Synergistic Cytotoxicity Exhibited by Combination Treatment of Selective Retinoid Ligands with Taxol (Paclitaxel)

Valerie Vivat-Hannah,1 Dan You, Cheryl Rizzo, Jean-Paul Daris, Philippe Lapointe, F. Christopher Zusi, Anne Marinier, Matthew V. Lorenzi, and Marco M. Gottardis


ABSTRACT

The focus of this study was to develop retinoic acid receptor (RAR) RARα/β selective agonists with anticancer efficacy and reduced toxicity associated with RARγ activity. In these studies, we report the identification and characterization of high-affinity RARα/β selective agonists with limited RARγ activity. These compounds inhibited human tumor cell line proliferation with similar efficacy to that observed for a pan-RAR agonist. However, for most tumor cell lines, the efficacy of these compounds was restricted to the micromolar range. To determine whether the RARα/H9251/H9252/RXR heterodimer allows protein interactions with large corepressor complexes, we investigated the observations indicate that potent RARα/β selective agonists may be of therapeutic benefit in combination with Taxol therapy.

INTRODUCTION

The retinoids are biologically active derivatives of vitamin A that can regulate the proliferation, differentiation, and apoptosis of a wide variety of both normal and malignant cells (1–3). The natural retinoids primarily consist of ATRA2 and 9-cis retinoic acid, which are processed from vitamin A through its irreversible oxidation (4, 5). In addition, several synthetic ligands have been designed that retain many of the biological properties of the natural ligands (6, 7). The biological activities of the retinoids are mediated by two classes of nuclear receptors, the RARs and the RXRs, each of which comprises three isoforms designated α, β, and γ (reviewed in Ref. 8). RAR and RXR act as ligand-dependent transcription factors, which can modulate target gene expression by binding as RAR/RXR heterodimeric complexes to a specific RAR element DNA sequence (9, 10). The activity of the RAR/RXR heterodimer can be repressive or stimulatory, depending upon its apo (unliganded) or holo (ligand-bound) conformation, respectively. The apo conformation of RAR/RXR heterodimers allows protein interactions with large corepressor multiprotein complexes that contain nuclear corepressor and histone deacetylases and act to maintain the chromatin in a condensed transcriptionally inactive state to repress target gene expression (11–13). In contrast, the binding of agonist ligand to RARs is associated with a conformational transition of the ligand binding domain (14), resulting in the destabilization of the co-repressor complex and simultaneous recruitment of coactivators, including p160 protein family, CBP/p300, and the multiprotein complexes thyroid hormone receptor associated proteins (TRAP), Vitamin D3 receptor interacting proteins (DRIP), activator recruited cofactor (ARC) (11). Some of these factors contain histone acetyltransferase activity, allowing the decondensation of the chromatin required for the subsequent link of the holo-RAR/RXR heterodimer with the basal transcriptional complex to initiate target gene expression (11, 13).

ATRA and synthetic retinoid receptor ligands have been shown to promote tumor regression in a number of animal models of carcinogenesis and have shown efficacy in patients afflicted with acute promyelocytic leukemia (15, 16). Among the three RAR isotypes, the RARβ2 isoform has been associated with the tumor-suppressive effects of the retinoids. RARβ2 arises from alternative promoter use of the RARβ gene promoter P2 (8), which contains a strong RAR element, suggesting that RARβ expression is up-regulated by retinoic acid and places RARβ2 as a potential target gene underlying the growth-suppressive effects of retinoic acid. Furthermore, a large number of reports have described the loss of the RARβ2 expression during the early phases of cancer development including breast and lung tumors (17–19). In contrast, the expression of RARα and RARγ as well as RXRα are mostly unaltered in malignant tissues. Consistent with a role in modulating cell growth, RARβ gene inactivation in several cell types has led to the loss of retinoic acid-dependent growth arrest (20, 21). In contrast, the induction of RARβ expression in different tumor cell lines has been shown to be associated with a strong enhancement of retinoic acid responsiveness (22, 23). These observations highlight RARβ as an important mediator of retinoid action and suggest that its modulation may be effective in reducing tumor cell growth.

A growing interest has focused recently on increasing the antitumor activity of the retinoids through combination with other types of agents, including IFN-α (24), tumor necrosis factor-related apoptosis-inducing ligand (25), HDAC inhibitors (26–28), as well as chemotherapeutic agents (29–32). For instance, in cell lines isolated from patients afflicted with acute myeloid leukemia, HDAC inhibitors potentiate the differentiation effects of the retinoids and show activity in retinoid-resistant acute myeloid leukemia cells (26). The molecular mechanisms underlying the effect of these agents are related to the derepression of the retinoid receptor transduction pathway (26). For instance, in breast cancer cells, HDAC inhibitors have been shown to restore the retinoic acid-inducible expression of RARβ, independently of the methylation status of the RARβ gene promoter (33). In contrast, the mechanisms supporting the synergistic effects observed in combination treatments using the retinoids and IFN-α, or with chemotherapeutic agents such as cisplatin or Taxol, remain largely undefined.

In the present study, we report the identification of selective
RARα/β agonists and the effects of these ligands in inhibiting tumor cell proliferation. The engineered RARα/β agonists were generally found to be weak inducers of cytotoxicity in a variety of tumor cell lines as single agents. However, when administered with Taxol, RARα/β agonists dramatically lowered the inhibitory concentration of the tubulin-polymerizing agent to induce a cytotoxic response. The ability of the RARα/β selective ligands to synergize with Taxol was correlated with effects on the antiapoptotic protein Bcl-2 and with the activities of JNK and AP-1, pathways common to both retinoid and Taxol signaling. These observations indicate common retinoid-Taxol signaling pathways mediating cell proliferation and suggest that RARα/β agonists may have potential for use in combination regimens with Taxol.

MATERIALS AND METHODS

Materials. The cell lines used in this study including T47D, HT-3, UM-SCC-25, MCF7, OVCAR3, HCT-116, and N87 were obtained from American Type Culture Collection and maintained in the media and serum concentration recommended by American Type Culture Collection. The H3396 cell line was obtained from Dr. K. E. Hellstrom (Bristol-Myers Squibb, Seattle, WA; Ref. 34), and maintained in RPMI supplemented with 10% FBS (Life Technologies, Inc.). MDA-PCA2.B and SQCC-Y1 were obtained from Drs. N. Navone and S. M. Lippman (M. D. Anderson Cancer Center, Houston, TX), respectively. A2780 and its p27 transgenic variant A2780p27, HCT-116.TPXK. (Taxol-resistant) and HCTVM46, SAN-1, M109 and its Taxol-resistant variant M109TAX, and PAT-7 (Taxol resistant) cell lines were engineered for drug resistance were also used. All cell lines were of human origin except SAN-1, M109, and its variant M109TAX, which were murine cell lines. All of the cytototoxic agents, with the exception of Taxol, and PMA were purchased from Sigma Chemical Co.-Aldrich. The mouse anti-

Synthetic RAR Ligands. A detailed description of the routes of syntheses and structures of chemical intermediates used to generate BMS-194753 and BMS-228987, respectively. The c-jun (1–169)-GST fusion protein was obtained from Upstate Biosource International, Sigma Chemical Co.-Aldrich, and BD-PharMingen, respectively. The c-jun (1–169)-GST fusion protein was obtained from Upstate Biotechnology.

Mitogenic Assay. Cellular proliferation was quantified using [3H]thymidine incorporation. Briefly, the cells were plated in 96-well plates in medium containing 10% FBS. Cell medium was removed the following day, and the indicated compounds were added to fresh medium supplemented with 10% of charcoal-stripped FBS (HyClone). Compound-containing medium was renewed after 3 days exposure. After 6 days of treatment, 4 μCi/ml of [3H]thymidine (NEN Life Science Products) were added to each well for 2–4 h. The cells were then trypsinized and harvested on GF/B glass fiber filters. [3H]Thymidine incorporated into the DNA was measured in a scintillation Topcount counter (Packard). The results were expressed as a percentage of inhibition compared with the DMSO vehicle. IC50 was defined as the concentration of the (AP1)5x-tk-Luc reporter gene (37). The reporter construct consists of the collagenase gene AP1 response element repeated five times in tandem ([AP1]5x), the minimal tk promoter, and the coding sequence of the luciferase gene (Luc). For AP1 activity measurements, MCF7 cells were plated in 12-well plates at a density of 400,000 cells/well in DMEM supplemented with 10% FBS and allowed to adhere overnight. The next day, the medium was removed; the cells were washed with PBS and supplemented with DMEM medium containing 5% charcoal-stripped FBS (HyClone). Twenty-four h later, the cells were treated for 6 h with PMA (10 nm) in the absence or the presence of ATRA, BMS-231974, or BMS-228987 (concentrations ranging from 0.1, 1, and 50 nm) combined with or without Taxol (1 μM). The luciferase activity was measured by using Luc-lite system from Packard according to manufacturer’s recommendations. Results are expressed as the percentage of activity compared with PMA (10 nm), which was assigned as 100% activity. The data presented are the mean ± SE of three independent experiments.

JNK Assay. Activity of JNK was measured as described (40) with minor modifications. Briefly, MCF7 cells were plated in six-well plates in DMEM supplemented with 10% FBS. The next day, the cells were switched to DMEM supplemented with 5% charcoal-stripped FBS (HyClone), and 100 nM indicated retinoids or 0.1% DMSO was added to the cells for 24 h before an additional 2–3 h incubation in the presence of Taxol. JNK was immunoprecipitated from cleared cellular lysates by incubating overnight at 4°C with 1 μg of anti-human JNK (PharMingen) and 25 μl of protein G-agarose beads (Life Technologies, Inc.) washed previously with the lysis buffer. Immune complexes were pelleted by centrifugation and processed as described for JNK activity using 2 μg of GST-c-Jun (1–169) fusion protein. Samples were resolved by 10% SDS-PAGE, and phosphorylated GST-c-Jun was visualized by autoradiography and quantified by densitometry. The results represent the mean ± SE of three independent experiments.

RESULTS

RARα/β Selective Agonists Display Efficacy at Inhibiting Tumor Cell Proliferation. Although RARβ has been described as a key player of retinoid-induced growth inhibition, this isoform is most often lost during the process of carcinogenesis, and thus, the use of a pure RARβ agonist (BMS-209641) is inactive in inhibiting tumor cell proliferation (unpublished data). Taking into account the undesirable RARγ-related toxicity (41, 42) and the possibility that RARβ might be induced by retinoid treatment (43), the compounds chosen for this study were two synthetic ligands with a high selectivity for human RARα and RARβ.

3 Unpublished observations.
study exhibited both α- and β-agonistic selectivities without significant RARγ activity. The structures of the compounds and their selectivity profile for retinoid receptors are presented in Fig. 1.

Because ATRA and synthetic retinoids have displayed the ability to arrest the growth of cancer cells, the selective RARα/β agonists, BMS-228987 and BMS-276393, were first compared with the natural and synthetic RAR pan-agonists, ATRA and BMS-231974, respectively. The proliferation of 6 of 18 tumor cell lines (e.g., T47D, H3396, HT3, SANI, SCC25, and MCF7) was inhibited by nanomolar concentrations of α/β selective retinoids (Table 1). The maximum effect on growth inhibition ranged from 55 to 100%, as compared with DMSO. The remaining tumor cell lines were less sensitive to the antiproliferative effect of these retinoid ligands with IC50 restricted to the high micromolar concentration. In most tumor cell lines tested, the α/β selective agonists appeared as efficient as the RAR pan-agonist at inhibiting tumor cell proliferation, suggesting that the γ isotype of RAR is not critically required for the tumor-suppressive activity of the retinoids. In the SQCC-Y1 head and neck squamous carcinoma cell line, the 2 log difference in ATRA IC50 compared with the synthetic retinoids might be attributed to the participation of RXR because ATRA can be converted in situ into 9-cis-RA which is a pan-RAR and RXR agonist. Conversely, the lack of activity of ATRA compared with the synthetic compounds in several cell lines might reflect a difference in the stability of natural versus synthetic retinoid because the rapid metabolism of ATRA in some cell lines has been observed (5, 20).

The ability of MCF7 cells to form colonies was assayed in the presence of the selective RARα/β agonist, BMS-228987, and compared with ATRA. The colonies grown in the presence of BMS-228987 revealed a strong differentiating effect of the retinoid that is similar to that observed with ATRA. In contrast, vehicle-treated cells formed very compact colonies, with cells showing a very restricted cytoplasm, and most of the colonies appeared very well segregated (Fig. 2a). The treatment of MCF7 cells with ATRA (Fig. 2b) or BMS-228987 (Fig. 2c) resulted in a dramatic change in the morphology of both the colonies and the cells. The colonies appeared dispersed, the cells gained in volume, exhibited a large cytoplasm, and tended to organize into an epithelium-like structures (Fig. 2b for ATRA and 2c for BMS-228987). The number of colonies was only slightly decreased by BMS-228987 treatment (15%; P < 0.05) compared with DMSO-treated colonies (Fig. 2d), suggesting that retinoids were inducing differentiation rather than frank apoptosis.

**Cytotoxicity of Taxol Is Strengthened by RARα/β Selective Retinoids.** To determine whether the effect of the RARα/β selective agonists on cell growth could be additive or synergistic with existing chemotherapeutic agents, we investigated the combined use of RAR selective ligands with various cytotoxic agents. The colony assays performed with MCF7 cells (Fig. 2d) showed that Taxol-induced apoptosis resulted in the dose-dependent decrease of the colony number (by 45% at 10 nM Taxol), which was even more dramatically pronounced when Taxol was combined with the α/β selective retinoid, BMS-228987, and resulted in a reduction of 80% of the MCF7 colony number (Fig. 2d). Similarly, cotreatment of a variety of cell lines with Taxol and the RARα/β selective agonist, BMS-228987, dramatically lowered the effective dose of Taxol to induce cytotoxicity. In the presence of BMS-228987, the IC50 of Taxol was decreased by 8–15 and 32-fold in MCF7, OVCAR3, and SQCC-Y1, respectively. These values were very similar to those obtained for the combination of Taxol with the RAR pan-agonist BMS-231974 (Fig. 3 and Table 2), which supports the major role of RARα/β isotypes in the tumor-suppressive activity of the retinoids. In contrast, the pure RARα agonist BMS-194753 was associated with only a 2–4-fold decrease in Taxol IC50 (Table 2), which is in agreement with its lower affinity for RARα compared with BMS-228987 (not shown). Interestingly, the pure agonist for RARβ, BMS-209641, the RARγ-selective, BMS-270394, or the pure RXR agonist, BMS-188649, did not affect Taxol cytotoxicity by more than 2–3-fold, suggesting that the synergistic effects of the RARα/β ligands with Taxol is mainly mediated by RARα (Table 2).

To determine whether the effect of retinoids on lowering the effective dose of Taxol could be extended to other classes of chemotherapeutic agents, a variety of anticancer cytotoxic compounds including other tubulin-modifying agents and DNA-modifying agents were also assayed for their potential to synergize with the retinoids at inhibiting cell proliferation. The mechanisms of action of the different drugs depicted in Table 3 include DNA cross-linking for cisplatin, inhibition of topoisomerases I and II for camptothecin and doxorubicin, respectively, depolymerization of tubulin microfilaments for colchicine and vinblastine, and inhibition of thymidylate synthase for 5-fluorouracil, associated with an incorporation of fluorouracil into.

**Fig. 1.** Structure and selectivity of the retinoid derivatives. A, chemical structure of the synthetic retinoids (left diagram) compared with ATRA (right diagram). B, transcriptional activity of ATRA and the synthetic retinoids. ATRA is a pan-agonist for RAR. All synthetic retinoids are RAR specific and do not display binding to RXR. BMS-231974 is a pan-RAR agonist with β > α > γ selectivity. BMS-228987 and BMS-276393 are α/β selective agonists with β ≥ α selectivity. Weak residual γ activity could be detected (~25% at 3 μM ligand) for BMS-228987 and BMS-276393. BMS-209641 is a pure RARβ agonist, and BMS-270394 is a γ-selective agonist with low β-agonist activity. BMS-313493 is a pan-RAR antagonist. The data are the means of three independent experiments, where each ligand was tested in duplicate. The SDs associated with EC50/IC50 were between 10 and 15%. –, no effect was observed.

<table>
<thead>
<tr>
<th>Transcriptional Activation</th>
<th>RARα</th>
<th>RARβ</th>
<th>RARγ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC50</strong></td>
<td><strong>Efficacy</strong></td>
<td><strong>IC50</strong></td>
<td><strong>Efficacy</strong></td>
</tr>
<tr>
<td>ATRA</td>
<td>0.5</td>
<td>105</td>
<td>0.1</td>
</tr>
<tr>
<td>BMS-231974</td>
<td>2.0</td>
<td>105</td>
<td>0.2</td>
</tr>
<tr>
<td>BMS-228987</td>
<td>1.2</td>
<td>126</td>
<td>0.4</td>
</tr>
<tr>
<td>BMS-276393</td>
<td>1.1</td>
<td>107</td>
<td>1.0</td>
</tr>
<tr>
<td>BMS-209641</td>
<td>-</td>
<td>-</td>
<td>28.7</td>
</tr>
<tr>
<td>BMS-270394</td>
<td>-</td>
<td>-</td>
<td>275</td>
</tr>
<tr>
<td>BMS-313493</td>
<td>8</td>
<td>100</td>
<td>24.5</td>
</tr>
</tbody>
</table>
Table 1 Inhibition of tumor cell proliferation by retinoids

<table>
<thead>
<tr>
<th>Growth inhibition ([3H]thymidine incorporation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-trans RA</td>
</tr>
<tr>
<td>IC50</td>
</tr>
<tr>
<td>T47D</td>
</tr>
<tr>
<td>H3396</td>
</tr>
<tr>
<td>HT3</td>
</tr>
<tr>
<td>SCC25</td>
</tr>
<tr>
<td>MCF7</td>
</tr>
<tr>
<td>SQCC-Y1</td>
</tr>
<tr>
<td>SAN1</td>
</tr>
<tr>
<td>A2780p27</td>
</tr>
<tr>
<td>HCTVM46</td>
</tr>
<tr>
<td>OVCA3</td>
</tr>
<tr>
<td>HCT116</td>
</tr>
<tr>
<td>HCT116TXPK</td>
</tr>
<tr>
<td>M109</td>
</tr>
<tr>
<td>A2780S</td>
</tr>
<tr>
<td>PAT7</td>
</tr>
<tr>
<td>N87</td>
</tr>
<tr>
<td>MDA-PCA.2B</td>
</tr>
<tr>
<td>M109TX</td>
</tr>
</tbody>
</table>

* = no inhibitory activity.

Retinoids Do Not Affect Taxol-induced Tubulin Polymerization. To better understand the molecular basis of the synergistic effect between Taxol and the RARα/β selective retinoids, we analyzed the effects of both compounds on various signal transduction pathways affected by these agents. The combination effect of Taxol and RARα/β agonists was analyzed at the level of the reporter construct. Accordingly, the soluble, nonpolymerized tubulin was separated from insoluble polymerized tubulin and analyzed by Western blotting using anti-α-tubulin antibodies. The results shown in Fig. 4 reveal a dose-dependent polymerization of tubulin induced by Taxol (Lanes 1–6), whereas the selective RAR agonists (Fig. 4, Lanes 7 and 13 versus Lanes 8 and 14) or antagonist (Fig. 4, Lane 19 versus Lane 20) had no effect. The combination of Taxol with any of the retinoids did not enhance the effect of Taxol alone (Fig. 4, compare Lanes 3–6 with Lanes 9–12, with Lanes 15–18, and with Lanes 21–24). These observations indicate that the retinoids do not synergize with the effects of Taxol on the cytoskeleton to induce cytotoxicity.

Cross-Regulation of AP1 Activity by Retinoids and Taxol. Several recent reports have described Taxol-induced stimulation of JNK activity, leading to an increase in c-Jun phosphorylation and associated increase activity of the AP1 transcription complex (44). Retinoids have been also shown to regulate AP1 transcriptional activity (37). To determine whether the observed synergy correlated with effects on the JNK-AP1 axis, we analyzed the combination effect of Taxol and retinoids on JNK activity. These effects on JNK were further analyzed at the level of the AP1 transcription complex activity by using MCF7 cell line stably expressing the AP1 reporter gene, (AP1)x5-luc. The basal activity of the reporter construct was not affected either by the retinoids or by Taxol (Fig. 5, B and C), suggesting that jun/fos heterodimers are not constitutively binding to the AP1 response elements and that effects of these agents on AP1 require activation of the complex. Accordingly,
MCF7 cells treated with PMA, a potent activator of AP1, resulted in a robust AP1 transcriptional activity yielding a 10-fold increase in luciferase activity (Fig. 5B). The PMA-induced AP1 activity was inhibited in a dose-dependent manner by various retinoids including ATRA, the pan-RAR agonist (BMS-231974), or the selective RARα/β agonist (BMS-228987) reaching a maximum of 45% at 50 nM compounds (Fig. 6B). As expected, Taxol further enhanced AP1 activity by 20% (P < 0.01; Fig. 5C). However, in combination with ATRA, BMS-231974, or BMS-228987, the effect of Taxol on AP1 was abrogated in a dose-dependent manner, resulting in an overall inhibition of the collagenase promoter activity by up to 50% (Fig. 5C). Taken together, these data show that the combination effect of Taxol and the α/β selective RAR agonists at stimulating JNK activity becomes dissociated from the subsequent increase in AP1 activity.

Combination Effects of Taxol and Retinoids on Bcl-2. We examined next the effects of Taxol/retinoid cotreatment on the expression levels and phosphorylation status of the antiapoptotic protein, bcl-2, a protein implicated in both retinoid and Taxol signaling. MCF7 cells treated with Taxol displayed a dose-dependent increase in the phosphorylation of bcl-2, which is characterized by a higher molecular weight upper band (Fig. 6, Lanes 1–6). The addition to the cells of RARα- or selective α/β agonists resulted in a down-regulation of the Bcl-2 protein levels (Fig. 6, compare Lanes 7 and 8; Lanes 13 and 14), whereas the RAR-selective antagonist alone had no effect on Bcl-2 levels or phosphorylation (Fig. 6, compare Lanes 19 and 20). The combination of Taxol with the selective RAR agonists resulted in a down-regulation of Bcl-2 levels and an increase in its phosphorylation status (Fig. 6, Lanes 9–12 and Lanes 15–18). In contrast, the combination of Taxol with the RAR antagonist (BMS-313493) was only associated with an increase in the phosphorylation of Bcl-2 (Fig. 6, Lanes 21–24) without a change in protein expression levels. These results indicate that the synergistic effect of Taxol and RARα/β agonists may in part be attributable to the additive effects at the level of down-regulation and phosphorylation of bcl-2 that may result in the release of proapoptotic Bax activity, mitochondria membrane permeabilization, and the induction of apoptosis. These observations, together with the effects on AP1, may underlie the potent synergistic cytotoxic effect of these agents.
**DISCUSSION**

Natural and synthetic retinoids have shown their efficacy at inhibiting cell progression in a wide variety of tumor models. The data presented in this report reveal that RARα/β selective retinoids are sufficient to trigger inhibition of cell proliferation and enhance tumor cell sensitivity to cytotoxic agents. On a panel containing 18 tumor cell lines of various origin, the effect of RARα/β selective agonists at inhibiting cell growth are similar to that of ATRA, demonstrating that the RARγ or the pan RXXR selectivity are not critically required for regulating tumor cell growth. These observations are of importance considering the severe mucocutaneous toxicity associated with the RARγ activity in pan retinoid ligands (41, 42), which have limited the therapeutic uses of these agents. It is possible that the weak sensitivity of many of the tumor cell lines used in this study to RAR-selective ligands is attributable to lower levels of expression of the retinoid receptors. However, recent reports have demonstrated that several lung cancer cell lines were resistant to the growth-inhibitory effects of ATRA, despite high levels of RXRs and the RARs, including RARβ (45, 46). This defect of the retinoids at inhibiting cell proliferation was attributed in part to a failure of the RARs at trans-repressing AP1 activity (45). Furthermore, it has been suggested that the desensitization of lung cancer cells to RARβ-mediated growth inhibition is related to the balance of orphan receptors NUR-77 and COUP-TF, which governs RARβ promoter responsiveness to ATRA-activated RAR (46). Taken together, this suggests that in addition to the retinoid receptor levels, the activity of the retinoids at inhibiting cell proliferation may also depend upon cross-regulation of the retinoid, AP1, COUP-TFs, and NUR77 signaling pathways.

Several reports have emphasized the cytotoxic effect of the RARγ-selective agonist, CD437, on a variety of tumor cell lines (47, 48). However, various lines of evidence suggest that the apoptotic pathway induced by CD437 is RAR independent (49–51). Similarly, the genetic inactivation of the retinoid receptors in F9 embryonic carcinoma cells showed an RAR-independent apoptotic response of the cells to the retinoid analogue fenretinide (52). Interestingly, DNA fragmentation or FACS measurements on a limited number of cell lines revealed that treatment of breast and ovarian cell lines for up to 6 days with the selective RAR pan-agonist (BMS-231974) or the α/β-selective agonists did not induce apoptosis (data not shown) but significantly inhibited the proliferation of these cells. Because the expression of the β-isotype of RAR is lost in most of the tumor cells, the RARα/β agonist activity is most likely mediated through the RARα isoform. Consistent with this hypothesis, a pure RARβ agonist (BMS-209641) had no effect on cell proliferation. However, it is conceivable that RARα might derepress RARβ expression through the RAR-specific response element located in RARB3 promoter and induces a RARβ-mediated growth-inhibitory effect. However, of the cell lines examined in this study, only T47D displayed strong induction of RARβ in response to the selective retinoids. In contrast, two other cell lines, H3396 (breast) and HT3 (cervix), did not show any induction of RARβ expression in response to ATRA or the selective α/β agonists (data not shown). This observation is in agreement with the observations that the sensitivity of various murine melanoma cell lines to the growth-inhibitory effect of retinoids was not systematically associated with an induction of RARβ (43). In contrast, the MCF7 clonogenic assays demonstrated the differentiating effect of the α/β selective retinoids. Taken together, these observations reveal that the growth-inhibitory activity of the α/β selective retinoids is not related to the induction of apoptosis and/or RARβ expression and suggest that the activity of these retinoids on cell proliferation might reflect an inhi-

---

**SELECTIVE RETINOIDS SYNERGIZE WITH TAXOL**

*IC₅₀* and efficacy of DNA-damaging agents (cisplatin, doxorubicin, and camptothecin), tubulin-modifying agents (colchicine and vinblastine), or 5-FU in the absence or the presence of selective retinoids as measured by [*³H*]thymidine incorporation. The cell lines included MCF7 (breast), SQCC-Y1 (head and neck), and OVCAR3 (ovary) and were processed as described in Tables 1 and 2. Efficacy is defined as the percentage of inhibition compared with DMSO. All *IC₅₀* are expressed in nanomolar values. The results are the means of two independent experiments.

**Table 3 Combination effect of retinoids and chemotherapeutic agents on tumor cell proliferation**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀</th>
<th>Efficacy</th>
<th>IC₅₀</th>
<th>Efficacy</th>
<th>IC₅₀</th>
<th>Efficacy</th>
<th>IC₅₀</th>
<th>Efficacy</th>
<th>IC₅₀</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>1875</td>
<td>100</td>
<td>6.5</td>
<td>100</td>
<td>1975</td>
<td>100</td>
<td>7</td>
<td>100</td>
<td>64</td>
<td>100</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>614</td>
<td>100</td>
<td>5.3</td>
<td>100</td>
<td>2760</td>
<td>100</td>
<td>6.5</td>
<td>100</td>
<td>2.2</td>
<td>100</td>
</tr>
<tr>
<td>5-FU</td>
<td>970</td>
<td>100</td>
<td>5.8</td>
<td>100</td>
<td>225</td>
<td>100</td>
<td>11.5</td>
<td>100</td>
<td>0.6</td>
<td>100</td>
</tr>
<tr>
<td>Colchicine</td>
<td>2135</td>
<td>100</td>
<td>5.8</td>
<td>100</td>
<td>1350</td>
<td>100</td>
<td>8</td>
<td>100</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>96</td>
<td>100</td>
<td>7.8</td>
<td>100</td>
<td>932</td>
<td>100</td>
<td>6</td>
<td>100</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>100</td>
<td>100</td>
<td>5</td>
<td>100</td>
<td>960</td>
<td>100</td>
<td>5</td>
<td>100</td>
<td>0.2</td>
<td>100</td>
</tr>
</tbody>
</table>

---

![Fig. 4. Taxol-induced tubulin polymerization is retinoid independent. The combined effects of retinoids and Taxol on the polymerization of tubulin in MCF7.](image-url)
bition of cell cycle progression and/or induction of a pathway of differentiation.

The combination of the selective RARα/β agonists with various cytotoxic agents revealed an enhanced cytotoxicity to the tubulin-modifying agent, Taxol, and to a much lesser extent with vinblastine. In contrast, no significant effects were observed with DNA-damaging agents such as cisplatin, doxorubicin, or camptothecin. Wang et al. (32) recently reported that pretreatment with ATRA-sensitized breast cancer cells to Taxol. The lack of synergy between the RAR-selective retinoids and the DNA-damaging agents, cisplatin and doxorubicin, in contrast to other reports (30, 31, 53), appears mostly related to the compound treatment schedules. It was shown that a pretreatment of breast cancer cells with ATRA is a prerequisite to the enhanced cell sensitization to cisplatin and doxorubicin (31, 53). Similar to our observations, ATRA had no beneficial effect on cisplatin or doxorubicin cell sensitivity in simultaneous treatment schedules (31). The specific synergistic effect observed with the combination of Taxol and RARα/β agonists suggests that the combination effect of Taxol and retinoids is linked to the modulation of common signaling pathways. Indeed, we show that combination of RARα/β agonists and Taxol results in a down-regulation of Bcl-2 and an increase of its phosphorylation, a posttranslational modification shown to abrogate the anti-apoptotic function of the protein (54). It has been proposed that cells undergoing mitosis are predisposed to apoptosis to ensure the elimination of cells with aberrant chromosomal segregation (55). This notion is consistent with our observations that the apoptosis resulting from the G2-M mitotic arrest and phosphorylation of Bcl-2 induced by Taxol is amplified by the retinoid-mediated down-regulation of Bcl-2 protein levels, which lowers the pool of Bcl-2 available to promote cell survival.

Retinoid ligands have also been shown to exert transrepression of AP1 activity (37, 56) by disrupting c-Jun/c-Fos dimerization (57). AP1 transcription factors can modulate proliferation, differentiation, or apoptosis, depending on the cell type. In fibroblasts and breast epithelial cells, AP1 activity is induced in response to multiple growth/mitogenic factors, resulting in the stimulation of cell proliferation, transformation, and tumoral progression (58, 59). We demonstrate here that the increase in AP1 activity observed in response to Taxol-induced JNK activity is dramatically inhibited in the presence of retinoids. These observations suggest that the enhanced activity of JNK induced by Taxol is uncoupled from the subsequent stimulation of AP1 activity in the presence of retinoids without affecting the capacity of Taxol at inducing Bcl-2 phosphorylation and cell apoptosis. Several reports have demonstrated that the effects of tubulin-modifying agents on both raf1/Bcl-2 (54, 60) and MEKK1/JNKK/JNK (61–63) signaling pathways are linked and necessary for the induction of apoptosis. In particular, impairment of the JNK pathway induced by a dominant-inhibitory mutant has been shown to prevent phosphorylation of Bcl-2 and the ability of Taxol at regulating apoptosis (55, 61). Similarly, mutation of Bcl-2 phosphorylation sites was shown to abolish paclitaxel-induced apoptosis (64). In agreement with our findings, Lee et al. (61) have shown that the combination of Taxol treatment with 1xBα expression, which dissociates JNK activation from subsequent AP1 activity, had no impact on Taxol-induced apoptosis. These observations indicate that the combined effect of Taxol and retinoids enhance Taxol-induced JNK activity and compromise cell survival through retinoid inhibition of AP1 activity. Together these data support the synergistic inhibition of tumor cell proliferation obtained by the combination of Taxol with selective retinoids at the level of Bcl-2 and JNK/AP1, two key regulators of cell survival and proliferation.

The benefit of the use of retinoids in cancer was first demonstrated in patients afflicted with acute promyelocytic leukemia (15, 16).
Subsequently, the combination of 13-cis-retinoic acid with paclitaxel or cisplatin and/or IFN-α has entered Phase II/II trials for the treatment of head and neck squamous cell carcinomas, non-small cell lung carcinomas, and prostate cancer (65, 66). Similarly, the pan-RAR agonist ALRT1550, a potent inhibitor of AP1, has been included in a Phase II/I study on a heterogeneous population of 15 patients with advanced cancer, including non-small cell lung, thyroid, kidney, uterine leiomyosarcoma, prostate, and adenoid cystic carcinoma (67). In this regard, the α,β selective retinoid ligands reported in this study may be useful in combination therapy with Taxol and offer the potential advantage of being devoid of adverse side effects such as mucocutaneous toxicity or chondrogenesis inhibition associated with RARγ activity.

ACKNOWLEDGMENTS

We are grateful to Drs. H. Gronemeyer and P. Chambon for providing us with the MCF7-API1 reporter cell line and for helpful discussion. We also thank Drs. A. Bianchi and F. Lee for providing cell lines and helpful discussion. We also acknowledge M.-L. Wen and M. Arico for providing us with helpful advice on colony and tubulin assays, D. Hawkins for the RARβ expression analysis, and S. Roy and M. Belema for providing us with BMS-313493 and BMS-270394.

REFERENCES


Downloaded from cancerres.aacrjournals.org on April 15, 2017. © 2001 American Association for Cancer Research.


Synergistic Cytotoxicity Exhibited by Combination Treatment of Selective Retinoid Ligands with Taxol (Paclitaxel)

Valerie Vivat-Hannah, Dan You, Cheryl Rizzo, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/24/8703

Cited articles
This article cites 65 articles, 32 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/24/8703.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/61/24/8703.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.