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ORIGINAL ARTICLE

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Activity of fenretinide plus chemotherapeutic agents in small-cell lung cancer cell lines

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Abstract *Purpose*: Fenretinide [*N*-(4-hydroxyphenyl)retinamide, 4HPR], a synthetic retinoid, is a potent inducer of apoptosis in small-cell lung cancer (SCLC) cell lines that may act through the generation of reactive oxygen species, suggesting that it may enhance the activity of other cytotoxic agents. In light of 4HPR's clinical potential and potent activity against SCLC cells, we evaluated the in vitro activity of 4HPR in combination with cisplatin, etoposide or paclitaxel. *Methods*: The growth-inhibitory activities of single-agent 4HPR, cisplatin, etoposide or paclitaxel, and combinations of 4HPR and individual chemotherapeutic agents, were evaluated using an MTT assay in two SCLC cell lines. Each two-drug combination was studied over a range of concentrations at a fixed ratio corresponding to the ratio of the IC_{50} values of the individual agents. Data were analyzed by median-effect analysis as previously applied to drug combination studies. *Results*: All four agents inhibited growth in a dose-dependent manner in the NCI-H82 and NCI-H446 SCLC cell lines. At clinically reported drug concentrations that resulted in over 50% growth inhibition, the activities of the combinations 4HPR and cisplatin and 4HPR and etoposide were more than additive in both cell lines, and the activity of 4HPR plus paclitaxel was more than additive in NCI-H446 cells. *Conclusion*: 4HPR's potent single-agent activity, minimal toxicity, and potential synergy with standard cytotoxic drugs will allow for the development of promising investigational regimens for the treatment of patients with SCLC.

Key words Fenretinide · Small-cell lung cancer · Synergy · Chemotherapy

Introduction

Fenretinide [*N*-(4-hydroxyphenyl)retinamide, 4HPR] is a synthetic retinoid with cytotoxic activity against a variety of malignant cell types, including relatively treatment-resistant head and neck [21] and non-small-cell-lung cancer [30] cell lines. We have previously demonstrated that 4HPR is a potent inducer of apoptosis in small-cell lung cancer (SCLC) cell lines and that this activity is not associated with cellular differentiation or alteration of cell cycle distribution [16]. In contrast, all-*trans*-retinoic acid (ATRA), a natural retinoid, inhibits SCLC growth through cytostatic mechanisms associated with increased neuroendocrine differentiation [6, 15]. Recent reports have suggested that the mechanism of action of 4HPR involves the generation of reactive oxygen species with subsequent activation of apoptotic pathways [22]. If this is the case, then 4HPR may enhance the activity of other cytotoxic agents which act through the induction of apoptosis. In support of this hypothesis, 4HPR has been reported to potentiate the activity of cisplatin in a human ovarian cancer xenograft model [7] and to be synergistic with tamoxifen in the prevention of rat mammary cancer [23]. Thus far, combinations of 4HPR and cytotoxic agents have not been evaluated in other tumor types. Clinically, 4HPR has been found to have favorable pharmacokinetic and toxicity profiles during prolonged administration both as a single agent and in combination with tamoxifen [5, 8]. In light of 4HPR's clinical potential and potent activity against SCLC cells, we evaluated the in vitro activity of 4HPR in combination with cisplatin, etoposide

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or paclitaxel, agents that are commonly used in the treatment of SCLC.

Materials and methods

Cell culture

NCI-H82 and NCI-H446 SCLC cell lines were purchased from the American Type Culture Collection (Rockville, Md.) and were propagated in RPMI-1640 medium (Gibco/BRL, Gaithersburg, Md.) supplemented with 2% fetal calf serum (Sigma Chemical Co., St. Louis, Mo.), 100 U/ml penicillin, 100 μg/ml streptomycin, and HITES (10 n*M* hydrocortisone, 15 μg/ml insulin, 100 μg/ml transferrin, 10 n*M* 17β-estradiol, 100 μg/ml selenium) in a humidified atmosphere containing 5% CO_2 at 37 °C. 4HPR was provided by the R.W. Johnson Pharmaceutical Research Institute, Raritan, N.J. A 5 m*M* solution of 4HPR in 100% ethanol was stored in the dark at −20 °C and diluted as needed. Cisplatin, etoposide and paclitaxel were obtained from Sigma.

Cell proliferation assay

A standardized MTT assay was utilized to determine growth inhibition. Briefly, cells were plated in 96-well plates at a density of 1–2×10^4 cells/ml with 200 μl/well and five wells per experimental condition. Cells were exposed 24 h after plating to a range of concentrations of 4HPR and/or chemotherapeutic agents. Control cells were treated with 0.1% ethanol and blank wells were loaded with medium only. After 5 days, 50 μl of 1.0 mg/ml MTT (Sigma) was added to each well and plates were incubated for 4 h at 37 °C prior to centrifugation, removal of supernatant, and dissolution of formazan crystals in 200 μl DMSO for 1 h at room temperature with agitation. Absorbance at 570 nm (A_{570}) was determined for each well, and the fractional survival for each study condition was calculated from the ratio: (mean A_{570} treated cells − mean A_{570} blank wells)/(mean A_{570} control cells − mean A_{570} blank wells). The 50% inhibitory concentration (IC_{50}) for each single agent was determined from at least three experiments with CalcuSyn software (Biosoft, Cambridge, UK).

Analysis of combined drug effects

All combination studies were performed with a range of concentrations of each individual agent and both agents together at a fixed ratio corresponding to the ratio of the IC_{50} values of each agent alone as identified in preliminary experiments. The fractional survival (f) after 5 days of treatment was calculated for each experimental condition as noted above. CalcuSyn software, based on the median-effect analysis method of Chou and Talalay [3], was then used to determine the effects of combination therapy. For each level of fractional survival (f = 0.80, 0.75, 0.70,. . .,0.20), a combination index (CI) was calculated according to the equation: $CI = (D)_1/(D_f)_1 + (D)_2/(D_f)_2 + \alpha[(D)_1(D_f)_2/(D_f)_1(D_f)_2]$, where $(D)_1$ and $(D)_2$ are the concentrations of the combination required to produce fractional survival f, $(D_f)_1$ and $(D_f)_2$ are the concentrations of the individual drugs required to produce f, and $\alpha = 0$ or 1 if the drugs' mechanisms of action are mutually exclusive or mutually nonexclusive, respectively. CI < 1.0 indicates synergy, CI = 1.0 indicates additivity, and CI > 1.0 indicates antagonism [3].

Results

Effects of individual agents on SCLC cells

Initially, the in vitro, single-agent growth-inhibitory effects of 4HPR, cisplatin, etoposide and paclitaxel were determined for the NCI-H82 and NCI-H446 SCLC cell lines. Cellular proliferation was assessed using a standardized MTT assay after 5 days of exposure to a broad range of concentrations of each agent and each experiment was repeated at least three times. All four agents exhibited significant cytotoxic activity with dose-response relationships up to 95% growth inhibition (IC_{95}) in preliminary experiments (data not shown) and the IC_{95} values for all agents were consistent with concentrations reported in clinical pharmacokinetic studies [1, 8, 9, 24, 28]. As previously reported, 4HPR inhibited growth through the induction of apoptosis at submicromolar concentrations in NCI-H82 and NCI-H446 cells [16].

Interaction of 4HPR and cisplatin in SCLC cells

In order to study the effects of combinations of 4HPR and other cytotoxic agents, NCI-H82 and NCI-H446 SCLC cells were exposed to 4HPR plus cisplatin, etoposide, or paclitaxel over a range of doses at a fixed ratio for 5 days prior to the determination of cell viability by the MTT assay. Three separate experiments were performed for each drug combination in each cell line and data from these experiments were pooled for further analysis. The effects of 4HPR, cisplatin and the combination of both agents on the survival of NCI-H82 and NCI-H446 cells are presented in Fig. 1A,D. While both 4HPR and cisplatin inhibited growth in a dose-dependent manner, the degree of inhibition achieved with the combination was greater than that of either agent alone. Median-effect analysis was utilized in order to fully appreciate the magnitude of this combination effect [3]. Initially, each dose-response curve was transformed into a linear median-effect plot in which the x-intercept represents the $\log(IC_{50})$ for the specified treatment condition (Fig. 1B,E). The nonparallel nature of the combination curves suggests that the agents interacted in a mutually nonexclusive manner. However, since the actual mechanism of 4HPR's activity is not known, further calculations were performed using both mutually exclusive and mutually nonexclusive assumptions.

The effect of combining 4HPR with cisplatin was then determined in NCI-H82 and NCI-H446 cells by calculating the CI over a range of fractional inhibitory values (Fig. 1C,F) using parameters derived from the median-effect plots. By definition, CI = 1 indicates additivity, CI < 1 indicates synergy, and CI > 1 indicates antagonism. For both cell lines, the CI values for the combination of 4HPR and cisplatin were less than 1.0 under both exclusive and nonexclusive assumptions at higher fractional inhibitory values, suggesting a synergistic effect (Fig. 1C,F; Table 1). The concentrations of both agents required to obtain fractional inhibitory values up to the IC_{95} have been reported in clinical pharmacokinetic studies [1, 8, 9].

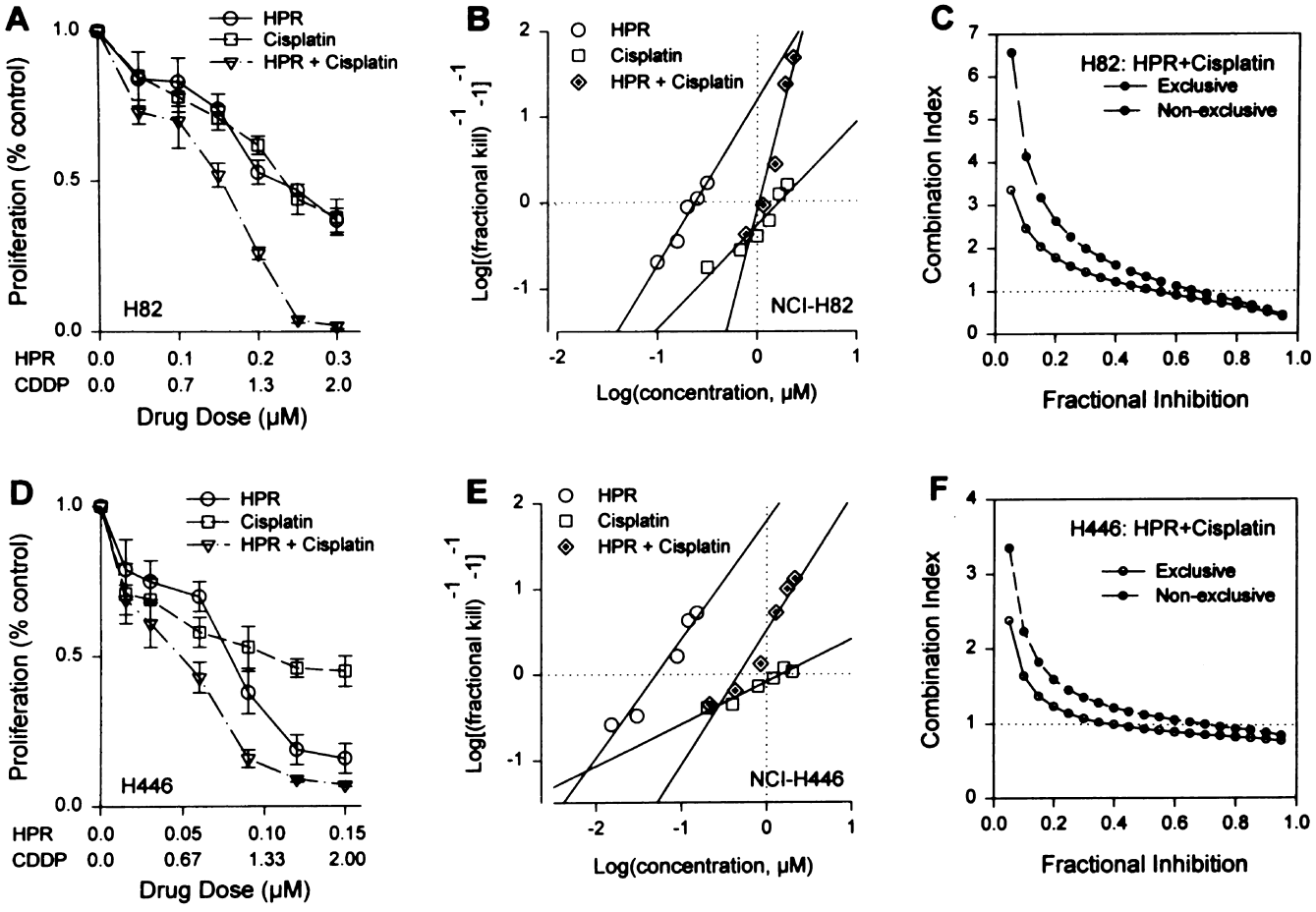

Fig. 1A–F Interaction of fenretinide (HPR) and cisplatin in NCI-H82 (**A–C**) and NCI-H446 (**D–F**) SCLC cell lines. **A, D** Inhibition of SCLC growth by HPR alone, cisplatin alone, or a combination of HPR and cisplatin at a fixed ratio (H82, 1:6.6; H446, 1:13.3). **B, E** Median-effect plots with regression lines based on the data in **A** and **D**, respectively. **C, F** Combination index plots for the combination of HPR and cisplatin calculated from the data in **B** and **E**, respectively, under assumptions that the agents are mutually exclusive (*open symbols*) or mutually nonexclusive (*closed symbols*) in their mechanisms of action

Interaction of 4HPR and etoposide in SCLC cells

The results of experiments with the combination of 4HPR and etoposide in both NCI-H82 and NCI-H446 cells are presented in Fig. 2. In both cell lines the combination resulted in a greater degree of growth inhibition than either drug alone, especially at higher fractional inhibitory values (Fig. 2A,D). The median-effect plots for 4HPR plus etoposide in both cell lines again suggests mutually nonexclusive mechanisms of action (Fig. 2B, E). CI values demonstrated that 4HPR and etoposide acted synergistically in both cell lines at levels of fractional inhibition that are achievable with clinically reported drug concentrations (Fig. 2C,F; Table 1) [8, 28].

Interaction of 4HPR and paclitaxel in SCLC cells

As a single agent, paclitaxel exhibited substantial activity against both cell lines with greater activity against NCI-H82 than NCI-H446 (Fig. 3A,D). Using mutually nonexclusive assumptions, the CI values of the

Table 1 Combination index (CI) values for 4HPR plus cisplatin, etoposide or paclitaxel in SCLC cell lines. Each value is the mean ± SEM of three experiments

Combination	CI values[a] for NCI-H82				CI values[a] for NCI-H446			
	Ratio	IC_{50}	IC_{75}	IC_{95}	Ratio	IC_{50}	IC_{75}	IC_{95}
4HPR + cisplatin	1:6.6	1.34 ± 0.28	0.85 ± 0.02	0.44 ± 0.11	1:13.3	1.05 ± 0.17	0.77 ± 0.24	0.59 ± 0.29
4HPR + etoposide	1:1.7	1.14 ± 0.01	0.91 ± 0.11	0.66 ± 0.21	1:5.7	1.17 ± 0.17	0.75 ± 0.09	0.63 ± 0.11
4HPR + paclitaxel	1:0.007	1.41 ± 0.05	1.26 ± 0.05	1.13 ± 0.19	1:0.05	1.33 ± 0.17	0.92 ± 0.11	0.58 ± 0.01

[a] CI < 1.0 indicates synergy, CI = 1.0, additivity, and CI > 1, antagonism, assuming mutually nonexclusive drug interactions

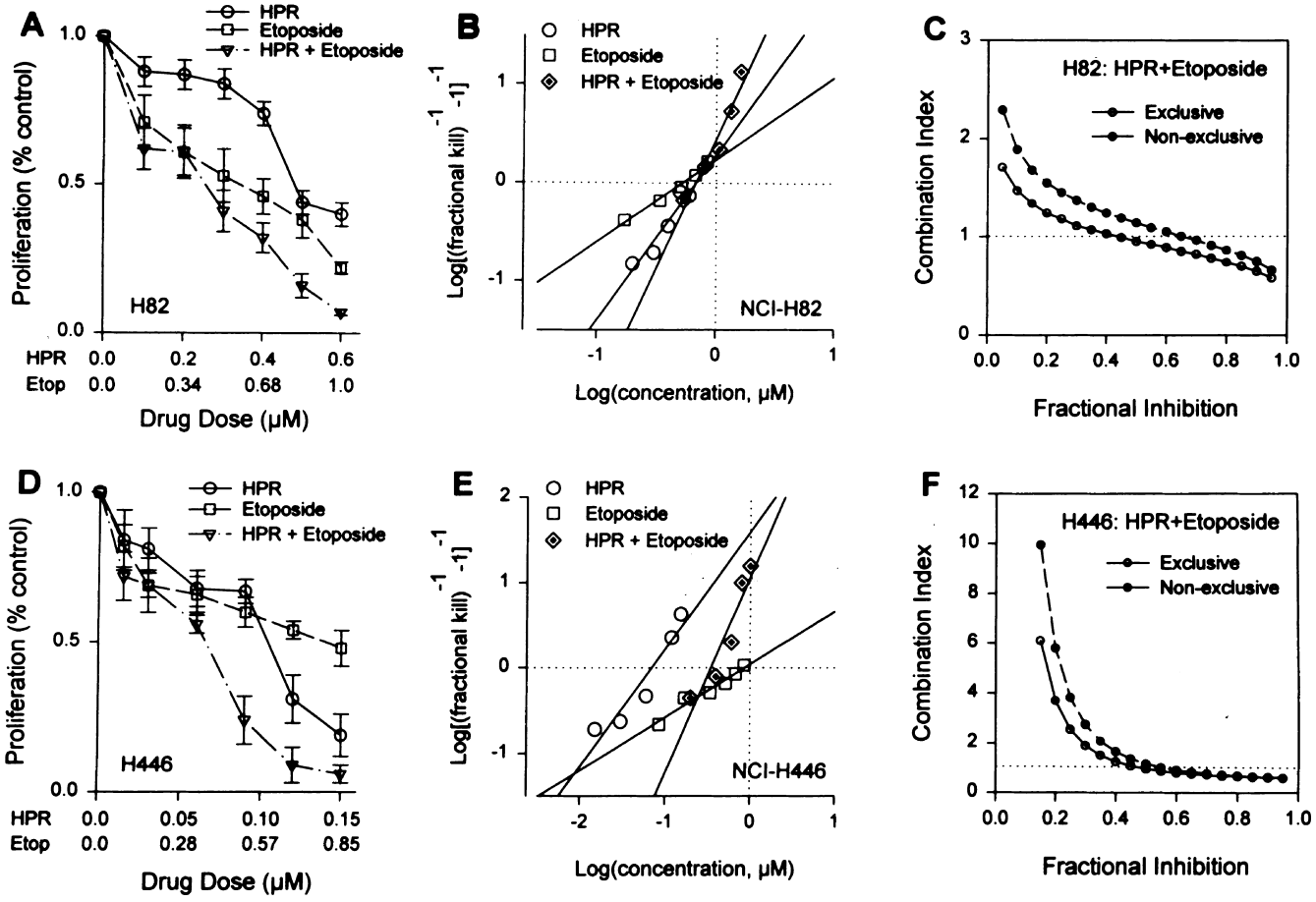

Fig. 2A–F Interaction of fenretinide (HPR) and etoposide in NCI-H82 (**A–C**) and NCI-H446 (**D–F**) SCLC cell lines. **A, D** Inhibition of SCLC growth by HPR alone, etoposide alone, or a combination of HPR and etoposide at a fixed ratio (H82, 1:1.7; H446, 1:5.7). **B, E** Median-effect plots with regression lines based on the data in **A** and **D**, respectively. **C, F** Combination index plots for the combination of HPR and etoposide calculated from the data in **B** and **E**, respectively, under assumptions that the agents are mutually exclusive (*open symbols*) or mutually nonexclusive (*closed symbols*) in their mechanisms of action

combination of 4HPR and paclitaxel remained greater than 1.0 at all concentrations in NCI-H82 cells, suggesting a less than additive interaction (Fig. 3C; Table 1). In contrast, the CI values were less than 1.0 in NCI-H446 cells at higher concentrations using either mutually exclusive or nonexclusive assumptions, suggesting a synergistic interaction (Fig. 3F; Table 1).

Discussion

The results of this study indicate that combinations of 4HPR and the chemotherapeutic agents most commonly used to treat SCLC can induce synergistic cytotoxicity in SCLC cells in vitro. Although numerous methods have been devised to analyze cytotoxic drug interactions, few have stood up to rigorous critical review [10, 20]. We chose to use the median-effect analysis, as applied to drug combinations [3], because of its extensive use in the published literature and its relative reproducibility [2, 4, 13, 17, 19]. Table 1 summarizes the CI values obtained over a range of inhibitory concentrations utilizing the more conservative mutually nonexclusive assumptions. Since the exact mechanism of action of 4HPR has not been defined, it is not possible to clearly state whether the experimental agents interacted in a mutually exclusive or mutually nonexclusive manner. However, since the cytotoxic effects of the four agents used in this study have been reported to involve the activation of apoptotic pathways, it is possible that some overlap in their mechanisms of action does exist. If this is the case, then the use of mutually exclusive assumptions would be appropriate. One clear limitation of the present study is that the results reported with the fixed-ratio concentrations used in our experiments would not necessarily be observed under other experimental conditions [29].

All of the combinations tested, except 4HPR plus paclitaxel in NCI-H82 cells, revealed a more than additive effect at higher growth-inhibitory concentrations. Although the mechanisms involved in this effect have not been determined, it is interesting to note that Oridate et al. demonstrated that 4HPR induces reactive oxygen species in cervical carcinoma cells in a dose-dependent manner with a half-maximal effect at 0.6 μM [22], a value that corresponds with the highest concentration of 4HPR used in the present study. The concentrations of

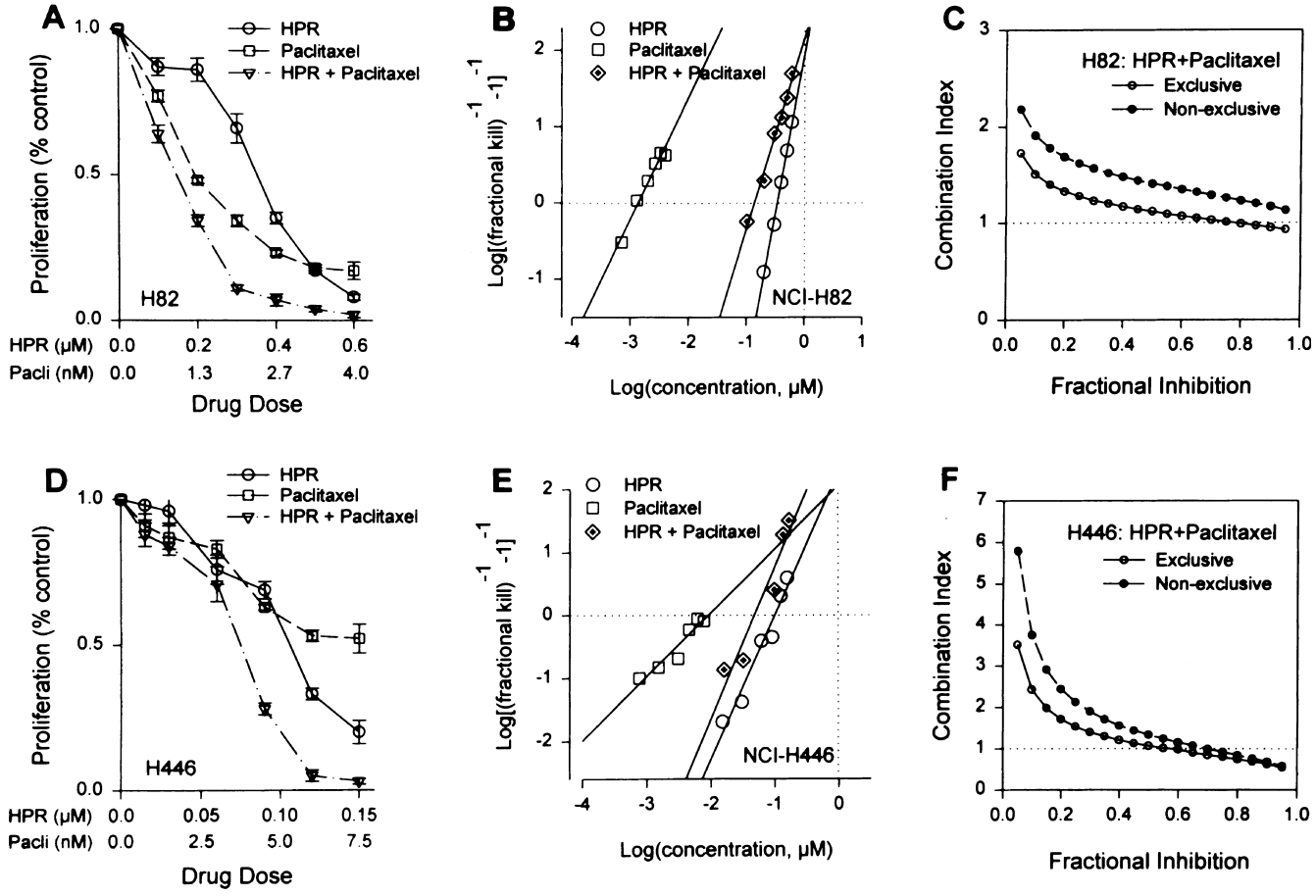

Fig. 3A–F Interaction of fenretinide (HPR) and paclitaxel in NCI-H82 (**A–C**) and NCI-H446 (**D–F**) SCLC cell lines. **A, D** Inhibition of SCLC growth by HPR alone, paclitaxel alone, or a combination of HPR and paclitaxel at a fixed ratio (H82, 1:0.007; H446, 1:0.05). **B, E** Median-effect plots with regression lines based on the data in **A** and **D**, respectively. **C, F** Combination index plots for the combination of HPR and paclitaxel calculated from the data in **B** and **E**, respectively, under assumptions that the agents are mutually exclusive (*open symbols*) or mutually nonexclusive (*closed symbols*) in their mechanisms of action

component agents required in each combination to achieve higher levels of fractional inhibition were all within the clinically reported range of serum concentrations for each agent under standard clinical conditions. However, the prolonged exposures used in this study may be applicable only to clinical situations in which prolonged infusional or oral dosing schemes are utilized. For cisplatin and paclitaxel, peak serum concentrations of 2.0–13.0 μ*M* [1, 9] and 2.0–8.1 μ*M* [24], respectively, have been reported after 6-h intravenous infusions, while for etoposide, serum levels of 5.1–32.6 μ*M* have been noted after either oral or intravenous dosing [28]. Likewise, steady-state serum concentrations of up to 2.0 μM have been observed with chronic oral administration of 4HPR [8].

The importance of the retinoids as regulators of cellular proliferation, differentiation, and death in many types of normal and malignant cells has led to renewed interest in their use as preventive and therapeutic anticancer agents [27]. Natural retinoids, such as ATRA, exhibit primarily cytostatic activity, with cell death occurring only as the result of differentiation in a limited number of cell types. In contrast, several synthetic retinoids, including 4HPR, are potent inducers of apoptosis in a variety of malignant cell types [14]. Although the pathways involved in retinoid-mediated cell death have not been fully defined, recent studies have shown that 4HPR directly induces oxidative stress with subsequent activation of apoptotic pathways, suggesting that 4HPR may enhance the activity of other cytotoxic agents that act through these pathways. Thus far, combinations of retinoids and standard chemotherapeutic agents have been evaluated in only a few tumor types. ATRA enhances the activity of both cisplatin and etoposide in teratocarcinoma cells [11], and ATRA and 9-*cis*-retinoic acid potentiates the activity of cisplatin in squamous cell head and neck cancer cells [25] and xenografts [26], respectively. In contrast, ATRA abrogates the activity of etoposide in head and neck cancer cells [18]. In the only study to evaluate 4HPR in combination with a chemotherapeutic agent, Formelli and Cleris found that 4HPR potentiates the activity of cisplatin in a xenograft model of human ovarian carcinoma [7].

SCLC is characterized by initial responsiveness to chemotherapy followed by relapse with treatment-resistant disease in the great majority of patients. The

combination of cisplatin and etoposide is the most commonly used regimen in SCLC, yielding response rates of well over 50% [12]. However, the overall 5-year survival rate for patients with SCLC remains at 5–10%. Efforts to improve survival with dose-intensive regimens or the addition of newer agents have been severely hampered by excessive toxicity. In contrast to most retinoids and chemotherapeutic agents, 4HPR has exhibited favorable pharmacokinetic and toxicity profiles in clinical trials [5, 8], suggesting that it could be added to chemotherapeutic regimens without excessive toxicity or the need to reduce the dose of other active agents. The identification of 4HPR as an agent with cytotoxic activity and potential synergy with standard cytotoxic drugs will allow the development of promising investigational regimens for the treatment of SCLC.

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