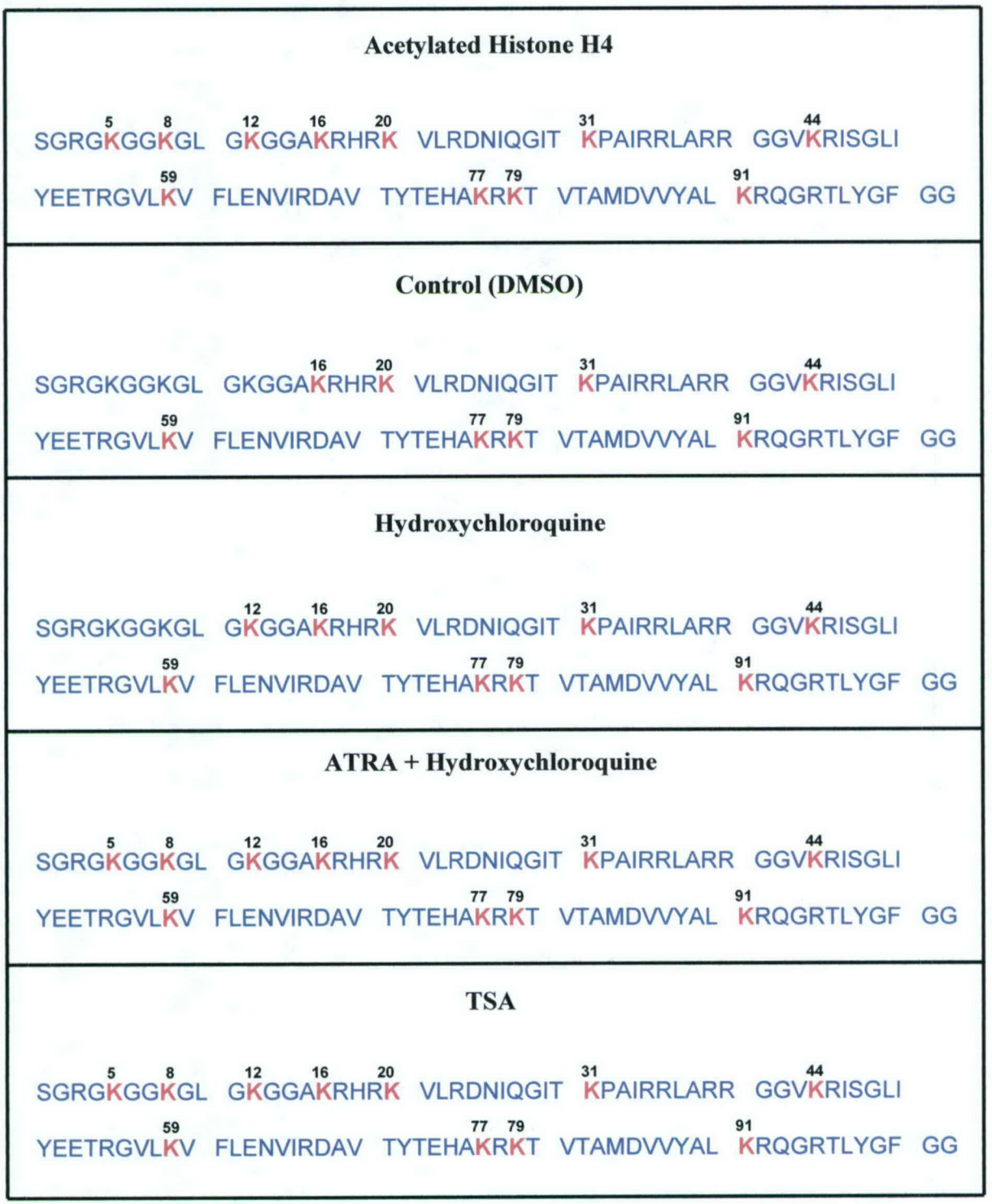
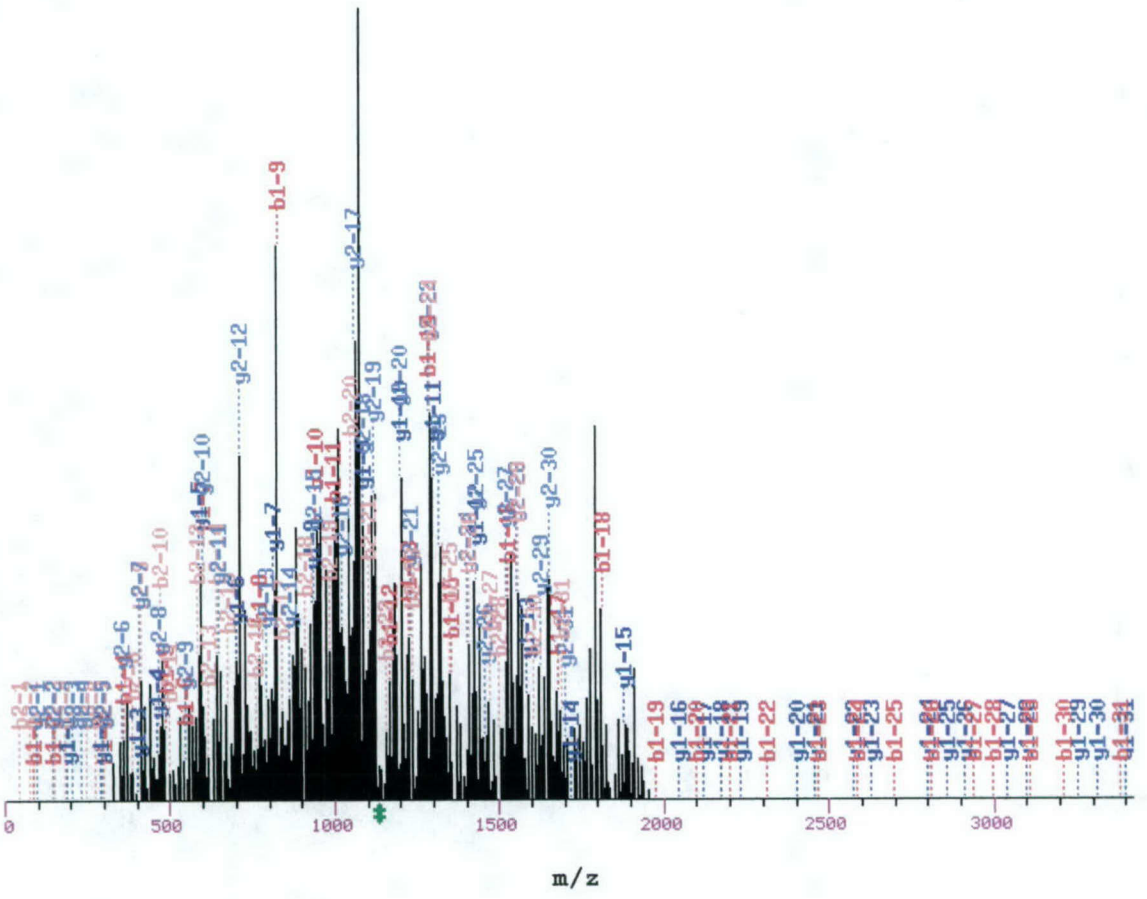

Acetylated Histone H4
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG
Control (DMSO)
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG
Hydroxychloroquine
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG
ATRA + Hydroxychloroquine
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG
TSA
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG

Figure 9B. ATRA + Hydroxychloroquine

Seq	#	b	y	(+1)
S	1	88.0	3397.9	31
G	2	145.1	3310.9	30
R	3	301.2	3253.8	29
G	4	358.2	3097.7	28
K	5	486.3	3040.7	27
G	6	543.3	2912.6	26
G	7	600.3	2855.6	25
*K	8	770.4	2798.6	24
G	9	827.4	2628.5	23
L	10	940.5	2571.5	22
G	11	997.5	2458.4	21
*K	12	1167.6	2401.4	20
G	13	1224.7	2231.3	19
G	14	1281.7	2174.2	18
A	15	1352.7	2117.2	17
*K	16	1522.8	2046.2	16

Seq	#	b	y	(+1)
R	17	1678.9	1876.1	15
H	18	1816.0	1720.0	14
R	19	1972.1	1582.9	13
K	20	2100.2	1426.8	12
V	21	2199.2	1298.7	11
L	22	2312.3	1199.7	10
R	23	2468.4	1086.6	9
D	24	2583.4	930.5	8
N	25	2697.5	815.5	7
I	26	2810.6	701.4	6
Q	27	2938.6	588.3	5
G	28	2995.7	460.3	4
I	29	3108.7	403.2	3
T	30	3209.8	290.2	2
*K	31	3379.9	189.1	1



Key Research Accomplishments:

- Conditions for cellular differentiation optimized. (Task #1)
- Optimization of histone isolation and Western blotting conditions. (Task #2)
- Optimization of qualitative assay using mass spectrometry. (Task #3)
- Analyzed most promising agents using mass spectrometry. (Task #4)

Reportable Outcomes:

Liu, C., Strobl, J.S., Bane, S., Schilling, J.K., McCracken, M., Chatterjee, S.K., Rahim-Bata, R., Kingston, D.G.I. (2003) **Design, synthesis, and bioactivities of steroid-linked taxol analogues as potential targeted drugs for prostate and breast cancer**. *Journal of Natural Products*. 67(2): 152-159. (copy enclosed)

Martirosyan, A.R., Rahim-Bata, R., Freeman, A.B., Clarke, C.D., Strobl, J.S. (2004) **Identification of differentiation-inducing quinolines as experimental breast cancer agents in the MCF-7 human breast cancer cell model.** *Biochemical Pharmacology* (in press).

Conclusions:

The proposed work focused on using two promising antitumor antimalarials in combination with the demethylating agent, 5-Aza-dC, or with the differentiating agent, ATRA in order to lower the threshold for chemotherapy-induced cell death in breast cancer cells by augmenting their differentiation response. Cell survival, cellular differentiation, histone H3 and/or histone H4 acetylation status, and HDAC protein and activity were measured in order to optimize conditions for a new and highly sensitive assay for histone acetylation by mass spectrometry. This new approach illustrated the specific lysine sites that get modified (acetylated/deacetylated) by the most promising combination of chemotherapeutic agents to generate an overall histone acetylation profile.

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Design, Synthesis, and Bioactivities of Steroid-Linked Taxol Analogues as Potential Targeted Drugs for Prostate and Breast Cancer

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Design, Synthesis, and Bioactivities of Steroid-Linked Taxol Analogues as Potential Targeted Drugs for Prostate and Breast Cancer[⊥]

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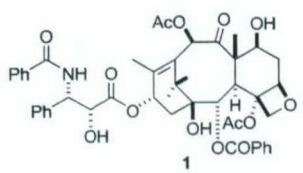
The female steroid hormone 3,17β-estradiol (**2**) was selected as an agent to target taxol (**1**) to estrogen receptor (ER) positive breast cancer cells. Estradiol−taxol conjugates (ETC) were synthesized through linkages from the 11- or 16-position of estradiol to the 2′-, 7-, or 10-position of taxol. All conjugates were cytotoxic to the A2870 ovarian cancer cell line, although less so than taxol. The MCF-7 breast cancer cell line (ER-α positive) and MDA-MB-231 breast cancer cell line (ER-α negative) were also used to evaluate the selectivity and cytotoxicity of these conjugates. One conjugate showed some selectivity for ER positive cells, but it was less potent than taxol. Two ETC hemisuccinates were also prepared to improve the solubility of the conjugates. The corresponding Na and triethanolammonium salts were slightly more cytotoxic than the acid form but were much less cytotoxic than the corresponding ETC.

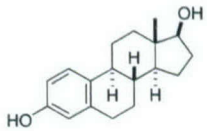
Taxol[1] (**1**) was first isolated from the bark of the Pacific yew about 35 years ago by Drs. Wall and Wani.[2] Although its development as an anticancer agent was delayed by numerous reasons, including its scarcity and insolubility,[3] the discovery of its tubulin-assembly activity[4] and other factors motivated oncologists to overcome these problems. It has since become one of the most important current drugs for the treatment of several cancers, including breast and ovarian cancers;[5] its importance in the treatment of breast cancer has been reviewed,[6] as has its chemistry.[7]

Like almost all anticancer drugs taxol does have some toxic side effects, such as bone marrow suppression and neutropenia,[8] and many tumors also show significant resistance to therapy with taxol.[9] One approach to improving its selectivity and efficacy is by targeting it to selected tumors through the use of various conjugates, and several taxol conjugates have been synthesized recently with improved selectivity and solubility.[10] Thus Safavy reported a water-soluble and tumor-recognizing conjugate of taxol and BBN[7-13], which retained binding ability to the BBN/GRP receptor compared to the free BBN[7-13] molecule.[10a] Huang used the binding ability of somatostatin (SST) to its receptors (SSTRs) to specifically target taxol to tumor cells.[10b] A report from Luo revealed that a conjugate of hyaluronic acid and taxol was selectively toxic toward the human cancer cell lines that are known to overexpress HA receptors.[10c] Fuchs and co-workers have reported the preparation and evaluation of taxol−folic acid conjugates.[10d] Finally, Ojima has reported a C-10 methyldisulfanylpropanoyl taxoid conjugated to monoclonal antibodies; these conjugates were shown to possess selective in vivo antitumor activity against EGFR-expressing A431 tumor xenografts.[10e]

One approach that has not yet been explored is that of targeting taxol to breast cancer by means of selected steroid hormone conjugates. The female hormone estradiol (**2**) plays an important role in breast cancer, and the hormone dependence of breast cancer was first reported by Beatson in the late 1800s.[11] Further studies revealed the interaction between steroid hormones and their receptors[12] and, thus, led to a better understanding of the hormone in controlling the growth of breast cancer.[13] The hormone dependence of breast cancer can also be used as a drug delivery target through the recognition and binding of estrogen to its receptor, and several studies have investigated the targeting of drug molecules into breast cancer cells by linking them to estradiol or other estrogens.[14] The potential benefits of this approach include the improvement of a drug's therapeutic effectiveness and bioavailability, coupled with a reduction in multidrug resistance (MDR) and toxic side-effects.



1



2

The goal of the present research was to target taxol to estrogen receptor (ER) positive breast cancer cells through the interaction between estradiol and its corresponding receptor, with the goal of developing new drug candidates against breast cancer, responsible for the second largest number of cancer deaths in women.[15] From previous studies of the structure−activity relationships (SAR) of estradiol, it is known that estradiol can be modified at the 16- and 11-positions without losing its ability to bind to the ER.[14] As for taxol, SAR studies have shown that the 10- and 7-positions can be acylated with only relatively minor effects on the drug's activity.[7a,16] Another position that can be used for targeting is the 2′-position, because ester linkages at this position can be hydrolyzed in vivo,[17] and hence an estradiol−taxol conjugate at the 2′-position could serve as a "targeted pro-drug" if the targeting occurred before hydrolysis. In this paper, we describe the synthesis and biological evaluation of estradiol−taxol

[⊥] Dedicated to the late Dr. Monroe E. Wall and to Dr. Mansukh C. Wani of Research Triangle Institute for their pioneering work on bioactive natural products.

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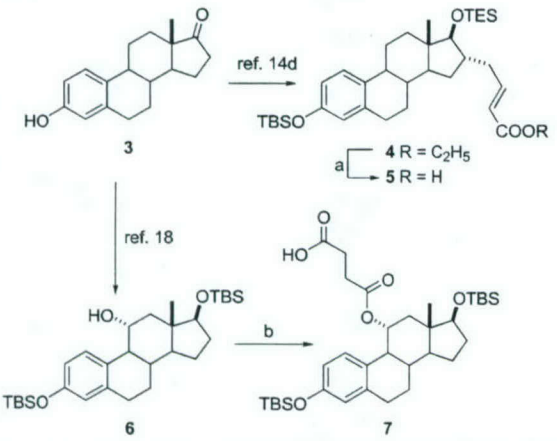
Scheme 1[a]



[a] Reagents and conditions: (a) LiOH, THF/H_2O, RT, 36 h, 72%; (b) LHMDS, THF, then succinic anhydride, RT, overnight, 70%.

conjugates through ester linkers from the 11- and 16-positions of estradiol to the 2′-, 7-, and 10-positions of taxol.

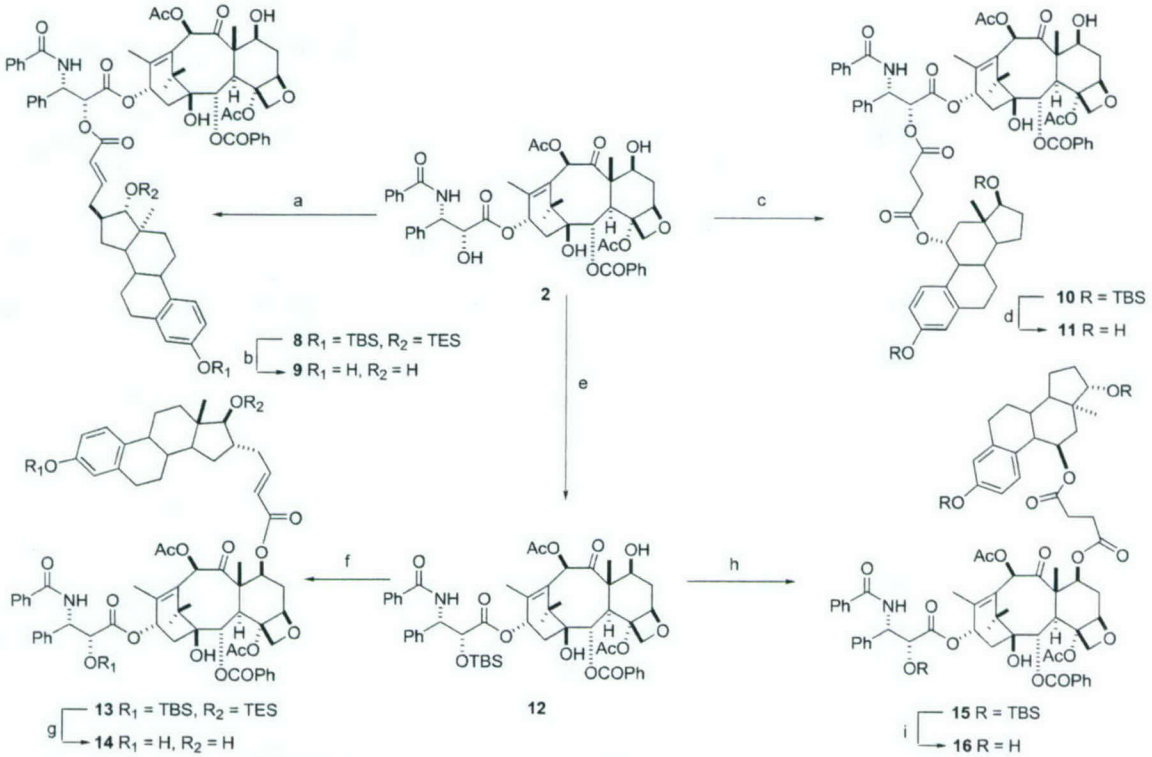
Results and Discussion

Synthesis. The synthesis of estradiol linkers **5** and **7** is outlined in Scheme 1. The commercially available estrone (**3**) was converted to compounds **4**[14d] and **6**[18] through reported procedures. Compound **4** was hydrolyzed to generate compound **5** with a free carboxyl group for coupling. The linker **7** was obtained by reacting **6** with succinic anhydride. The use of pyridine as solvent only gave a 30% yield, but deprotonation of **6** with LHMDS in THF followed by addition of succinic anhydride gave **7** in 70% yield based on unrecovered starting materials.

With the two estradiol linkers **5** and **7** in hand, the estradiol–taxol conjugates could be assembled. According to SAR studies, the most reactive hydroxyl group in taxol is the 2′-OH, followed by the 7- and 10-OH groups; the 1-OH group is inert to ester formation under normal conditions.[19] Direct acylation of taxol with compounds **5** and **7** thus yielded the 2′-acyl derivatives **8** and **10** (Scheme 2), respectively. Protection of the 2′-hydroxyl group as its *tert*-butyldimethylsilyl ether **12**, followed by acylation with compounds **5** and **7**, gave the 7-acyl analogues **13** and **15**. In general, conjugate formation occurred in low yield, with conversion percentages of 25–35%, and with significant amounts of unreacted taxol; the yields based on unrecovered taxol were in the range 60–70%. Deprotection of the silyl groups with HF–pyridine proceeded in good yields to give the estradiol–taxol complexes **9**, **11**, **14**, and **15**.

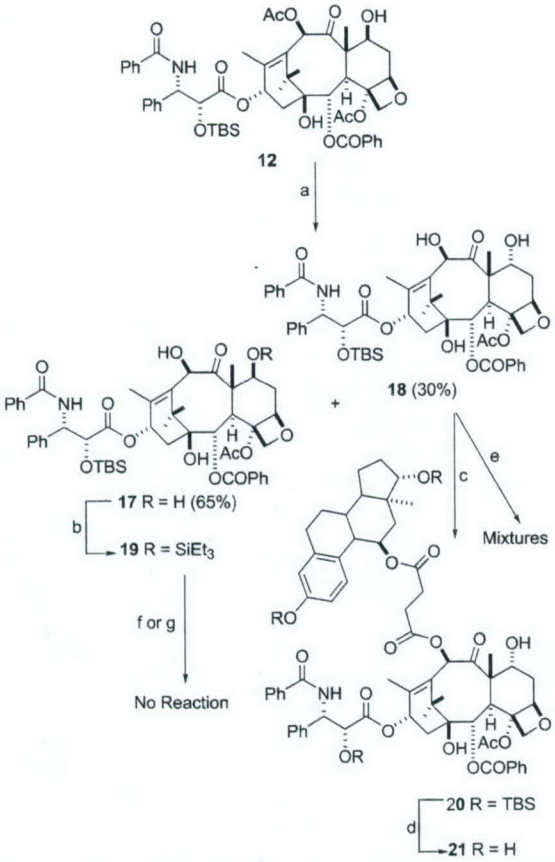
The synthesis of estradiol–taxol conjugates at the 10-position was achieved by converting 2′-(*tert*-butyldimethylsilyl)taxol (**12**) to 2′-(*tert*-butyldimethylsilyl)-10-deacetyltaxol (**17**) and hence to 2′-(*tert*-butyldimethylsilyl)-10-deacetyl-7-(triethylsilyl)taxol (**19**) through a known procedure.[20] During the deacetylation of **12** using hydrazine monohydrate in ethanol, a byproduct of 30% of 2′-(*tert*-butyldimethylsilyl)-7-*epi*-taxol (**18**) was observed (Scheme 3). Unfortunately, compound **19** did not undergo ester formation using standard EDC/DMAP conditions. One possibility is that the 10-position was too sterically hindered to accept the relatively short linkage to estradiol because the bulky 7-TES group might somehow block this position. To test this hypothesis, 2′-(*tert*-butyldimethylsilyl)-7-*epi*-taxol was used as a substrate, since this not only lacked the bulky 7-TES group but also had an unreactive 7-*epi*-hydroxyl group.[21] Compound **18** reacted with estradiol **7** smoothly under EDC/DMAP conditions in CH_2Cl_2 to give product **20** in 79% yield. Deprotection of **20** by HF–pyridine gave **21** in good yield. Coupling of linker **5** with **18** was also attempted under the same conditions, but two

Scheme 2[a]



[a] Reagents and conditions: (a) **5**, EDC/DMAP, toluene, 60 °C, 24 h, 73%; (b) HF–pyridine, THF, RT, overnight, 97%; (c) **7**, EDC/DMAP, toluene, 60 °C, 24 h, 78%; (d) HF–pyridine, THF, RT, overnight, 92%; (e) TBSCl, imidazole, DMF, 65 °C, 3 h, 95%; (f) **5**, EDC/DMAP, toluene, 60 °C, 48 h, 65%; (g) HF–pyridine, THF, RT, overnight, 82%; (h) **7**, EDC/DMAP, toluene, 60 °C, 48 h, 65%; (i) HF–pyridine, THF, RT, overnight, 91%.

Scheme 3[a]



[a] Reagents and conditions: (a) hydrazine monohydrate, EtOH, RT, 1.5 h; (b) TESCl, imidazole, DMF, RT, 10 min, 89%; (c) **7**, EDC/DMAP, CH_2Cl_2, RT, 24 h, 79%; (d) HF–pyridine, THF, RT, overnight, 90%; (e) **5**, EDC/DMAP, toluene or CH_2Cl_2, RT, 24 h; (f) **5**, EDC/DMAP, toluene, or CH_2Cl_2, RT, 72 h; (g) **7**, EDC/DMAP, toluene, or CH_2Cl_2, RT, 72 h.

inseparable products were obtained as determined by NMR spectroscopy.

It is well known that taxol has very low solubility in water, and the estradiol–taxol conjugates would be expected to be even less soluble, since estradiol is hydrophobic. We thus synthesized two estradiol–taxol conjugates with improved water solubility. It is been reported that a hemisuccinate at the 2′-position of taxol can improve the drug's solubility when the free carboxyl group was neutralized as its sodium or (triethanol)ammonium salts.[19a] Scheme 4 shows the synthesis of two estradiol–taxol conjugates with either a 2′-hemisuccinate or a 7-hemisuccinate ester group. Compound **22** was prepared by reaction of taxol with monobenzyl succinate using EDC/DMAP conditions to protect the 2′-position. This was followed by introduction of the estradiol linker **7** at the 7-position using the conditions described previously. The desired compound **25** was obtained after desilylation and hydrogenolysis; its sodium and triethanolamine salts (**26** and **27**) were also prepared. The 7-hemisuccinate **30** was obtained using the reverse order of steps, with initial acylation of taxol with estradiol **7** followed by acylation with monobenzyl succinate at C-7 and deprotection; its sodium and triethanolamine salts (**31** and **32**) were also prepared by a previously reported procedure.[19a]

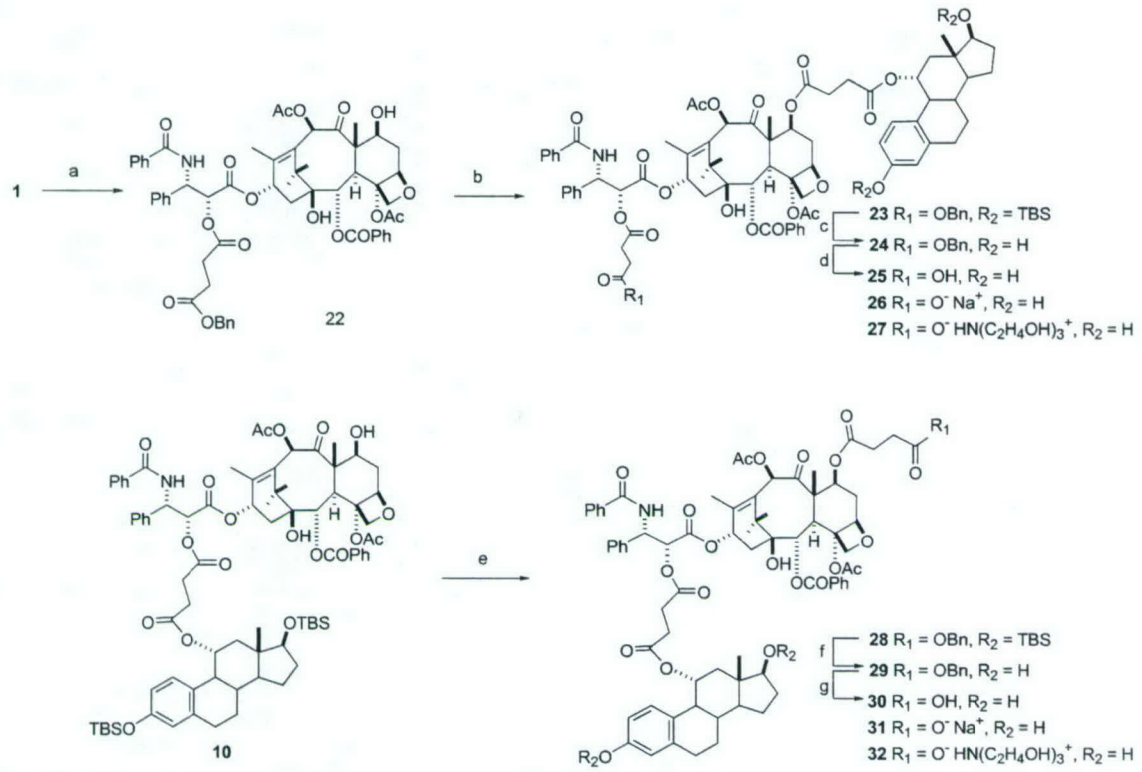
Biological Results. The biological activities of taxol and of the estrogen conjugates **9**, **11**, **14**, **16**, and **21** were compared in a tubulin-assembly assay, for cytotoxicity to estrogen receptor (ER) negative A2780 ovarian cancer cells, ER (beta) positive PC-3 prostate cancer cells, and two lines of human breast tumor cells (Table 1). The taxol IC_{50} value estimated by nonlinear regression analysis was similar in the ER-α positive MCF-7 and ER-α negative MDA-231 lines, 4.9 and 4.5 nM, respectively, and both these values were lower by over an order of magnitude than the IC_{50} value in the PC-3 prostate cancer cell line. The 2′-substituted taxol conjugates **9** and **11** were both about as active as taxol in the PC-3 cell line, but were less active than taxol in the breast cancer lines. They were also less active than taxol in the tubulin-assembly assay. These results are explicable by postulating that the 2′-derivatives undergo slow conversion to taxol under the conditions of the cell culture, with the conversion being more rapid in the PC-3 assay than in the two breast cancer cell lines; the lower activity of both compounds in the tubulin-assembly assay is consistent with this hypothesis. Similar results were obtained for the activity of 2′-acetyltaxol.[22] Neither compound **9** nor **11** showed any significant selectivity for the ER-α positive cell line MCF-7 as compared with the ER-α negative line MDA-MB-231; this result is also consistent with hydrolysis under cell culture conditions. Interestingly, the MDA-MB-231 breast tumor cell line which expresses ER-β receptors was more sensitive to compounds **9** and **11** than the ER-α positive MCF-7 breast tumor line. In addition, the steroid conjugate **11** may show improved activity compared with taxol against the PC-3 line. Recently, clinical samples of prostate cancer as well as certain prostate cell lines (PC-3) have been found to express ER-β receptors, and expression is correlated with tumor aggressiveness on the Gleason scale.[23] These data raise the possibility that these derivatives target the beta form of the estrogen receptor.

Table 1. Cytotoxicity and Tubulin-Assembly Activity of Steroid-Linked Taxol Derivatives

compound	% assembly, 0.2 μM[a]	% assembly, 1.0 μM[a]	A2780 IC_{50} (nM)	PC-3 IC_{50} (nM)	MDA-MB-231 IC_{50} (nM)	MCF-7 IC_{50} (nM)
taxol (**1**)	100	100	25	77 ± 3	4.5 ± 1.2	4.9 ± 1.8
9	45	55	180	73 ± 12	22 ± 4.5	39 ± 0.6
11	60	60	680	40 ± 10	51 ± 4.6	62 ± 12
14	100	100	8300	120 ± 20	2200 ± 800	1600 ± 90
16	100	100	2900	320 ± 80	780 ± 100	557 ± 117
21	100	100	1900	68 ± 7	304 ± 12[b]	103 ± 3.4[b]
25	NT	NT	15 000	NT	NT	NT
26	NT	NT	10 000	NT	NT	NT
27	NT	NT	13 000	NT	NT	NT
30	NT	NT	15 000	NT	NT	NT
31	NT	NT	12 000	NT	NT	NT
32	NT	NT	10 000	NT	NT	NT

[a] The extent of tubulin assembly induced by 0.2 and 1.0 μM taxol and by each compound with 10 μM tubulin was determined. The extent of tubulin assembly in the presence of taxol is defined as 100%, and the extent of tubulin assembly with each ligand was compared with this value. [b] $n = 3$ experiments in quadruplicate, $p < 0.001$.

Scheme 4[a]



[a] Reagents and conditions: (a) $BnOCOCH_2CH_2COOH$, EDC/DMAP, CH_2Cl_2, RT, 24 h, 40%; (b) **7**, EDC/DMAP, CH_2Cl_2, RT, 48 h, 70%; (c) HF–pyridine, THF, RT, overnight, 98%; (d) H_2, Pd–C, EtOAc, 30 psi, 24 h, 80%; (e) $BnOCOCH_2CH_2COOH$, EDC/DMAP, CH_2Cl_2, RT, 48 h, 90%; (f) HF–pyridine, THF, RT, overnight, 99%; (g) H_2, Pd–C, EtOAc, 50 psi, 24 h, 50%.

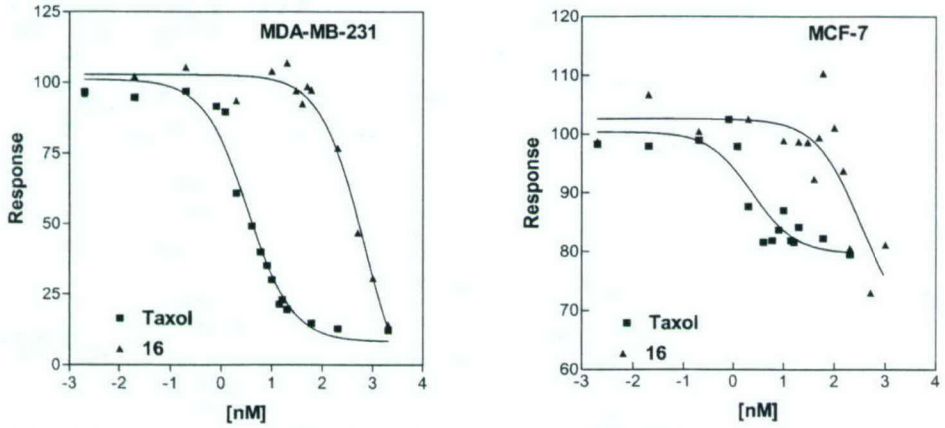


Figure 1. Inhibition of breast tumor cell survival in vitro by taxol and compound **16**. Human breast tumor cell lines were incubated for 48 h with the indicated drug concentrations. The cell survival response in estrogen-receptor negative MDA-MB-231 cells and estrogen-receptor positive MCF-7 cells was determined using the MTS assay. Response (A_{490} nm) is plotted as a fraction of control cells, which was set to 100%. A nonlinear regression fit to a sigmoidal dose–response equation is shown. Data are representative of $n = 2$ independent experiments performed in quintuplicate.

The two C-7-substituted derivatives **14** and **16** were both significantly less potent cytotoxic agents than taxol in the two breast cancer cell lines, although both compounds were comparable to taxol in their tubulin-assembly activity and were only less active in the PC-3 cell line by factors of 1.6 and 4.2, respectively. Although both compounds showed modest selectivities toward the ER-α positive cell line MCF-7, the observed differences were not statistically significant.

The dose–response curves for taxol and compound **16** are shown in Figure 1. The maximal antiproliferative response to either taxol or **16** was 85–90% inhibition of cell survival by 48 h; thus the efficacy of both compounds was equivalent. In MCF-7 cells, the maximal reduction in cell survival elicited by either taxol or **16** in a 48 h incubation was 20–30%. When MCF-7 cells were incubated with taxol or **16** for 7 days, the maximal decrease in cell survival was approximately 70%. The difference in the efficacy of taxol and the steroid-conjugated derivative between MDA-MB-231 cells and MCF-7 cells is most likely the differences in the cell doubling time. For MDA-MB-231 cells with a cell doubling time of approximately 22 h, nearly all cells are exposed to taxol or **16** during a sensitive stage of the cell cycle during the 48 h incubation period. However, MCF-7 cells, with a doubling time of nearly 60 h, require a much longer period of drug exposure before a similar fraction of the cells enter or transit the taxol-sensitive phase of the cell cycle.

The 10-substituted derivative **21** gave the most interesting results. It had comparable activity to taxol in both the tubulin-assembly and PC-3 assays, and it also showed a 3-fold greater activity ($p < 0.001$) toward the ER-α positive MCF-7 cell line than the ER-α negative MDA-MB-231 cell

line. It was however significantly less potent than taxol to both these cell lines. Our results do, however, suggest that future efforts at targeting taxol to ER-α positive breast cancer cells would be most fruitful if centered around modifications at the C-10 position.

The hemisuccinates **25**−**27** and **30**−**32** were tested only in the A2780 ovarian cancer cell line; they were all found to be significantly less active that taxol, and so were not subjected to further testing.

Experimental Section

General Experimental Procedures. Chemicals were obtained from Aldrich Chemical Co. and were used without further purification. All solvents were of reagent grade or HPLC grade. THF was distilled over sodium/benzophenone, and CH_2Cl_2 was distilled over calcium hydride. All ^1H NMR spectral data were obtained in $CDCl_3$ or CD_3OD on a Varian Unity 400 spectrometer (operating at 399.951 MHz for ^1H and 100.578 MHz for ^{13}C). Mass spectra were obtained at Analytical Service in the Department of Chemistry (HRFABMS) or the Department of Biochemistry (MALDI-TOFMS) at Virginia Tech.

7-{4-[3-*tert*-Butyldimethylsilyloxy-17β-triethylsilyloxyestra-1,3,5(10)-triene-16α-yl]-2*E*-but-2-enoic Acid (5). To a solution of **4** (836 mg, 1.36 mmol) in THF (8 mL) was added LiOH (131 mg, 5.44 mmol) in water (8 mL). After stirring at room temperature for 36 h, the reaction mixture was quenched with saturated ammonium chloride and extracted three times with ethyl acetate (50 mL). The combined organic phase was washed through water and brine, dried over sodium sulfate, and concentrated in a vacuum. The residue was purified by column chromatography (10% EtOAc/hexane) to give **5** (560 mg, 72%): ^1H NMR ($CDCl_3$) δ 0.21 (6H, s), 0.64 (6H, q, J = 7.9 Hz), 0.82 (3H, s), 1.00 (9H, s), 1.01 (9H, t, J = 7.9 Hz), 1.20−2.90 (16H, steroid skeleton), 3.33 (1H, d, J = 7.3 Hz), 5.88 (1H, d, J = 15.6 Hz), 6.56 (1H, d, J = 2.7 Hz, Ar), 6.63 (1H, dd, J = 8.5, 2.7 Hz, Ar), 7.07−7.16 (2H, overlapped); ^{13}C NMR ($CDCl_3$) δ −4.1, 5.7, 7.3, 12.4, 18.4, 26.0, 26.5, 27.5, 29.4, 29.8, 37.5, 37.8, 38.8, 43.1,44.2, 44.6, 48.6, 87.8, 117.4, 120.2, 121.7, 126.3, 133.2, 138.1, 151.7, 153.5, 172.3; HRFABMS *m/z* 584.3707 [$M^{+\cdot}$] (calcd for $C_{34}H_{56}O_4Si_2$, 584.3717).

Succinic Acid, Mono-3,17β-di-*tert*-butyldimethylsilyloxyestra-1,3,5(10)-triene-11β-yl Ester (7). To a solution of **6** (275 mg, 0.532 mmol) in 20 mL of dry THF was added LHMDS (1 M, 0.80 mL, 0.798 mmol) at 0 °C. After stirring for 1 h, succinic anhydride (1.06 g, 10.64 mmol) was added in one portion. The reaction mixture was allowed to stir overnight at room temperature. The reaction mixture was then poured into 200 mL of water, and EtOAc (150 mL) was used to extract the product. The extract was washed through water and brine, dried over sodium sulfate, and concentrated in a vacuum. The residue was purified by column chromatography (25% EtOAc/hexane) to give **7** (164 mg, 50%) and recovered **6** (55 mg, 20%). **7**: ^1H NMR ($CDCl_3$): δ 0.02 (3H, s), 0.03 (3H, s), 0.19 (6H, s), 0.79 (3H, s), 0.89 (9H, s), 0.98 (9H, s), 1.10−2.85 (17H, steroid skeleton), 3.67 (1H, t, J = 8.5 Hz), 5.45 (1H, td, J = 10.6, 5.2 Hz), 6.58−6.63 (2H, m, Ar), 6.93 (1H, d, 8.1 Hz, Ar); ^{13}C NMR ($CDCl_3$) δ −4.5, −4.3, −4.17, −4.15, 12.2, 18.3, 18.4, 23.4, 26.0, 26.1, 27.1, 28.4, 29.2, 29.6, 31.3, 37.7, 42.6, 44.5, 46.6, 49.7, 74.8, 81.2, 117.3, 120.0, 125.5, 132.4, 139.3, 153.9, 171.9, 179.0; HRFABMS *m/z* 616.3619 [$M^{+\cdot}$] (calcd for $C_{34}H_{56}O_6Si_2$, 616.3615).

General Procedure for Preparation of Estradiol−Taxol Conjugates. To a solution of estradiol derivative **5** (13.7 mg, 0.0234 mmol) in 2 mL of toluene was added EDC (4.5 mg, 0.0234 mmol). After 15 min stirring, DMAP (2 mg, cat.) was added and stirring continued for 5 min before taxol (20 mg, 0.0234 mmol) was added. The reaction mixture was allowed to stir at 60 °C for 24−48 h. Then, 50 mL of EtOAc was added to the reaction mixture, and the organic phase was washed with sodium bicarbonate, water, and brine, dried over sodium sulfate, and concentrated in a vacuum. The residue was applied to preparative TLC (50% EtOAc/hexane) to give silyl-protected estradiol−taxol conjugate **8** (15.1 mg, 73%). A similar procedure was applied to estradiol derivative **7** to give **10** and to the reaction of 2′-*tert*-butyldimethylsilyltaxol (**12**) with estradiols **5** and **7** to give the 7-acyl analogues **13** and **14**, respectively.

2′-{4-[3-*tert*-Butyldimethylsilyloxy-17β-triethylsilyloxyestra-1,3,5(10)-triene-16α-yl]-2*E*-but-2-enoyl}taxol (8): ^1H NMR ($CDCl_3$) δ 0.19 (6H, s), 0.61 (6H, q, J = 7.9 Hz), 0.79 (3H, s), 0.97 (9H, t, J = 7.9 Hz), 0.98 (9H, s), 1.13 (3H, s), 1.24 (3H, s), 1.68 (3H, s), 1.95 (3H, brs), 2.23 (3H, s), 2.44 (3H, s), 1.20−2.90 (20H, taxol and steroid skeletons), 3.29 (1H, d, J = 7.4 Hz), 3.82 (1H, d, J = 7.4 Hz), 4.20 (1H, d, J = 8.4 Hz), 4.32 (1H, d, J = 8.4 Hz), 4.46 (1H, m), 4.98 (1H, dd, J = 9.6, 2.0 Hz), 5.56 (1H, d, J = 3.6 Hz), 5.58 (1H, d, J = 7.2 Hz), 5.93 (1H, d, J = 15.6 Hz), 5.96 (1H, dd, J = 9.3, 3.6 Hz), 6.26 (1H, t, J = 9.1 Hz), 6.30 (1H, s), 6.55 (1H, d, J = 2.6 Hz, Ar), 6.61 (1H, dd, J = 8.5, 2.6 Hz, Ar), 6.93 (1H, d, J = 9.3 Hz), 7.10 (1H, d, J = 8.5 Hz, Ar), 7.30−7.70 (11H, m, Ar), 7.75 (2H, m, Ar), 8.13 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ −4.2, 5.6, 7.3, 9.8, 12.3, 15.1, 18.4, 21.1, 22.9, 25.9, 26.4, 26.5, 27.1, 29.3, 29.8, 35.7, 35.8, 37.4, 37.9, 38.8, 43.1, 43.4, 44.2, 44.5, 45.8, 48.4, 53.2, 58.7, 72.0, 72.4, 74.0, 75.3, 75.8, 76.6, 79.5, 81.2, 84.7, 87.8, 117.4, 120.1, 126.3, 126.9, 127.3, 128.7, 128.9, 129.0, 129.3, 129.4, 130.4, 132.2, 132.9, 133.1, 133.92, 133.95, 137.3, 138.0, 143.2, 152.3, 153.5, 165.6, 167.25, 167.29, 168.5, 170.0, 171.5, 204.1; HRFABMS *m/z* 1442.6644 [M + Na^+] (calcd for $C_{81}H_{105}NO_{17}Si_2Na$, 1442.6819).

Succinic acid, 3,17β-di-*tert*-butyldimethylsilyloxyestra-1,3,5(10)-triene-11β-yl ester 2′-taxol ester (10): ^1H NMR ($CDCl_3$) δ 0.02 (6H, brs), 0.17 (6H, s), 0.77 (3H, s), 0.88 (9H, s), 0.96 (9H, s), 1.13 (3H, s), 1.23 (3H, s), 1.68 (3H, s), 1.94 (3H, brs), 2.22 (3H, s), 2.45 (3H, s), 1.15−2.90 (21H, taxol and steroid skeletons), 3.66 (1H, t, J = 8.4 Hz), 3.82 (1H, d, J = 7.2 Hz), 4.20 (1H, d, J = 8.6 Hz), 4.32 (1H, d, J = 8.6 Hz), 4.45 (1H, dd, J = 9.7, 7.2 Hz), 4.97 (1H, dd, J = 9.6, 1.7 Hz), 5.41 (1H, td, J = 10.5, 5.2 Hz), 5.54 (1H, d, J = 3.0 Hz), 5.68 (1H, d, J = 7.0 Hz), 6.00 (1H, dd, J = 9.2, 3.0 Hz), 6.24−6.31 (2H, overlapped), 6.54−6.61 (2H, overlapped, Ar), 6.85 (1H, d, J = 8.3 Hz, Ar), 6.99 (1H, d, J = 9.2 Hz), 7.30−7.65 (11H, Ar), 7.76 (2H, m, Ar), 8.15 (2H, m, Ar); ^{13}C NMR (100 MHz, $CDCl_3$) δ −4.5, −4.3, −4.1, 9.8, 12.2, 15.1, 18.3, 18.4, 21.1, 22.4, 22.9, 23.4, 25.9, 26.1, 27.0, 27.1, 28.4, 29.1, 29.7, 31.2, 35.7, 35.8, 37.7, 42.6, 43.4, 44.6, 45.7, 46.6, 49.7, 53.0, 59.7, 72.0, 72.4, 74.3, 75.0, 75.3, 75.8, 76.6, 79.4, 81.2, 81.3, 84.7, 117.4, 120.2, 125.2, 126.8, 127.4, 128.7, 128.9, 129.0, 129.3, 129.4, 130.5, 132.2, 132.4, 132.9, 133.8, 133.9, 137.2, 139.4, 143.1, 153.9, 167.28, 167.30, 168.1, 170.0, 171.47, 171.54, 204.1; HRFABMS *m/z* 1452.6803 [M + H^+] (calcd for $C_{81}H_{106}NO_{19}Si_2$, 1452.6898).

7-{4-[3-*tert*-Butyldimethylsilyloxy-17β-triethylsilyloxyestra-1,3,5(10)-triene-16α-yl]-2*E*-but-2-enoyl}-2′-*tert*-butyldimethylsilyl taxol (13): ^1H NMR ($CDCl_3$) δ −0.30 (3H, s), −0.02 (3H, s), 0.18 (6H, s), 0.61 (6H, q, J = 7.9 Hz), 0.79 (3H, s), 0.80 (9H, s), 0.98 (9H, t, J = 7.9 Hz), 1.18 (3H, s), 1.21 (3H, s), 1.86 (3H, s), 2.01 (3H, brs), 2.11 (3H, s), 2.59 (3H, s), 1.10−2.85 (20H, taxol and steroid skeletons), 3.29 (1H, d, J = 7.1 Hz), 4.00 (1H, d, J = 7.1 Hz), 4.23 (1H, d, J = 8.1 Hz), 4.36 (1H, d, J = 8.1 Hz), 4.68 (1H, d, J = 2.0 Hz), 5.00 (1H, d, J = 9.4 Hz), 5.64 (1H, dd, J = 10.5, 7.2 Hz), 5.68−5.82 (3H, overlapped), 6.26 (1H, t, J = 9.2 Hz), 6.35 (1H, s), 6.54 (1H, d, J = 2.5 Hz, Ar), 6.61 (1H, dd, J = 8.5, 2.5 Hz, Ar), 6.89 (1H, m), 7.09 (1H, d, J = 8.8 Hz), 7.11 (1H, d, J = 8.5 Hz, Ar), 7.28−7.66 (11H, Ar), 7,76 (2H, m, Ar), 8.14 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ −5.6, −4.9, −4.2, 5.7, 7.3, 11.2, 12.4, 14.9, 18.35, 18.40, 20.8, 21.6, 23.3, 25.8, 25.9, 26.6, 27.5, 29.3, 29.8, 33.6, 35.8, 37.5, 37.6, 38.8, 43.0, 43.6, 44.2, 44.5, 47.0, 48.4, 55.9, 56.4, 71.52, 71.55, 74.8, 75.2, 75.3, 76.7, 78.9, 81.2, 84.3, 87.8, 117.3, 120.2, 122.0, 126.3, 126.6, 127.2, 128.2, 128.97, 129.02, 129.3, 130.4, 132.0, 133.0, 133.4, 134.0, 134.3, 138.1, 138.5, 141.1, 149.1, 153.5, 165.6, 167.20, 167.21, 168.6, 170.0, 171.7, 202.3; HRFABMS *m/z* 1534.7910 [M + H^+] (calcd for $C_{87}H_{120}NO_{17}Si_3$, 1534.7864).

Succinic acid, 3,17β-di-*tert*-butyldimethylsilyloxyestra-1,3,5(10)-triene-11α-yl ester 2′-*tert*-butyldimethylsilyl-7-taxol ester (15): ^1H NMR ($CDCl_3$) δ −0.31 (3H, s), −0.03 (3H, s), 0.01 (3H, s), 0.02 (3H, s), 0.19 (6H, s), 0.77 (3H, s), 0.80

(9H, s), 0.88 (9H, s), 0.97 (9H, s), 1.15 (3H, s), 1.21 (3H, s), 1.81 (3H, s), 1.97 (3H, brs), 2.11 (3H, s), 2.58 (3H, s), 1.10−2.80 (21H, taxol and steroid skeletons), 3.65 (1H, t, J = 8.4 Hz), 3.97 (1H, d, J = 6.8 Hz), 4.21 (1H, d, J = 8.5 Hz), 4.34 (1H, d, J = 8.5 Hz), 4.67 (1H, d, J = 2.1 Hz), 4.97 (1H, d, J = 9.3 Hz), 5.40 (1H, td, J = 10.4, 5.2 Hz), 5.61 (1H, dd, J = 10.6, 7.1 Hz), 5.70 (1H, d, J = 7.0 Hz), 5.73 (1H, dd, J = 8.9, 1.7 Hz), 6.25 (1H, s), 6.27 (1H, t, J = 9.4 Hz), 6.57 (1H, d, J = 2.5 Hz, Ar), 6.61 (1H, dd, J = 8.5, 2.5 Hz, Ar), 6.91 (1H, d, J = 8.5 Hz, Ar), 7.08 (1H, d, J = 8.9 Hz), 7.30−7.65 (11H, m, Ar), 7.75 (2H, m, Ar), 8.13 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ −5.6, −4.9, −4.5, −4.2, −4.15, −4.13, 11.1, 12.2, 14.8, 18.28, 18.34, 18.39, 20.9, 21.7, 23.2, 23.4, 25.7, 25.9, 26.1, 26.6, 27.0, 28.5, 29.5, 29.9, 31.1, 33.5, 35.8, 37.7, 42.7, 43.6, 44.6, 46.7, 47.0, 49.7, 55.9, 56.2, 71.5, 71.8, 74.5, 74.7, 75.3, 75.4, 76.6, 77.6, 78.9, 81.2, 84.2, 117.4, 119.9, 125.6, 126.6, 127.2, 128.2, 128.96, 129.01, 129.3, 130.4, 132.0, 132.5, 132.8, 134.0, 134.3, 138.5, 139.2, 141.2, 153.9, 167.15, 167.17, 169.2, 170.0, 171.6, 171.7, 172.2, 202.2; HRFABMS m/z 1566.7789 [M + H$^+$] (calcd for $C_{87}H_{120}NO_{19}Si_3$, 1566.7762).

Succinic acid, 3,17β-di-*tert*-butyldimethylsilyloxyestra-1,3,5(10)-triene-11α-yl ester 2′-*tert*-butyldimethylsilyl-10-deacetyl-7-*epi*-taxol ester (20): ^{1}H NMR ($CDCl_3$) δ −0.30 (3H, s), −0.04 (3H, s), 0.02 (3H, s), 0.03 (3H, s), 0.19 (6H, s), 0.77 (3H, s), 0.78 (9H, s), 0.88 (9H, s), 0.98 (9H, s), 1.13 (3H, s), 1.19 (3H, s), 1.67 (3H, s), 1.88 (3H, brs), 2.67 (3H, s), 1.10−2.96 (21H, taxol and steroid skeletons), 3.67 (1H, t, J = 8.3 Hz), 3.71 (1H, m), 3.92 (1H, d, J = 7.5 Hz), 4.40 (2H, brs), 4.66 (1H, d, J = 2.2 Hz), 4.71 (1H, d, J = 11.7 Hz), 4.94 (1H, dd, J = 8.9, 3.5 Hz), 5.44 (1H, td, J = 10.3, 5.2 Hz), 5.75 (1H, d, J = 7.5 Hz), 5.78 (1H, dd, J = 9.0, 1.8 Hz), 6.30 (1H, t, J = 8.9 Hz), 6.58 (1H, d, J = 2.6 Hz, Ar), 6.61 (1H, dd, J = 8.4, 2.6 Hz, Ar), 6.86 (1H, s), 6.91 (1H, d, J = 8.4 Hz, Ar), 7.07 (1H, d, J = 9.0 Hz), 7.30−7.63 (11H, Ar), 7.72 (2H, m, Ar), 8.17 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ −5.7, −5.0, −4.5, −4.3, −4.1, 12.2, 15.2, 16.5, 18.3, 18.38, 18.39, 21.9, 23.1, 23.4, 25.7, 25.9, 26.12, 26.14, 27.1, 28.4, 29.3, 30.1, 31.3, 35.6, 36.5, 37.7, 40.5, 42.6, 42.9, 44.5, 46.6, 49.6, 55.8, 57.7, 71.1, 74.7, 75.52, 75.56, 76.0, 77.9, 78.5, 79.5, 81.2, 82.3, 83.0, 117.4, 119.9, 125.4,126.6, 127.2, 128.2, 128.9, 129.0, 129.1, 129.5, 130.5, 132.0, 132.6, 133.1, 133.9, 134.3, 138.5, 139.3, 140.6, 153.9, 167.1, 167.4, 170.9, 171.2, 171.9, 172.5, 207.3; HRFABMS m/z 1524.7583 [M + H$^+$] (calcd for $C_{85}H_{118}NO_{18}Si_3$, 1524.7657).

2′-(3-Benzyloxycarbonylpropanoyl)taxol (22): ^{1}H NMR ($CDCl_3$) δ 1.14 (3H, s), 1.23 (3H, s), 1.68 (3H, s), 1.88 (1H, m), 1.93 (3H, brs), 2.16 (1H, m), 2.22 (3H, s), 2.38 (1H, m), 2.45 (3H, s), 2.55 (1H, m), 2.66 (2H, m), 2.77 (2H, m), 3.81 (1H, d, J = 7.0 Hz), 4.20 (1H, d, J = 8.4 Hz), 4.31 (1H, d, J = 8.4 Hz), 4.44 (1H, dd, J = 10.9, 6.6 Hz), 4.97 (1H, dd, J = 9.6, 2.0 Hz), 5.51 (1H, d, J = 3.1 Hz), 5.69 (1H, d, J = 7.0 Hz), 5.99 (1H, dd, J = 9.2, 3.1 Hz), 6.25 (1H, t, J = 9.0 Hz), 6.30 (1H, s), 7.11 (1H, d, J = 9.2 Hz), 7.25−7.65 (16H, Ar), 7.80 (2H, m, Ar), 8.14 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ 9.8, 15.0, 21.1, 22.4, 22.9, 27.0, 29.4, 29.5, 35.76, 35.80, 43.3, 45.8, 53.0, 58.7, 67.0, 72.1, 72.4, 74.5, 75.3, 75.8, 76.7, 79.3, 81.2, 84.7, 126.8, 127.5, 128.4, 128.6, 128.7, 128.8, 128.9, 129.0, 129.3, 129.4, 130.5, 132.2, 133.0, 133.8, 133.9, 135.7, 137.2, 143.0, 167.2, 167.5, 168.1, 170.0, 171.3, 171.5, 172.2, 204.1; HRFABMS m/z 1044.4032 [M + H$^+$] (calcd for $C_{58}H_{62}NO_{17}$, 1044.4018).

Succinic acid, 3,17β-di-*tert*-butyldimethylsilyloxyestra-1,3,5(10)-triene-11α-yl ester 2′-(3-benzyloxycarbonylpropanoyl)-7-taxol ester (23): ^{1}H NMR ($CDCl_3$) δ 0.00 (3H, s), 0.01 (3H, s), 0.17 (6H, s), 0.76 (3H, s), 0.86 (9H, s), 0.95 (9H, s), 1.14 (3H, s), 1.19 (3H, s), 1.79 (3H, s), 1.97 (3H, brs), 2.10 (3H, s), 2.44 (3H, s), 1.10−2.80 (25H, taxol and steroid skeletons), 3.64 (1H, t, J = 8.5 Hz), 3.94 (1H, d, J = 6.9 Hz), 4.18 (1H, d, J = 8.5 Hz), 4.31 (1H, d, J = 8.5 Hz), 4.94 (1H, d, J = 9.3 Hz), 4.99 (2H, s), 5.39 (1H, td, J = 10.4, 5.2 Hz), 5.52 (1H, d, J = 3.0 Hz), 5.58 (1H, dd, J = 10.5, 7.2 Hz), 5.68 (1H, d, J = 7.0 Hz), 5.99 (1H, dd, J = 9.2, 3.0 Hz), 6.22 (1H, t, J = 9.4 Hz), 6.23 (1H, s), 6.56 (1H, d, J = 2.6 Hz, Ar), 6.60 (1H, dd, J = 8.5, 2.6 Hz, Ar), 6.91 (1H, d, J = 8.5 Hz, Ar), 7.11 (1H, d, J = 9.2 Hz), 7.25−7.65 (16H, Ar), 7.80 (2H, m, Ar), 8.13 (2H, m, Ar); ^{13}C NMR (100 MHz, $CDCl_3$) δ −4.6, −4.3, −4.2, 11.0, 12.1, 14.6, 18.2, 18.3, 20.8, 21.5, 22.8, 23.3, 25.9, 26.0, 26.6, 27.0, 28.4, 29.2, 29.4, 29.5, 29.8, 31.2, 33.4, 35.6, 37.6, 42.6, 43.4, 44.5, 46.6, 46.9, 49.6, 52.9, 56.1, 66.9, 71.7, 74.3, 74.4, 74.7, 75.3, 76.4, 78.8, 81.0, 81.1, 84.1, 117.3, 119.8, 125.5, 126.7, 127.4, 128.4, 128.5, 128.6, 128.7, 128.8, 128.9, 129.2, 129.3, 130.4, 132.1, 132.4, 132.6, 133.7, 133.8, 135.6, 137.1, 139.1, 141.4, 153.8, 167.1, 167.4, 168.1, 169.1, 169.7, 171.0, 171.3, 172.2, 202.2; MALDI-TOFMS m/z 1665 [M + Na$^+$] (calcd for $C_{92}H_{115}NO_{22}Si_2Na$, 1664.8).

Succinic acid, 3,17β-di-*tert*-butyldimethylsilyloxyestra-1,3,5(10)-triene-11α-yl ester 7-(3-benzyloxycarbonylpropanoyl)-2′-taxol ester (28): ^{1}H NMR ($CDCl_3$) δ 0.02 (3H, s), 0.03 (3H, s), 0.17 (6H, s), 0.77(3H, s), 0.88 (9H, s), 0.96 (9H, s), 1.15 (3H, s), 1.20 (3H, s), 1.79 (3H, s), 1.98 (3H, brs), 2.13 (3H, s), 2.45 (3H, s), 1.10−2.83 (25H, taxol and steroid skeletons), 3.67 (1H, t, J = 8.5 Hz), 3.95 (1H, d, J = 6.9 Hz), 4.19 (1H, d, J = 8.5 Hz), 4.32 (1H, d, J = 8.5 Hz), 4.93 (1H, d, J = 9.4 Hz), 5.12 (2H, AB, J = 12.4 Hz), 5.41 (1H, td, J = 10.4, 5.3 Hz), 5.58 (1H, d, J = 3.1 Hz), 5.60 (1H, dd, J = 10.5, 7.1 Hz), 5.68 (1H, d, J = 7.0 Hz), 6.01 (1H, dd, J = 9.3, 3.1 Hz), 6.22 (1H, s), 6.24 (1H, t, J = 9.1 Hz), 6.55−6.59 (2H, overlapped, Ar), 6.85 (1H, d, J = 8.0 Hz, Ar), 7.03 (1H, d, J = 9.3 Hz), 7.27−7.65 (16H, Ar), 7.77 (2H, m, Ar), 8.14 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ −4.5, −4.3, −4.2, 11.0, 12.1, 14.6, 18.2, 18.3, 20.9, 21.5, 22.8, 23.3, 25.9, 26.0, 26.6, 26.9, 28.1, 28.3, 29.2, 29.3, 29.7, 31.2, 33.3, 37.2, 37.6, 42.5, 43.3, 44.5, 46.5, 47.0, 49.6, 52.9, 56.1, 66.5, 71.7, 71.9, 74.1, 74.7, 74.9, 75.4, 76.5, 78.8, 81.00, 81.04, 84.2, 117.3, 119.9, 125.1, 126.7, 127.3, 128.31, 128.35, 128.37, 128.67, 128.71, 128.8, 129.25, 129.30, 130.4, 132.1, 132.4, 132.5, 133.7, 133.8, 136.1, 137.1, 139.3, 141.3, 153.9, 167.1, 167.4, 168.2, 169.0, 169.7, 171.2, 171.3, 171.6, 172.6, 202.2; HRFABMS m/z 1642.8468 [M + H$^+$] (calcd for $C_{92}H_{116}NO_{22}Si_2$).

General Procedure for Deprotection of Silyl Group. To a solution of silyl-protected estradiol−taxol conjugate **8** (15.1 mg, 0.0106 mmol), in 0.6 mL of dried THF, was added 0.1 mL of anhydrous pyridine, then the solution was cooled to 0 °C, and 0.1 mL of HF−pyridine was added. The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was then diluted with EtOAc, and the organic phase was washed with sodium bicarbonate, water, and brine, dried over sodium sulfate, and concentrated in a vacuum. The residue was purified by preparative TLC (50% EtOAc/hexane) to give **9** (12.3 mg, 97%). Compounds **11**, **14**, **16**, **21**, **24**, and **27** were prepared similarly.

2′-{4-[3,17β-Dihydroxyestra-1,3,5(10)-triene-16α-yl]-2*E*-but-2-enoyl}taxol (9): ^{1}H NMR (CD_3OD) δ 0.78 (3H, s), 1.11 (3H, s), 1.13 (3H, s), 1.65 (3H, s), 1.93 (3H, brs), 2.16 (3H, s), 2.40 (3H, s), 2.15−2.80 (20H, taxol and steroid skeletons), 3.23 (1H, d, J = 8.0 Hz), 3.81 (1H, d, J = 7.2 Hz), 4.18 (2H, brs), 4.34 (1H, dd, J = 11.0, 6.7 Hz), 4.99 (1H, dd, J = 9.6, 1.9 Hz), 5.50 (1H, d, J = 6.8 Hz), 5.63 (1H, d, J = 7.1 Hz), 5.85 (1H, d, J = 6.8 Hz), 5.99 (1H, d, J = 15.6 Hz), 6.06 (1H, t, J = 9.1 Hz), 6.45 (1H, s), 6.47 (1H, d, J = 2.6 Hz, Ar), 6.53 (1H, dd, J = 8.5, 2.6 Hz, Ar), 7.05 (1H, d, J = 8.5 Hz, Ar), 7.16 (1H, td, J = 15.6, 7.1 Hz), 7.23−7.70 (11H, Ar), 7.81 (2H, m, Ar), 8.11 (2H, m, Ar); ^{13}C NMR (CD_3OD) δ 9.3, 11.3, 13.8, 19.6, 21.2, 22.1, 25.7, 26.2, 27.3, 29.1, 29.5, 35.2, 36.3, 36.8, 37.6, 39.0, 42.0, 43.4, 43.9, 44.1, 46.7, 48.3, 54.2, 58.0, 71.1, 71.7, 74.7, 75.1, 75.6, 76.3, 77.8, 81.0, 84.7, 86.7, 112.5, 114.9, 120.3, 126.0, 127.4, 127.5, 128.4, 128.5, 128.9, 130.0, 130.2, 131.3, 131.7, 133.4, 133.7, 134.4, 137.2, 137.6, 141.3, 151.6, 154.7, 165.8, 166.4, 169.3, 169.4, 170.1, 170.4, 204.0; HRFABMS m/z 1192.5267 [M + H$^+$] (calcd for $C_{69}H_{78}NO_{17}$, 1192.5270).

Succinic acid, 3,17β-dihydroxyestra-1,3,5(10)-triene-11β-yl ester 2′-taxol ester (11): ^{1}H NMR (CD_3OD) δ 0.74 (3H, s), 1.15 (3H, s), 1.16 (3H, s), 1.65 (3H, s), 1.97 (3H, brs), 2.17 (3H, s), 2.42 (3H, s), 1.24−2.86 (21H, taxol and steroid skeletons), 3.59 (1H, t, J = 8.7 Hz), 3.84 (1H, d, J = 7.2 Hz), 4.20 (2H, brs), 4.36 (1H, dd, J = 11.1, 6.7 Hz), 5.02 (1H, dd, J = 9.6, 2.0 Hz), 5.29 (1H, td, J = 10.4, 5.3 Hz), 5.48 (1H, d, J = 5.2 Hz), 5.65 (1H, d, J = 7.1 Hz), 5.89 (1H, d, J = 5.2 Hz), 6.17 (1H, t, J = 9.1 Hz), 6.43 (1H, s), 6.52 (1H, d, J = 2.7 Hz, Ar), 6.54 (1H, dd, J = 8.4, 2.7 Hz, Ar), 6.83 (1H, d, J = 8.4 Hz, Ar), 7.25−7.69 (11H, Ar), 7.77 (2H, m, Ar), 8.13 (2H, m, Ar); ^{13}C NMR (CD_3OD) δ 9.3, 11.0, 14.1, 19.8, 21.2, 22.7, 25.9, 26.8,

28.1, 28.2, 29.2, 29.5, 35.3, 36.3, 37.9, 41.6, 43.5, 43.9, 45.7, 46.6, 49.5, 53.7, 58.0, 71.2, 71.6, 74.6, 75.09, 75.11, 75.7, 76.3, 77.8, 80.4, 81.1, 84.7, 112.5, 114.8, 125.0, 127.3, 127.4, 128.3, 128.54, 128.56, 128.9, 130.0, 130.2, 130.9, 131.6, 133.4, 133.8, 134.4, 137.1, 139.0, 141.3, 155.2, 166.5, 168.9, 169.6, 170.2, 170.4, 171.9, 172.3, 204.0; HRFABMS *m/z* 1224.5200 [M + H$^+$] (calcd for $C_{69}H_{78}NO_{19}$, 1224.5168).

7-{4-[3,17β-Dihydroxyestra-1,3,5(10)-triene-16α-yl]-2*E*-but-2-enoyl}taxol (14): ^{1}H NMR (CD_3OD) δ 0.82 (3H, s), 1.11 (3H, s), 1.15 (3H, s), 1.81 (3H, s), 1.90 (3H, brs), 2.13 (3H, s), 2.37 (3H, s), 1.20−2.80 (20H, taxol and steroid skeletons), 3.27 (1H, d, *J* = 7.7 Hz), 3.92 (1H, d, *J* = 7.1 Hz), 4.20 (2H, brs), 4.75 (1H, d, *J* = 5.2 Hz), 5.00 (1H, d, *J* = 9.3), 5.60 (1H, dd, *J* = 10.6, 7.4 Hz), 5.63−5.68 (2H, overlapped), 5.75 (1H, d, *J* = 15.6 Hz), 6.15 (1H, t, *J* = 9.1 Hz), 6.31 (1H, s), 6.37 (1H, d, *J* = 2.5 Hz, Ar), 6.53 (1H, dd, *J* = 8.5, 2.5 Hz, Ar), 6.92 (1H, td, *J* = 15.6, 7.3 Hz), 7.26−7.69 (11H, Ar), 7.85 (2H, m, Ar), 8.11 (2H, m, Ar); ^{13}C NMR (CD_3OD) δ 10.3, 11.3, 13.5, 19.5, 20.9, 22.0, 25.55, 25.58, 26.3, 27.3, 29.50, 29.55, 35.3, 36.9, 39.1, 42.2, 43.4, 44.0, 44.1, 46.8, 48.3, 56.2, 56.5, 71.0, 71.7, 73.6, 74.7, 75.4, 76.1, 77.7, 80.8, 84.0, 86.5, 112.5, 114.9, 121.6, 126.0, 127.3, 127.8, 128.4, 128.55, 128.57, 130.0, 130.1, 131.4, 131.7, 133.3, 133.5, 134.4, 137.6, 138.8, 140.9, 149.1, 154.7, 165.6, 166.4, 169.1, 169.7, 170.8, 173.3, 202.5; HRFABMS *m/z* 1192.5237 [M + H$^+$] (calcd for $C_{69}H_{78}NO_{17}$ 1192.5270).

Succinic acid, 3,17β-dihydroxyestra-1,3,5(10)-triene-11α-yl ester 7-taxol ester (16): ^{1}H NMR (CD_3OD) δ 0.76 (3H, s), 1.11 (3H, s), 1.15 (3H, s), 1.78 (3H, s), 1.89 (3H, brs), 2.11 (3H, s), 2.38 (3H, s), 1.17−2.81 (21H, taxol and steroid skeletons), 3.67 (1H, t, *J* = 8.7 Hz), 3.91 (1H, d, *J* = 7.1 Hz), 4.18 (1H, d, *J* = 8.5 Hz), 4.22 (1H, d, *J* = 8.5 Hz), 4.77 (1H, d, *J* = 5.3 Hz), 5.00 (1H, d, *J* = 9.5 Hz), 5.32 (1H, td, *J* = 10.6, 5.2 Hz), 5.68−5.78 (3H, overlapped), 6.16 (1H, t, *J* = 9.1 Hz), 6.21 (1H, s), 6.53 (1H, d, *J* = 2.7 Hz, Ar), 6.58 (1H, dd, *J* = 8.6, 2.7 Hz, Ar), 6.91 (1H, d, *J* = 8.6 Hz), 7.26−7.69 (11H, Ar), 7.85 (2H, m, Ar), 8.11 (2H, m, Ar); ^{13}C NMR (CD_3OD) δ 10.3, 11.0, 13.7, 19.5, 20.9, 22.0, 22.7, 25.6, 26.8, 28.1, 29.0, 29.3, 29.4, 33.0, 35.3, 37.9, 42.3, 43.4, 43.9, 46.3, 46.9, 49.7, 56.0, 56.5, 71.0, 72.0, 73.6, 74.59, 74.63, 75.5, 76.1, 77.7, 80.4, 80.8, 84.1, 112.6, 114.8, 125.4, 127.3, 127.8, 128.4, 128.56, 128.59, 130.0, 130.1, 130.6, 131.7, 133.1, 133.5, 134.4, 138.8, 139.0, 141.0, 155.2, 166.4, 169.1, 169.7, 170.9, 171.8, 172.8, 173.3, 202.4; HRFABMS *m/z* 1224.5176 [M + H$^+$] (calcd for $C_{69}H_{78}NO_{19}$, 1224.5168).

Succinic acid, 3,17β-dihydroxyestra-1,3,5(10)-triene-11α-yl ester 10-deacetyl-7-*epi*-taxol ester (21): ^{1}H NMR ($CDCl_3$) δ 0.74 (3H, s), 1.12 (3H, s), 1.14 (3H, s), 1.63 (3H, s), 1.77 (3H, brs), 2.47 (3H, s), 1.20−2.94 (21H, taxol and steroid skeletons), 3.6−3.74 (2H, overlapped), 3.87 (1H, d, *J* = 7.2 Hz), 4.22 (1H, brs), 4.37 (2H, brs), 4.78 (1H, d, *J* = 1.8 Hz), 4.84−4.92 (2H, overlapped), 5.38 (1H, td, *J* = 10.3, 5.5 Hz), 5.73 (1H, d, *J* = 7.2 Hz), 5.77 (1H, dd, *J* = 9.0, 2.0 Hz), 6.19 (1H, t, *J* = 8.7 Hz), 6.46−6.55 (2H, Ar), 6.76 (1H, s), 6.88 (1H, d, *J* = 8.1 Hz, Ar), 7.28 (1H, d, *J* = 8.9 Hz, Ar), 7.29−7.63 (12H, Ar and −NH), 7.72 (2H, d, *J* = 7.9 Hz, Ar), 8.14 (2H, d, *J* = 7.9 Hz, Ar); ^{13}C NMR ($CDCl_3$) δ 11.8, 15.0, 16.7, 21.6, 22.8, 23.2, 26.1, 27.0, 28.4, 29.4, 30.0, 30.3, 35.4, 36.3, 37.7, 40.5, 42.2, 42.8, 44.2, 46.3, 49.8, 55.4, 57.7, 72.3, 73.4, 74.6, 75.5, 76.1, 77.9, 78.7, 79.1, 81.1, 82.3, 82.9, 112.9, 115.3, 125.9, 127.1, 127.4, 128.4, 128.9, 129.0, 129.2, 129.5, 130.4, 131.3, 132.2, 133.4, 133.8, 133.9, 138.3, 139.4, 140.3, 154.4, 167.2, 167.9, 171.2, 172.1, 172.6, 173.1, 207.2; MALDI-TOFMS *m/z* 1204.5 [M + Na$^+$] (calcd for $C_{67}H_{75}NO_{18}Na$, 1204.5).

Succinic acid, 3,17β-dihydroxyestra-1,3,5(10)-triene-11α-yl ester 2′-(3-benzyloxycarbonylpropanoyl)-7-taxol ester (24): ^{1}H NMR ($CDCl_3$) δ 0.76 (3H, s), 1.16 (3H, s), 1.20 (3H, s), 1.83 (3H, s), 1.99 (3H, brs), 2.12 (3H, s), 2.47 (3H, s), 3.66 (1H, t, *J* = 8.6 Hz), 3.95 (1H, d, *J* = 6.8 Hz), 4.19 (1H, d, *J* = 8.4 Hz), 4.35 (1H, d, *J* = 8.4 Hz), 4.96 (2H, s), 5.00 (1H, d, *J* = 9.4 Hz), 5.37 (1H, td, *J* = 10.6, 5.3 Hz), 5.59 (1H, d, *J* = 3.1 Hz), 5.67−5.75 (2H, overlapped), 6.01 (1H, dd, *J* = 9.1, 3.1 Hz), 6.17−6.25 (2H, overlapped), 6.54 (1H, d, *J* = 2.5 Hz, Ar), 6.68 (1H, dd, *J* = 8.5, 2.5 Hz, Ar), 7.05 (1H, d, *J* = 8.5 Hz, Ar), 7.19 (1H, d, *J* = 9.1 Hz), 7.23−7.65 (16H, Ar), 7.83 (2H, m, Ar), 8.12 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ 11.2, 11.9, 14.8, 20.9, 21.3, 23.0, 23.1, 26.6, 27.1, 28.5, 29.3, 29.5, 29.6, 29.7, 30.2, 33.5, 35.5, 37.8, 42.7, 43.5, 44.1, 46.5, 47.4, 49.8, 53.0, 56.2, 67.0, 71.6, 72.0, 74.46, 74.55, 74.57, 75.7, 76.7, 78.6, 81.1, 84.5, 113.1, 115.2, 126.6, 126.8, 127.5, 128.4, 128.6, 128.7, 128.8, 128.9, 129.0, 129.28, 129.34, 130.4, 131.1, 132.3, 132.6, 133.8, 134.0, 135.7, 137.0, 139.2, 141.4, 154.6, 167.1, 167.8, 168.2, 169.1, 170.6, 171.6, 171.8, 172.4, 173.0, 202.2; HRFABMS *m/z* 1436.5613 [M + Na$^+$] (calcd for $C_{80}H_{87}NO_{22}Na$, 1436.5617).

Succinic acid, 3,17β-dihydroxyestra-1,3,5(10)-triene-11α-yl ester 7-(3-benzyloxycarbonylpropanoyl)-2′-taxol ester (29): ^{1}H NMR ($CDCl_3$) δ 0.77 (3H, s), 1.15 (3H, s), 1.19 (3H, s), 1.78 (3H, s), 2.01 (3H, brs), 2.12 (3H, s), 2.42 (3H, s), 1.10−2.95 (25H, taxol and steroid skeletons), 3.71 (1H, t, *J* = 8.5 Hz), 3.93 (1H, d, *J* = 6.8 Hz), 4.17 (1H, d, *J* = 8.5 Hz), 4.32 (1H, d, *J* = 8.5 Hz), 4.95 (1H, d, *J* = 9.3 Hz), 5.11 (2H, AB, *J* = 12.3 Hz), 5.39 (1H, td, *J* = 10.4, 5.2 Hz), 5.44 (1H, d, *J* = 3.1 Hz), 5.63 (1H, dd, *J* = 10.6, 7.0 Hz), 5.69 (1H, d, *J* = 6.8 Hz), 5.92 (1H, dd, *J* = 9.0, 2.9 Hz), 6.22 (1H, t, *J* = 9.0 Hz), 6.24 (1H, s), 6.55−6.60 (2H, overlapped, Ar), 6.86 (1H, d, *J* = 8.2 Hz, Ar), 7.10 (1H, d, *J* = 9.0 Hz), 7.26−7.64 (16H, Ar), 7.77 (2H, m, Ar), 8.11 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ 11.0, 11.9, 14.7, 21.0, 21.4, 22.9, 23.2, 26.6, 26.9, 28.3, 28.6, 29.2, 29.51, 29.54, 30.6, 33.3, 35.6, 37.7, 42.1, 43.5, 44.1, 46.2, 47.2, 49.9, 53.0, 56.2, 66.7, 71.8, 72.0, 74.2, 74.6, 74.7, 75.6, 76.5, 78.8, 81.0, 81.2, 84.1, 112.8, 115.5, 125.6, 126.9, 127.4, 128.4, 128.5, 128.7, 128.8, 128.9, 129.0, 129.2, 129.3, 130.4, 131.2, 132.2, 132.6, 133.8, 134.0, 136.1, 137.1, 139.4, 141.7, 154.7, 167.1, 167.6, 168.3, 169.4, 170.2, 171.6, 171.7, 172.3, 172.6, 202.2; HRFABMS *m/z* 1436.5562 [M + Na$^+$] (calcd for $C_{80}H_{87}NO_{22}Na$, 1436.5617).

General Procedure for Deprotection of the Benzyl Group. To a solution of benzyl-protected estradiol−taxol conjugate **24** (38.3 mg, 0.0266 mmol), in 10 mL of EtOAc, was added 10 mg of Pd−C (10%), and the mixture was hydrogenated at 30 psi at room temperature for 24 h. The reaction mixture was filtered, and the organic phase was concentrated in a vacuum. The residue was purified by preparative TLC (70% EtOAc/hexane) to give **25** (27.0 mg, 74%).

Succinic acid, 3,17β-dihydroxyestra-1,3,5(10)-triene-11α-yl ester 2′-(3-carboxypropanoyl)-7-taxol ester (25): ^{1}H NMR ($CDCl_3$) δ 0.75 (3H, s), 1.14 (3H, s), 1.17 (3H, s), 1.81 (3H, s), 1.96 (3H, brs), 2.11 (3H, s), 2.43 (3H, s), 1.20−2.80 (25H, taxol and steroid skeletons), 3.67 (1H, t, *J* = 8.7 Hz), 3.91 (1H, d, *J* = 6.8 Hz), 4.17 (1H, d, *J* = 8.7 Hz), 4.32 (1H, d, *J* = 8.7 Hz), 4.97 (1H, d, *J* = 9.5 Hz), 5.36 (1H, td, *J* = 10.4, 7.8 Hz), 5.68 (1H, d, *J* = 6.7 Hz), 5.96 (1H, dd, *J* = 9.1, 3.9 Hz), 6.17 (1H, t, *J* = 8.7 Hz), 6.20 (1H, s), 6.55 (1H, d, *J* = 2.7, Ar), 6.67 (1H, dd, *J* = 8.5, 2.7 Hz, Ar), 7.02 (1H, d, *J* = 8.5 Hz, Ar), 7.24 (1H, d, *J* = 9.2 Hz), 7.28−7.65 (11H, Ar), 7.77 (2H, m, Ar), 8.10 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ 11.2, 11.8, 14.8, 21.0, 21.3, 22.9, 23.1, 26.6, 27.1, 28.5, 28.77, 28.80, 29.13, 29.15, 29.2, 33.5, 35.4, 37.8, 42.6, 43.5, 44.1, 46.5, 47.4, 49.8, 53.3, 56.2, 71.7, 72.0, 74.4, 74.54, 74.56, 75.7, 76.6, 78.7, 81.2, 81.3, 84.4, 113.1, 115.1, 126.9, 127.4, 128.8, 129.0, 129.25, 129.34, 130.4, 131.3, 132.3, 132.6, 133.7, 134.0, 136.9, 139.3, 141.4, 154.3, 167.0, 167.7, 168.3, 169.2, 170.7, 171.8, 172.0, 172.9, 175.0, 202.2; HRFABMS *m/z* 1346.5162 [M + Na$^+$] (calcd for $C_{73}H_{81}NO_{22}Na$, 1346.5148).

Succinic acid, 3,17β-dihydroxyestra-1,3,5(10)-triene-11α-yl ester 7-(3-carboxypropanoyl)-2′-taxol ester (30): (Hydrogenation was carried out at 50 psi.) ^{1}H NMR ($CDCl_3$) δ 0.77 (3H, s), 1.15 (3H, s), 1.19 (3H, s), 1.80 (3H, s), 2.01 (3H, brs), 2.13 (3H, s), 2.41 (3H, s), 1.23−2.93 (25H, taxol and steroid skeletons), 3.73 (1H, t, *J* = 8.6 Hz), 3.92 (1H, d, *J* = 6.9 Hz), 4.17 (1H, d, *J* = 8.4 Hz), 4.32 (1H, d, *J* = 8.4 Hz), 4.97 (1H, d, *J* = 9.3 Hz), 5.40 (1H, dt, *J* = 10.7, 5.2 Hz), 5.44 (1H, d, *J* = 3.2 Hz), 5.63 (1H, dd, *J* = 10.4, 7.2 Hz), 5.69 (1H, d, *J* = 6.9 Hz), 5.91 (1H, dd, *J* = 9.1, 3.0 Hz), 6.21 (1H, t, *J* = 9.3 Hz), 6.23 (1H, s), 6.55−6.62 (2H, overlapped, Ar), 6.86 (1H, d, *J* = 8.2 Hz, Ar), 7.15 (1H, d, *J* = 9.1 Hz), 7.31−7.65 (11H, Ar), 7.77 (2H, m, Ar), 8.11 (2H, m, Ar); ^{13}C NMR ($CDCl_3$) δ 11.0, 11.9, 14.7, 21.0, 21.3, 22.9, 23.2, 26.6, 26.9, 28.3, 28.5, 28.7, 29.4, 29.5, 30.5, 33.3, 35.6, 37.7, 42.1, 43.4, 44.1, 46.2, 47.2, 49.9, 53.0, 56.3, 71.8, 72.1, 74.3, 74.6, 74.7, 75.7, 76.5, 78.8, 81.1, 81.2, 84.1, 112.8, 115.5, 125.5, 126.9, 127.4, 128.7,

128.95, 128.98, 129.24, 129.27, 130.4, 131.3, 132.3, 132.6, 133.8, 134.0, 137.1, 139.5, 141.7, 154.6, 167.1, 167.7, 168.4, 169.7, 170.2, 171.67, 171.71, 172.3, 176.1, 202.1; HRFABMS *m/z* 1346.5078 [M + Na^+] (calcd for $C_{73}H_{81}NO_{22}Na$, 1346.5148).

Cell Lines. MDA-MB-231 and MCF-7 human mammary carcinoma cells were propagated in Dulbecco's Modified Eagle's Medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Summit Biotechnology, Fort Collins, CO) and 0.04 mg/mL gentamicin in a 7.5% CO_2 atmosphere at 37 °C.

MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Cell Proliferation Assay. Experiments were performed using replicate plated cells growing in 96-well sterile culture plates and the Cell Titer96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) as the source of MTS. Concentrated stocks of the test compounds dissolved in DMSO were added to the cells by diluting into culture medium supplemented with 5% serum. The DMSO concentration in each well was 0.1%. After 48 h incubation, MTS was added to the culture medium. Cell survival was assayed using the metabolic reduction MTS to a colored product by intact viable cells as the end point. Product formation at 37 °C, monitored by the increase in absorbance at 490 nM, was linear for 3 h. Cell survival curves were transformed to log-linear concentration response curves using Prism3.0 (GraphPad Software, Inc., San Diego, CA) and fit by nonlinear regression to the equation describing a sigmoidal dose−response relationship. Statistically significant differences in IC_{50} values between MCF-7 and MDA-MB-231 cells were determined by the Student's *t*-test.

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References and Notes

(1) The name taxol was assigned to the chemical compound **1** by Drs. Wall and Wani in 1971, and this name remained in general use until 1991. The name Taxol was then trademarked by Bristol-Myers Squibb for their formulation of the chemical compound taxol on the basis of an existing French trademark for a laxative compound with this name. Since this paper has been submitted in honor of Drs. Wall and Wani, the original name that they assigned for compound **1** is used, rather than the alternative name of paclitaxel. No infringement of the Bristol-Myers Squibb trademark is implied by this usage.

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