Enhanced Antitumor Efficacy of Cisplatin in Combination with ALRT1O57 (9-cis Retinoic Acid) in Human Oral Squamous Carcinoma Xenografts in Nude Mice


Departments of Retinoid and Endocrine Research, Ligand Pharmaceuticals, Inc., San Diego, California 92121 [D. R. S., E. D. B., M. L. G., W. W. L., R. A. H., J. S. H.], and Department of Pharmacology, University of Texas at Houston, Houston, Texas 77030 [V. T., P. J. A. D.]

ABSTRACT

Cisplatin (DDP) is commonly used to treat head and neck tumors. Therapy frequently fails due to development of DDP resistance or toxicities associated with DDP therapy. In this study, effects of ALRT1O57 [9-cis retinoic acid (9-cis RA)] on DDP cytotoxicity were studied in a human oral squamous carcinoma xenograft model. Mice bearing xenografts were dosed p.o. daily 5 days/week with 30 mg/kg 9-cis RA and/or i.p. twice weekly with 0.3–0.9 mg/kg DDP. Maximum tolerated doses of 9-cis RA and DDP were approximately 60 and ≥2.9 mg/kg, respectively, under their dosing schedules and routes of administration. Control tumors grew rapidly with mean doubling times of 4 ± 1 days and reached mean volumes of 1982 ± 199 (SE) mm³ after 24 days. DDP at doses of 0.3, 0.45, and 0.9 mg/kg inhibited tumor growth by 28, 47, and 86%, respectively, 24 days after tumor cell implantation. Thirty mg/kg 9-cis RA inhibited tumor growth by 25%. In combination, 0.3 mg/kg DDP + 30 mg/kg 9-cis RA inhibited tumor growth by 68%; 0.45 mg/kg DDP + 30 mg/kg 9-cis RA inhibited growth by 78%. These decreases were greater than those that would have been produced by either agent consumed separately. Of importance, at doses of 9-cis RA that enhanced DDP cytotoxicity, no change in dose tolerance was observed as compared to tolerances observed for either agent alone, indicating that 9-cis RA increased sensitivity to DDP without altering systemic toxicity. In addition, 9-cis RA profoundly altered squamous cell carcinoma phenotypes by suppressing squamous cell differentiation, resulting in tumors with increased numbers of basal cells. In contrast, DDP selectively depleted proliferating basal cells from carcinomas. In combination, morphological changes produced by 9-cis RA alone predominated, suggesting a possible basis for enhanced DDP sensitivity in tumors exposed to both agents. These data demonstrate that 9-cis RA enhances tumor sensitivity to DDP, and suggest that this combination should be tested in Phase I–II clinical trials for its potential for improving anticancer therapy of squamous cell cancers.

INTRODUCTION

DDP¹ is a chemotherapeutic agent used to treat head and neck, testicular, ovarian, and lung carcinomas (1, 2). Although tumors generally respond to DDP, resistance to DDP frequently develops and most patients ultimately fail chemotherapy (2, 3). In addition, DDP therapy is limited by cumulative neurotoxicities, myelosuppression, renal and otoxicities, and emesis (2, 3). It would be desirable to improve the therapeutic index of this commonly used cytotoxic agent by either improving efficacy or decreasing toxic side effects. One of the strategies to achieve this goal would be to use DDP in combination with agents that exhibit low or no toxicity.

Squamous cell cancers, which comprise 90% of head and neck tumors (4), represent one of the classes of tumors that often fail chemotherapy to DDP (2, 3). Retinoids have been used to treat a variety of squamous cell neoplasms, including head and neck tumors, in which prominent chemopreventive activities have been reported (4–9). Retinoids regulate squamous cell differentiation and proliferation in normal and neoplastic epithelial cells (10–12), suggesting that retinoids would be ideal candidates to combine with DDP to improve chemotherapy. This hypothesis is supported by Sacks et al. (13), who have reported that ATRA inhibits monolayer growth of head and neck cell lines and multicellular HNSCC spheroids in culture without altering cell cycle (specifically S-phase) or thymidine incorporation (13). Because of these effects and the fact that retinoid toxicities do not overlap with those produced by DDP (2, 4), retinoids appear to represent an excellent class of agents to combine with DDP to improve therapy of solid tumors.

There is a paucity of preclinical in vitro or in vivo data in which retinoids have been studied in combination with cytotoxic agents. Preliminary in vitro reports have recently appeared suggesting that combination chemotherapy results in enhanced ef-

Received 9/13/95; revised 11/13/95; accepted 11/28/95.

¹ This work was supported by the Allergan-Ligand joint venture. Portions of this work were presented at the 86th Annual Meeting of the American Association for Cancer Research, Toronto, Ontario, Canada, March 18–22, 1995 (Proc. Am. Assoc. Cancer Res., 36: 296, 1995).

² To whom requests for reprints should be addressed, Ligand Pharmaceuticals, Inc., 10255 Science Center Drive, San Diego, CA 92121. Phone: (619) 550-7836; Fax: (619) 550-7730; E-mail: dshalinsky@ligand.com.

³ The abbreviations used are: DDP, cis-diamminodichloroplatinum (cis-platin); ATRA, all-trans retinoic acid; 9-cis RA, 9-cis retinoic acid; MTD, maximum tolerated dose; HNSCC, head and neck squamous cell carcinoma; RAR, retinoic acid receptor; RXR, retinoid X receptor; BrdU, 5’-bromo-2’-deoxyuridine.
ficiacy (14–16). In vivo in a preclinical model, a single report demonstrated that the synthetic retinoid 4-hydroxyphenylretinamide enhanced activity of DDP to increase survival of nude mice bearing ovarian carcinoma xenografts (17). Encouraging clinical results have also been reported for using retinoids and cytotoxic agents in combination (reviewed in Ref. 13).

We have previously reported that ALR1057 (9-cis RA) suppresses squamous cell differentiation of human oral carcinoma xenografts (line HNSCC 1483) in nude mice (18). Based on these data and the fact that 9-cis RA binds to and transactivates RARs and RXRs with high affinities (19), we have chosen to study effects of DDP and 9-cis RA in combination in HNSCC xenografts. In this model, drugs are subjected to in vivo pharmacokinetics and squamous carcinomas develop a three-dimensional cytoarchitecture that more closely resembles oral cancers observed clinically (20–22). Furthermore, 30 mg/kg 9-cis RA inhibited tumor growth modestly but markedly increased the number of basal cells in the carcinomas (18), which represent the cell population that is theoretically susceptible to the cytotoxic effects of DDP. Based on these phenotypic changes, the current study was designed to test whether 9-cis RA would enhance activity of DDP in human HNSCC xenografts.

We report here that 9-cis RA enhanced the cytotoxic activity of DDP and improved antitumor efficacy without increasing toxicities of either agent, suggesting that this combination has potential for improving anticancer therapy of squamous cell carcinomas.

MATERIALS AND METHODS

Reagents and Chemicals. ALR1057 (9-cis RA; lot 000X018) was synthesized at Ligand Pharmaceuticals, Inc. (San Diego, CA) and recrystallized to >99% purity. Compound purity was verified by elemental analysis, nuclear magnetic resonance spectra, and high-performance liquid chromatography–UV assay. DDP [Platinol (clinical formulation); Bristol-Myers Squibb, Princeton, NJ] was purchased from an oncology pharmacy. Reconstituted to 1 mg/ml in saline, and stored at room temperature protected from light. BrdU, (Sigma, St. Louis, MO) was solubilized in sterile saline (100 mg/kg) prior to use.

Cell Line. The 1483 cell line was derived from a patient with squamous cell cancer of the retromolar trigone. Histopathology showed that line 1483 was aggressive and had spread to one lymph node (23). Cells were routinely cultured in DMEM: F12 media (Bio-Whittaker, Walkersville, MD) supplemented with 10% FCS (Hyclone, Logan, UT), 2 mm L-glutamine, and 5 units/ml penicillin/5 μg/ml streptomycin (GIBCO-BRL, Grand Island, NY) in 95% air/5% CO₂ as previously described (18). Cells were tested and found to be free of Mycoplasma.

In Vivo Tumor Xenograft Studies. Cells in log phase were harvested by trypsinization, resuspended in DMEM:F12 medium, centrifuged at 1000 rpm for 10 min, and resuspended in culture media at a concentration of 1 × 10⁷ cells/ml prior to s.c. implantation into mice.

Mice were quarantined for 1 week prior to study and allowed access to food (Purina Chow 5010; Purina, St. Louis, MO) and water ad libitum. Female athymic NCrl nude (nu/nu) mice, 6–7 weeks old (20 ± 2 g; Taconic Labs, Germantown, NY), were implanted with 1 × 10⁶ cells bilaterally into axial regions with a 24-gauge needle/1-ml tuberculin syringe (Becton Dickinson, Rutherford, NJ). Animals were randomized 48 h after tumor implantation into treatment groups. Each group consisted of five to six animals bearing two tumors/animal; experiments were repeated twice. 9-cis RA (30 mg/kg) was administered p.o. beginning 2 days after tumor cell implantation with a 20-gauge intragastric feeding tube (Popper & Sons, New Hyde Park, NY) daily, 5 days/week, in 0.1 ml super refined sesame oil (Croda, Inc., Parsippany, NJ). The MTD in this tumor biology model was defined as a dose that produced significant decreases (≤7%) in body weight or body weight gains and mild mucocutaneous toxicities. We have previously reported the MTD for 9-cis RA as 60 mg/kg in athymic mice dosed for 4 weeks daily (5 days/week) based on this definition (18). A 30-mg/kg dose of 9-cis RA was used in this study. DDP is typically dosed i.p. in rodents (24); DDP was diluted in 0.1 M Tris-HCl buffer (pH 7.4) to an appropriate concentration (~0.06–0.58 mg/ml) and injected in 0.1 ml in mice. DDP was administered twice weekly (Monday and Thursday), concurrently with 9-cis RA, to increase the time tumors would be exposed to both DDP and 9-cis RA. Studies were conducted for 24 days in accordance with a protocol for human animal care approved by the Institutional Animal Care and Use Committee. Tumors were measured with electronic calipers (Mitutoyo, Japan) once or twice weekly, and tumor volumes were calculated using the formula for volume (l × w × h). Animal weights were recorded at least once per week using an O’Haus Model C305-S balance (Florham Park, NJ). Clinical signs such as overall health status and potential mucocutaneous toxicities were also recorded at least weekly.

Xenograft Tumor Biomarker Analyses. On day 24 (Friday, the fifth day of a 5-day/week dosing schedule) of tumor biology studies, animals were given i.p. injections of 100 mg/kg BrdU (0.1 ml in PBS, pH 7.4) 1 h before tumor collection. Animals were anesthetized with isoflurane and euthanized by cervical dislocation immediately before tumor collection. Tumors were surgically excised, and cross-sections were cut using sterile scalpels. Samples were fixed in 10% neutral buffered formalin for 24 to 48 h before embedding in paraffin blocks. Hematoxylin and eosin staining of tumors was performed on 5-μm cross-sections cut from these blocks.

BrdU Staining and Analyses of Tumor Sections. Five-μm sections of paraffin-embedded tumor tissues were stained using an anti-BrdU antibody (Becton Dickinson, San Jose, CA) with the Vectastain ABC enzyme immunoperoxidase kit (Vector Labs, Burlingame, CA). Positive cells were stained blue-gray using a Vector SG substrate kit for peroxidase (Vector Labs), and tissues were counterstained with nuclear fast red to enhance visualization of positive BrdU-staining cells. Analyses were performed by counting the number of BrdU-labeled cells per 0.0625-mm² field from three different tissue sections per tumor. Four randomly chosen fields per section were examined (two to four tumors/treatment group).

Data Analyses. Data were plotted as mean ± SE. Statistical analyses of data were performed using the unpaired Student’s t test with two-tail comparison. Differences of P < 0.05 were considered to be significantly different from control.
RESULTS

Effects of DDP and 9-cis RA as Single Agents on Weights and Health of Nude Mice. To determine tolerated doses of DDP, dose-ranging studies were conducted. Table I gives the effects of DDP, 9-cis RA, and DDP + 9-cis RA on animal weights and health after 24 days when administered as described in "Materials and Methods." DDP was well tolerated when administered i.p. twice weekly at doses up to 0.9 mg/kg. Doses of 2.9 mg/kg were tolerated with minimal weight loss after 24 days. The MTD for DDP was assumed to have been at least 2.9 mg/kg. We have previously reported the MTD for 9-cis RA in tumor biology studies in nude mice as 60 mg/kg; this dose produces a 4.2% decrease in body weight and mild mucocutaneous irritations after 24 days of 5 days/week dosing (18). In this study, 9-cis RA, administered p.o. daily 5 days/week was well tolerated at 30 mg/kg. For xenograft studies, well-tolerated doses of DDP (<1 mg/kg) and 9-cis RA (30 mg/kg) were chosen to assess the potential for synergy in combination studies.

Effects of DDP on Tumor Growth. Prior to studying effects of DDP and 9-cis RA in combination, tumor response to DDP as a single agent was determined. Fig. 1 shows the effects of DDP on the growth of 1483 xenografts. Control tumors had a 10-day lag phase after implantation and then grew rapidly with doubling times of 4–6 days. Mean volumes of 1982 ± 199 mm³ were measured after 24 days. 1483 xenografts had a take rate of >95%, and this rate was not affected by treatments with DDP, 9-cis RA, or 9-cis RA + DDP. DDP at 0.3, 0.45, and 0.9 mg/kg inhibited tumor growth in a dose-dependent manner by 28, 47, and 86% after 24 days (P < 0.001 for doses ≥0.45 mg/kg). At 2.9 mg/kg, DDP completely inhibited tumor growth. DDP produced a 50% inhibitory concentration for tumor growth inhibition of 0.48 mg/kg (Fig. 1, inset). Morphologically, after 24 days, control tumors formed well-differentiated squamous cell carcinomas (Fig. 2A). Tumors were comprised of well-differentiated squamous cell carcinoma exhibiting pronounced keratinization (keratin pearls), cellular stratification (suprabasal and basal cells), and abundant stroma. Morphologies of tumors in mice treated with 0.45 and 0.9 mg/kg DDP are also shown in Fig. 2 (B and C). DDP did not suppress keratin pearl formation, and both suprabasal and basal cell compartments were present. However, fewer basal cells were observed with increasing doses of DDP in which tumor growth was progressively decreased. Morphological assessments indicated that DDP selectively depleted the proliferating basal cell pool.

Effects of 9-cis RA, and DDP + 9-cis RA, on Tumor Growth. We have previously reported that 1483 xenograft tumors are growth inhibited by 9-cis RA and ATRA (18). These retinoids did not decrease the rate of exponential tumor growth, but delayed the latent period until exponential growth began. In this study, similar data were produced by 9-cis RA; 9-cis RA decreased mean tumor volumes by 25 ± 9% to 1493 ± 186 mm³ after 24 days (Fig. 3A). Growth inhibition experiments have been performed seven times, and the effects of 9-cis RA on growth have been shown to reach statistical significance when all studies are included in statistical analyses (18). In two experiments reported herein, in which DDP and 9-cis RA were compared head-to-head, 9-cis RA inhibited tumor growth but this decrease was not statistically significant (P < 0.08). As we have previously reported (18), 9-cis RA treatment suppressed epidermoid differentiation of 1483 xenografts (Fig. 4A). After 9-cis RA treatment, keratin pearls were not present and suprabasal and basal cell layers were indistinguishable as compared to control tumors in which these cell layers were cytologically distinct (Fig. 2A). Thus, the effects of 9-cis RA were more prominent on tumor morphology than tumor growth; 9-cis RA decreased squamous cell differentiation and increased the number of basal cells in tumors.

Tumor growth after combination treatment with DDP + 9-cis RA is shown in Fig. 3. Tumor volumes were decreased by

Table I Effects of DDP, 9-cis RA, or DDP + 9-cis RA on animal body weights

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>% Change</th>
</tr>
</thead>
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<tr>
<td>Control</td>
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<td>19.9 ± 1.5</td>
<td>22.0 ± 1.1</td>
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<td>DDP</td>
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<td>21.5 ± 0.8</td>
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<td>18.5</td>
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<tr>
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<td>19.8 ± 1.1</td>
<td>21.5 ± 1.1</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>18.4 ± 0.9</td>
<td>17.7 ± 1.6</td>
<td>-3.8</td>
</tr>
<tr>
<td>9-cis RA</td>
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<td>20.7 ± 2.1</td>
<td>4.5</td>
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<tr>
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<td>19.4 ± 2.0</td>
<td>19.8 ± 2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>9-cis RA + DDP</td>
<td>0.9</td>
<td>19.7 ± 1.1</td>
<td>20.5 ± 1.7</td>
<td>4.1</td>
</tr>
</tbody>
</table>

* Mean ± SD from two to three experiments conducted with three to five animals/group, except 0.6 mg/kg DDP (n = 1).

* p value, mean group initial weight (day 0) vs. final weight (day 24). P < 0.05, significantly different final vs. initial weight.
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68% to 636 ± 122 mm³ (P < 0.001) after 24 days by the combination of 0.3 mg/kg DDP + 30 mg/kg 9-cis RA (Fig. 3B); a decrease that was 28% greater than that calculated by summing inhibitions produced by each agent alone. The combination of 0.45 mg/kg DDP + 30 mg/kg 9-cis RA decreased tumor volumes by 78% to 444 ± 48 mm³ (P < 0.001; Fig. 3C) after 24 days. At doses of 0.9 mg/kg DDP + 30 mg/kg 9-cis RA, complete tumor inhibition was observed (data not shown). Residual tumors observed after 24 days of combination treatment (Fig. 4A), looking similar to tumors treated with 9-cis RA alone (Fig. 4A), suggesting that the effects of 9-cis RA on tumor morphologies predominated to maintain suppression of squamous cell differentiation during combination treatment.

Tumor growth rates under control, single agent, and combination treatments were analyzed using linear regression over days 13–24 of the study (Fig. 5A). Control tumors had mean growth rates of 145 mm³/day. DDP at 0.3 and 0.45 mg/kg decreased mean rates by 30.3 and 50.1%, respectively. 9-cis RA decreased it by 26.6%. In combination, 0.3 mg/kg DDP + 9-cis RA decreased growth rates by 75.0% (P < 0.001), and for 0.45 mg/kg DDP + 9-cis RA, it was decreased by 81.1% (P < 0.001). The magnitude of inhibition produced by 0.3 mg/kg DDP + 9-cis RA on growth rates was 44% greater than that produced by 0.3 mg/kg DDP and 18% greater than the theoretical additive effect (P < 0.01). To highlight the effects of combination treatment on tumor growth as compared to DDP alone, growth as reflected by mean tumor volumes is shown in Fig. 5B. These data show that 9-cis RA shifted the dose-response curve for DDP to the left, demonstrating enhanced cytotoxic activities of DDP with 9-cis RA treatment.

Effects of 9-cis RA or DDP on BrdU Incorporation in Tumors. Tumor proliferation rates were estimated by assessment of incorporation of BrdU into tumor cells. Divergent results were produced in two separate studies. In one study, control tumors had an average of 43.1 ± 2.3 (SE) positive cells/field, whereas 9-cis RA treated tumors had 32 ± 1 positive cells/field. Thus, treatment with 9-cis RA showed a significant 27 ± 3% (P < 0.001) decrease from controls. DDP also decreased the percentage of BrdU-positive cells by 31 ± 3 and 40 ± 3% (P < 0.001) at 0.45 and 0.9 mg/kg, respectively. In combination, 0.45 mg/kg DDP + 9-cis RA also significantly inhibited BrdU incorporation by 36 ± 5% (P < 0.001). Decreases produced by DDP + 9-cis RA were not different from those produced by either DDP or 9-cis RA as single agents. In a subsequent study, only the effects of 9-cis RA were studied, and no effect on BrdU incorporation was observed. The control labeling index was 52.5 ± 2 positive cells/field, whereas 9-cis RA-treated tumors had values of 51.6 ± 2 positive cells/field. These data show that both DDP and 9-cis RA can decrease BrdU incorporation in the 1483 tumors, but the effects of 9-cis RA were not consistently observed.

In conjunction with assessing antitumor efficacies, animal health and weights were monitored to assess potential toxicities of combination therapy. Control animals gained 10.6% body...
weight over the 24-day course of the study. Weight gain increases after treatment with 30 mg/kg 9-cis RA were 4.5%, but the difference between control and 9-cis RA-treated animals was not statistically significant ($P < 0.1$), and occurred in the presence of mild mucocutaneous irritations such as dry and slightly flaky skin. Body weight increases and mucocutaneous

**Fig. 2 Continued.**
symptomologies observed after 24 days with DDP + 9-cis RA treatment were comparable to those observed after treatment with 9-cis RA alone (Table 1). Thus, combination treatments did not exacerbate mild mucocutaneous toxicities observed in mice treated with 9-cis RA alone, demonstrating that dosing regimens of DDP + 9-cis RA were well tolerated.

**DISCUSSION**

The present study was conducted to determine: (a) whether 9-cis RA can enhance cytotoxic activities of DDP and (b) the degree of DDP and 9-cis RA-associated toxicities, as single agents and in combination, in an in vivo model of oral cancer.

This study demonstrated that DDP is active in a solid tumor model in which 9-cis RA inhibits growth by 25%. In comparison, DDP dose dependently inhibited tumor growth; complete inhibition was observed at doses approximating 3 mg/kg. 9-cis RA profoundly suppressed squamous cell differentiation, resulting in increased numbers of basal cell tumors. DPP targeted tumors differently than 9-cis RA, selectively decreasing the number of basal cell tumors in tumors (Fig. 2), consistent with inhibiting the growth of proliferating tumor cells (1–3). The paradigm enabled a good test of whether 9-cis RA would enhance cytotoxicity of DDP. Our conclusions are that 9-cis RA indeed enhanced sensitivity to DDP in this model, and the basis for this enhancement may have been alteration of tumor phenotype by 9-cis RA.

DDP is a cytotoxic agent commonly used to treat solid tumors, notably head and neck, ovarian, testicular, and lung cancers (1, 2). DDP exerts antitumor effects by killing dividing cancer cells (1, 2) but is not selective toward neoplastic cells and can produce significant neuro-, renal, oto-, and gastrointestinal toxicities (2). DDP therapy is limited by these toxicities and development of resistance during chemotherapy (2, 3). Circumvention of toxic side effects or development of resistance would be desirable to improve the utility of DDP as a chemotherapeutic agent.

Retinoids modulate cellular growth and differentiation of normal and neoplastic epithelial cells (11, 12) by presumably activating RARs and/or RXRs, members of the steroid/thyroid/vitamin D superfamily of intracellular receptors (19). ALR1057 (9-cis RA) is an endogenous pan agonist retinoid that activates both RARs and RXRs (19). Based on the fact that pan agonist retinoids have not been systematically studied in HNSCC models and that 9-cis RA suppresses growth and differentiation of 1483 tumors (18), 9-cis RA was selected as a physiologically relevant retinoid for the current study.

Our results implicate activation of RXRs and RARs in modulation of tumor growth and differentiation produced by 9-cis RA based on its high affinity for these receptors (19). Although studies with 9-cis RA implicate retinoid receptor activation in these processes, they do not discriminate between potential contributions made by either receptor class subtype. To address this issue, additional xenograft studies would have to be conducted with retinoids that selectively activate RARs or RXRs, and by extension to data reported herein, in combination modalities. Based on the results of preliminary studies, RXR-selective retinoids have not been observed to suppress squamous cell differentiation morphologies or inhibit the growth of 1483 tumors, suggesting that RXR:RXR homodimers do not mediate these effects. These data suggest that retinoids that selectively activate RARs or RXRs within the context of forming RAR:RXR heterodimers play the most prominent roles in modulating cellular differentiation and growth. By inference, therefore, RAR-selective and/or pan agonist retinoids may have the most utility for use in combination chemotherapy regimens based on a rationale of modulating tumor differentiation state.

Retinoid-induced suppression of squamous cell differentiation in HNSCC has been well-documented preclinically in established tumor cell lines in vitro and in vivo (12, 18, 25). An
Fig. 4  Morphologies of HNSCC 1483 xenografts after treatment with 30 mg/kg 9-cis RA (A) or 0.45 mg/kg DDP + 30 mg/kg 9-cis RA (B) as assessed by hematoxylin and eosin staining after 24 days of growth. Distinct basal and suprabasal cell regions and keratin pearls are not evident. Morphologies are representative of at least two separate experiments. ×100.
important question is whether retinoids will have similar effects in clinical squamous cell cancers. To address this question, primary head and neck tumors have been passaged into nude mice directly after surgical excision and treated with 9-cis RA. Prominent growth inhibition was observed in two of three primary tumors studied in this paradigm (26). Differentiation state was assessed, and 9-cis RA-induced suppression of squamous cell differentiation was observed in at least one of the three tumors, suggesting that the results reported herein may have clinical implications for treating squamous cancers. Ultimately, this question will need to be addressed in cancer patients.

Retinoids have shown promise for use in squamous cell carcinoma therapy as single agents and in combination with cytotoxic agents (4–9). This contention is supported by reports by Sacks et al. (13) and others (14, 15), in which ATRA synergistically inhibited growth of head and neck and ovarian cancer cell lines with DDP in vitro. Formelli and Cleris (17) have also reported that 4-hydroxyphenylretinamide enhanced antitumor activities of DDP against ovarian carcinoma xenografts. Other preclinical studies (reviewed in Ref. 13) demonstrate promise for retinoids/retinol in combination with cytotoxics, including 1,3-bis(2-chloroethyl)-1-nitrosourea, 5-fluorouracil, and etoposide. Promising effects of ATRA and 5-fluorouracil have been also been reported in radiotherapy of head and neck cancers (27).

Because epithelial cancers comprise a substantial portion of malignancies of the oral cavity, retinoids, primarily 13-cis RA, have been extensively studied clinically in patients with oral premalignancies such as oral leukoplakia. 13-cis RA exerts significant efficacy as a chemopreventive agent in these patients (4–8). However, vitamin A-associated toxicities such as headaches, dry skin, mucositis, and cheilitis limit therapy. In contrast to its effects on oral premalignancies, 13-cis RA has had only minor effects on improving chemotherapy of frank oral malignancies (4, 5, 9), indicating a need to develop more effective retinoids for oncology.

Morphological changes provided by 9-cis RA provided a rationale for combining DDP and 9-cis RA. It might reasonably be hypothesized that tumors consisting of greater numbers of basal cells would be more sensitive to DDP. It might also be expected that tumors with increased numbers of basal cells would grow more rapidly than untreated tumors, but this has not been the case. 9-cis RA and ATRA reproducibly decrease HNSCC 1483 tumor growth in this xenograft model (18). Possible explanations to help understand the ability of 9-cis RA to suppress cellular differentiation and tumor growth are: (a) induction of another mechanism(s) leading to overall tumor cell kill and (b) despite greater numbers of basal cells, cellular growth rates were actually decreased. There are data to support both possibilities. Supporting the former are in vitro data demonstrating that ATRA decreases growth of HNSCC spheroids (line MDA 886Ln; Ref. 28) and 1483 cells in culture without affecting the cell cycle (specifically S-phase) (13); induction of apoptosis as an alternate mechanism has been proposed (28). To address the latter, preliminary estimates of mitotic activity were obtained in 1483 tumors by assessing BrdU incorporation. In two experiments, 9-cis RA produced either small (27%) or negligible effects on BrdU incorporation into tumors. Small decreases in incorporation would be consistent with small growth inhibitory effects produced by 9-cis RA alone and could reflect decreases in cellular division rates. However, small decreases in BrdU incorporation were also observed in tumors that were markedly growth inhibited by higher doses of DDP alone or by combinations of DDP + 9-cis RA. This apparent inconsistency may be due to the possibility that BrdU incorporation observed in residual surviving cells did not reflect effects produced in cells that were presumably killed by treatments. If this were the case, BrdU incorporation would not reflect growth inhibitions. However, based on the fact that the S-phase of 1483 cells is not decreased by exposure to ATRA (13), a minor effect of 9-cis RA on BrdU incorporation would not be surprising. In this case, an alternative hypothesis would be that 9-cis RA did not prominently inhibit cellular division but produced its chemosensitizing effects with DDP by altering tumor morphologies. Thus, BrdU incorporation into DDP and DDP + 9-cis

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* M. M. Gottardis, manuscript in preparation.

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Fig. 5 Effects of DDP and 9-cis RA on tumor growth rates (A) and volumes (B). A, effects of single-agent DDP or 9-cis RA on tumor growth rates are compared with rates produced by combination treatment of DDP + 9-cis RA. B, dose–response curves for DDP and DDP + 9-cis RA (C). Effect of 30 mg/kg 9-cis RA alone (■), * P < 0.05, statistically different from control; **, P < 0.05, combination treatments were statistically different from single-agent treatments. Bars, SE.
RA-treated tumors did not correlate with growth suppression and did not clarify the potential association between increased chemosensitivity and 9-cis RA induction of basal cell morphology.

It is notable that health, mild mucocutaneous symptoms, and weight gain changes of animals treated with 9-cis RA or DDP + 9-cis RA were similar. Combination therapy with DDP + 9-cis RA enhanced efficacy without increasing the side effects of either agent, suggesting that the therapeutic index was increased by the combination. This finding, if translated clinically, would be significant because DDP has produced response rates of 30% at best as a single agent (1-3), and retinoids, primarily 13-cis RA, have generally not been efficacious against established neoplasms (4, 5, 7, 9). Furthermore, DDP and retinoid-associated toxicities limit doses that can be used clinically. A combination regimen that produced an additive or synergistic anticancer response would theoretically lead to improved efficacies and decreased toxicities.

This report demonstrated that 9-cis RA profoundly suppressed squamous cell differentiation, resulting in poorly differentiated tumor morphologies that predominated in combination with DDP. In combination, they produced a clear decrease in tumor growth that was highly statistically significant and greater than what would have been expected for either agent alone. The enhanced efficacy of DDP in combination with 9-cis RA may have been due to changes in the cell population reflected by altered tumormorphologies. Of importance, enhanced tumor growth inhibition occurred without increasing toxicities associated with either 9-cis RA or DDP. It therefore appears that the combination of DDP + 9-cis RA offers potential for improving anticancer therapy. Results of this study form a basis for recommending Phase I-II clinical testing of 9-cis RA in combination with DDP in head and neck cancers.

ACKNOWLEDGMENTS

We are grateful to Dr. Marcus Boehm for synthesis of 9-cis RA. Paula Sicurello for histological assistance, Kristin Spath and Lisa Sheeter for technical assistance, Dr. Marco Gottardis for helpful discussions, and Dr. Reuben Lotan for critical review of the manuscript and for supplying HNSCC 1483 cells.

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Enhanced antitumor efficacy of cisplatin in combination with ALRT1057 (9-cis retinoic acid) in human oral squamous carcinoma xenografts in nude mice.


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