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All-trans retinoic acid in combination with cisplatin and paclitaxel enhances apoptosis and up-regulates retinoic acid receptors in lung adenocarcinoma cells

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ABSTRACT

Purpose: Loss of expression of retinoic acid receptors (RAR) results in lack of response to treatment and cancer progression. All-trans retinoic acid (ATRA) is a potential chemotherapeutic and chemopreventive agent with differentiation-inducing, anti-proliferative and pro-apoptotic effects that up-regulates retinoic acid receptors. The aim of this study was to assess a possible synergistic combination of ATRA with chemotherapy in terms of pro-apoptotic and anti-proliferative effects as well as on RAR expression in non-small cell lung cancer (NSCLC). Methods: The human lung cancer cell line H1666, was treated with ATRA, cisplatin (CIS) and paclitaxel (PACL) at different combinations. We analyzed cell viability by the MTT assay while cell cycle and apoptosis were determined by flow cytometry. RAR and RXR expression was determined by RT-qPCR. Results: ATRA combined with CIS or PACLI inhibited cell growth showing a synergistic effect at 24 hours. All Paclitaxel combined treatments arrested cells in the G2-M phase whereas ATRA-CIS increased the sub G0 population. Furthermore, ATRA alone enhanced apoptosis, and this effect was greater when combining ATRA-CISPACL. Additionally, ATRA in all combinations promoted up-regulation of RARα, β and γ. Conclusion: The addition of ATRA to CIS or PACLI enhanced their antitumor effect in lung cancer cells. This effect was more evident with the ATRA-PACLI combination.

Keywords: Non-small cell lung cancer, All-trans Retinoic Acid, Cisplatin, Paclitaxel, Retinoic Acid Receptors, Apoptosis, Cell Cycle.
INTRODUCTION

ABBREVIATIONS


Lung cancer is the leading cause of cancer deaths worldwide. The non-small-cell lung cancer subtype (NSCLC) accounts for more than 80% of the cases (Jemal et al., 2010). In Mexico, NSCLC is the first cause of death from cancer, with over 6,697 deaths per year (Arrieta et al., 2013), and a 5-year survival rate of only about 16% (Groome et al., 2007). Chemotherapy (CT) for advanced NSCLC has limited efficacy, with 20%-35% of global response and 35% of one-year survival rate (Scaglotti et al., 2002). A study comparing third-generation CT regimes, showed no differences in survival rate at one and two years, thus, cisplatin and paclitaxel remain the standard treatment for NSCLC (Schiller et al., 2002).

Carcinogenesis of the respiratory epithelium is a multifactorial process including inherited and acquired genetic changes, chromosomal rearrangements and epigenetic factors (Karamouzis and Papavassiliou, 2005). Retinoids are required for normal epithelial cell growth and regulation; however, their deficiency contributes to signaling dysfunction and has been associated with bronchial metaplasia and increased risk for lung cancer development (Bogos et al., 2008). Alteration or loss of expression of retinoic acid receptors (RAR), either RARα, β or γ, are found in premalignant and malignant tissues, a feature that is associated with malignant transformation of human cells (Xu et al., 1994; Xu et al., 1997).

All-trans retinoic acid (ATRA) is an active metabolite that regulates the expression of target genes through binding and activation of RAR receptors, inhibition of proliferation, and differentiation of cell cultures (Liu et al., 1996). ATRA induces RARβ expression in peripheral nerves, inducing regeneration (Zhelyaznik and Mey, 2006), and in breast cancer cells ATRA also up-regulates this receptor causing growth inhibition (Liu et al., 1996). Retinoids have been used as potential chemotherapeutic adjuvants since they promote differentiation and because of their anti-proliferative, proapoptotic, and anti-oxidant effects (Altucci et al., 2001). In premalignant lesions, retinoids inhibit the development of second primary cancers in patients with upper aerodigestive tract and lung cancers (Hong et al., 1990; Hansen et al., 2000). However, large randomized trials using retinoids for prevention of tobacco-related cancer, showed a higher incidence of lung cancer among smoking participants who received retinoids (Pastorino, 1997; Khuri and Lotan, 2004). We demonstrated previously that adding ATRA to chemotherapy increases the response rate (RR) and progression free survival (PFS) in patients with advanced NSCLC (Arrieta et al., 2011). Additionally, preclinical data and phase II clinical trials have suggested that platinum-based chemotherapy plus bexarotene improves overall survival (OS), but a subsequent phase III clinical trial showed benefits only for OS in patients with increased triglycerides (Tyagi, 2005; Edelman et al., 2005). Based on these facts, the use of retinoids in clinical trials remains controversial.

This study aimed at describing the effects of ATRA in combination with Paclitaxel (PACLI) and Cisplatin (CIS), on proliferation, apoptosis, and the expression of retinoic acid receptors in lung cancer cells.

MATERIAL AND METHODS

Cell culture

The NSCLC cell line H1666 (Adenocarcinoma line), was obtained from the ATCC (American Tissue Culture Collection, Rockville, MD, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, GibCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO BRL), 4 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were grown and maintained under sterile conditions at 37°C in a humid environment with 5% CO2. The assays were carried out in triplicate.

Cellular growth rates were determined using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Cells were grown in 96-well plates in 10% FBS-containing DMEM medium at a density of 1×104 cells/well. After 24 hr, cells were treated with 1 μM retinoic acid (ATRA) (Sigma Aldrich, Mexico), 35 μM Cisplatin (CIS) (PISA Pharmaceuticals), 0.008 μM Paclitaxel (PACL) (Dabur Pharmaceuticals Ltd) or combinations thereof at the same concentrations. After 24, 48h or 72h, 10 μl of the MTT labeling reagent (Roche applied, Mexico) was added and plates were incubated overnight. Cell viability was measured at 570 nm using a microplate reader (BioRad, Hercules, CA, U.S.A.).

Combination index (CI)-isobol method

Chou and Talalay introduced a scientific term “combination index” (CI) which is used to determined synergistic or antagonistic effects (Chou and Talalay, 1984; Chou, 2010). The combination index (CI)-isobol method provides a quantitative assessment of synergism between drugs. A CI is estimated from dose-effect data of
single and combined drug treatments. A value of CI less than 1 indicates synergism; CI = 1 indicates additive effect; and CI > 1 indicates antagonism. Drug interaction (synergism or antagonism) is more pronounced the farther a CI value is from 1. According to the CI range, the synergism could be defined as slight (CI: 0.85-0.90), moderate (0.7-0.85), synergism (CI: 0.3-0.7), strong (CI: 0.1-0.3), and very strong (CI<0.1) (Chou, 2010; Chou, 2006).

Formally, the combination index (CI) of a combined drug treatment is defined as CI = D1/ Dx1 + D2/ Dx2

Here D1 and D2 are the doses of drug 1 and drug 2, respectively, in the combination; Dx1 and Dx2 each is the dose of a treatment with only drug 1 and drug 2 that would give the same effect as that of the combination, respectively. The doses Dx1 and Dx2 need to be estimated from the dose-effect data of single drug treatments. In our study, the dose-effect relationship of individual and combined treatments was measured as the percentage cell growth inhibition to obtain the combination index (CI) for the multiple drugs tested i.e. ATRA, CIS and PACLI.

In vitro assays

To determine the cytotoxic effects of ATRA combined with CT (CIS and/or PACLI), cells were grown in 24-well plates in 10% FBS-containing DMEM medium at a density of 3×10^5 cells/well. After 24 hours, cells were treated with ATRA, CIS and PACLI alone or in different combinations for 24, 48 and 72 hours. The combinations used were Control (no treatment), ATRA, CIS, PACLI, ATRA-CIS, ATRA-PACLI, CIS-PACLI and ATRA-CIS-PACLI. After treatment, cells were incubated with trypsin and collected to determine RAR expression, cell cycle stage and induction of apoptosis.

Cell cycle analysis

The distribution of the cell cycle stage in cells was determined by flow cytometry using propidium iodide (PI) staining. After experimental treatment, 1×10^6 cells were rinsed with PBS, collected and fixed in cold 70% ethanol. The samples were stored at -20°C until analyzed. Then, cells were washed with PBS, centrifuged, resuspended in permeabilization buffer (0.2 M Na_2HPO_4, 0.1 M acetic acid) and incubated for 30 min at 37°C. Afterwards, cells were centrifuged and resuspended in RNase (100 µg/ml) and PI (25 µg/ml). Cells were incubated in the dark for 30 min at room temperature. Data were collected in the FACSCalibur Flow cytometer (Becton Dickinson, Carlsbad, CA, USA) and 10,000 events were evaluated. The cell cycle was determined on an FL-4 region. Measurements were obtained at 488 nm gating out, doublets and clumps for each sample were discarded. Cell QuestPro and Flow Jo ver. 7.6.1 software were used for data analysis.

Detection of apoptosis by annexin V-7AAD

The Annexin V assay is based on the observation that phosphatidylserine (PS), a phospholipid normally confined in the inner plasma membrane, translocates to the cell surface during apoptosis. After pharmacological treatments, cells were rinsed with PBS, resuspended in 1ml filtered PBS, and adjusted to a concentration of 1×10^6 cells/ml. Cell suspensions were incubated with 200 µl of annexin V-binding buffer (0.1 HEPEs (pH 7.4), 1.4 M NaCl and 25 mM CaCl solution) containing Annexin VFITC (1 µg/ml) (Annexin V Apoptosis Detection Kit I, BD Pharmingen) and 7 AAD. After gently vortexing, cells were incubated for 15 min at room temperature in the dark. Data were collected for 10,000 events by flow cytometry (FACSCalibur instrument, BD Biosciences) and analyzed using Cell QuestPro and Flow Jo ver. 7.6.1 software.

Gene expression analysis by real-time PCR

RNA extraction was performed using TRIzol® RNA Isolation Reagent (Gibco, Life Technologies Corporation), according to manufacturer’s instructions. Briefly, 500 µl of TRIzol® reagent were added to cells and the mix was kept at -70°C until used. Cells were then thawed at room temperature and 200 µl of chloroform were added, samples were kept on ice for 3 min on a plate shaker. At the end of the incubation period the tubes were centrifuged at 14,000 rpm for 5 minutes, the aqueous phase was then removed and RNA was precipitated with an equal volume (v/v) of isopropyl-alcohol (Merck Darnstadt Germany), tubes were centrifuged, the supernatant was discarded, and the pellet was resuspended in 50 µl DEPC water.

Reverse transcriptase quantitative PCR (RT-qPCR) was performed using master mix reagents (Applied Biosystems® Mexico), following manufacturer’s instructions. TaqMan® Probes (Applied Biosystems, Carlsbad, CA, USA), were coupled with Standard Dye Sets, VIC (Green) or FAM (Blue), to detect the relative expression of RAR α (Hs00940446_m1), RAR β (Hs00977140_m1), RAR γ (Hs01559231_gH), RXR α (Hs01067640_m1), RXR β (Hs00232774_m1) and RXR γ (Hs02519543_s1). Mean threshold values for target genes were normalized for the endogenous control

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MTT Viability Assay

The effect of ATRA, PACLI and CIS on H1666 cancer cell viability was determined using the MTT assay. Values are shown as percentage of viability relative to untreated cells. Data represent the means of three independent experiments. *p=0.005 comparing PACLI vs. Control, **p=0.001 comparing ATRA, CIS, ATRA-CIS, ATRA-PACLI, CIS-PACLI and ATRA-CIS-PACLI vs. control at 72 hours.

Table 1. Combination index of treatments in the H1666 cell line. The CI values are shown as means ± standard deviation (SD) for each combination: ATRA-CIS (A+C), ATRA-PACLI (A+P), CIS-PACLI (C+P), ATRA-CIS-PACLI (A+C+P).

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Statistical analysis

For descriptive purposes, continuous variables were summarized as arithmetic means, medians, and standard deviations (SD). According to data distribution (normal and non-normal) determined control RNAse P. The analysis was performed PRISM 7500.
by the Kolmogorov-Smirnov test, inferential comparisons among groups were conducted with One-way analysis of variance (ANOVA according to manufacturer’s instructions using ABI) and post-hoc (Tukey) test or with Kruskal-Wallis test. Statistical significance was determined with $p \leq 0.05$ in a two-sided test. SPSS software package (IBM SPSS, Inc., Chicago, IL, USA version 20) was used for data analysis.

**RESULTS**

**In Vitro cytotoxicity**

Cell viability was evaluated by the MTT assay in H1666 cells treated with ATRA, CIS, PACLI and their combinations. Cell growth was evaluated at 24, 48 and 72 hours after exposure to each drug. ATRA alone reduced cell viability up to 42% and 58% at 48 hours and 72 hours ($p=0.005$ and $p=0.001$, respectively). ATRA-CIS diminished cell viability at 24, 48 and 72 h (17%, 38%, 63%; $p=0.05$, $p=0.001$, $p=0.001$, respectively), better than CIS alone, showing a synergistic effect. ATRA-PACLI also showed a progressive decrease in cell viability at 24, 48 and 72 h (34%, 58%, 64%; $p=0.001$, respectively) better than PACLI alone. No statistically significant differences were found between ATRA-PACLI vs ATRA-CIS-PACLI ($p=0.834$) (Figure 1).

**Combination index**

CIS-PACLI displayed a strong synergism at 24 hours measured by the Combination Index (CI) ($0.23 \pm 0.01$). We also found synergistic effects at 24h in ATRA-CIS ($0.61 \pm 0.06$), ATRA-PACLI ($0.40 \pm 0.02$); and ATRA-CIS-PACLI ($0.56 \pm 0.10$). Conversely, at 48 and 72 hours we found antagonistic effects in all combinations (Table 1).

**Cell cycle**

As shown in Figure 2 (A, B and C) each of the three drugs used produced different effects on the cell cycle. ATRA increased the sub-G0 population at 48 and 72 hours. ATRA-CIS behaved like ATRA at all times. ATRA-PACLI arrested cells in S-phase, with increased G2/M arrest at 24 hours. At 48 hours all cells were arrested in S-phase, while at 72 hours sub-G0 cell population increased. ATRA-CIS-PACLI arrested cells in S-phase at 24 and 72 hours, in contrast, at 48 and 72 hours increased the sub-G0 population.

**Apoptosis**

After 24 hours of treatment, we did not find differences in apoptosis between CONTROL, ATRA, CIS and PACLI treatments. However, at this time ATRA-CIS, ATRA-PACLI and CIS-PACLI showed almost a two-fold increase in apoptosis ($p=0.033$; $p=0.015$, $p=0.023$). At 48 hours there a two to four-fold increase in apoptosis in almost all groups ($p<0.05$). However, the PACLI treatment showed an increase in both apoptosis and cell necrosis. At 72 hours, the death process by apoptosis continued, however, we also found an increase in necrosis in all groups as compared with controls ($p<0.05$) (Figure 3A, B and C).

**RAR and RXR expression after Chemotherapy plus ATRA treatment**

RAR and RXR expression were measured by RT-qPCR in NSCLC cells under treatment with CT-ATRA (Figure 4 A, B and C). RARα expression increased around 2-fold by ATRA alone after 48 and 72 hours while the combinations up-regulated RARα since 24 hours. RARβ increased expression after 24 hours only in the ATRA-CIS group and after 48 and 72 hours in ATRA, CIS-PACLI and ATRA-CIS-PACLI. Overall, treatments containing ATRA up-regulated RARβ except for ATRA-PACLI that had no effect on expression. RARγ levels increased at 24 hours for ATRA-CIS and ATRA-CIS-PACLI. After 48 hours, all treatments induced a 2-fold increase in RARγ levels, and this effect was maintained until the end of treatment. Consistently, the ATRA-CIS combination upregulated all three RAR receptors at all times, showing a better induction of these receptors over other treatments. No significant modifications were observed in RXRα, β and γ mRNA levels.

**DISCUSSION**

Retinoids and their natural or synthetic analogues are closely related to vitamin A, and play major roles in cell growth regulation. They are promising anti-neoplastic agents with therapeutic and chemopreventive effects as they regulate cell growth, differentiation and apoptosis. Retinoids suppress carcinogenesis in a variety of cell types (Clarke et al., 2004; Kim and Kim, 2011), also showing chemopreventive effects in clinical trials of breast cancer, renal cell carcinoma, oral premalignant lesions, bronchial epithelium of chronic smokers, skin premalignant lesions, and cervical cancer (Hansen et al., 2000; Fields et al., 2007).

In this study, we found that combining ATRA with
Figure 2. A, B and C Flow cytometry analysis of cell cycle distribution in H1666 cells. Cells were treated with either ATRA, CIS, PACLI or their combinations for 24, 48 and 72 h. At the specified time, they were harvested and stained with PI and analyzed by flow cytometry. Percentages of cells in the G1, S and G2/M phases of cell cycle are indicated.
Figure 3. A, B and C Induction of Apoptosis. Apoptosis analysis using Annexin-V-PE/7-AAD double staining. Bar graphs show the percentage of dead, living, and apoptotic cells according to treatment. *p=0.033; p=0.015 and p=0.023 comparing ATRA-CIS, ATRA-PACLI and CIS-PACLI vs. control.
Figure 4. A, B and C Expression of RARα, β and γ by Real Time PCR. Quantification of RAR-α, β and γ in the H1666 cell line as expressed in logarithm base 10 compared to the expression of RARs in controls. The expression of the receptors was significantly increased in all ATRA-treated groups.
PACLI and CIS inhibited cell growth in the H1666 cell and enhanced antitumor activity when compared to single drug therapy. Also, the combinations ATRA-CIS, ATRAPACLI, CIS-PACLI and ATRA-CIS-PACLI exhibited synergistic effects at 24 hours. Multiple studies have shown synergistic, antiproliferative effects of the ATRAPACLI combination in glioma, lung cancer, breast and colon carcinoma cells, as well as in glioma xenografts in mice (Karmakar et al., 2007; Karmakar et al., 2008; Hong et al., 2011). Besides, ATRA-CIS exerted additive effects in many experimental settings, including ovarian, head and neck, squamous cell carcinoma, cervical and human skin squamous cell carcinoma (Aebi et al., 1997; Masuda et al., 2002).

These observations may be derived from the mechanism of action of each compound. ATRA binds to RARs and modulate transcription of target genes through retinoic acid response elements (RAREs). CIS induces apoptosis by downregulation of Bcl-2 and upregulation of BAX. ATRA alone reduced Bcl-2/BAX ratios after prolonged exposure (Aebi et al., 1997). In the same manner, PACLI and retinoid signaling revealed that synergy is related in part due to phosphorylation of Bcl-2 induced by Paclitaxel (Vivat-Hannah et al., 2001).

Concerning cell cycle, we found the major effects after treatment with PACLI and its combinations. PACLI, ATRA-PACLI, CIS-PACLI and ATRA-CIS-PACLI treatments caused arrest at both, the G2/M-phase and the S-phase. Previous studies have shown that paclitaxel-induced apoptosis blocks lung cancer cells in the G2/M phase (Jordan et al., 1993; Haldar et al., 1997; Noh et al., 2014; Singh and Dash, 2009). Previous studies have shown that PACLI exerts a synergistic effect with ATRA by mitotic arrest of cancer cells in the G2-M phase (Vivat-Hannah et al., 2001).

Additionally, the cell groups treated with ATRA, CIS and ATRA-CIS showed an increase in the sub-G0/G1 population. This is supported by previous studies showing that cells treated with CIS are arrested in the G0/G1 and G2/M phases, accompanied by a Sub-G0/G1 peak (Moreno et al., 2008). CIS is considered a nonspecific cell cycle inhibitor, however, cells in-S phase are more sensitive to this drug. CIS induces irreversible cell damage during G2 phase leading to apoptosis (Evans et al., 1994; Gonzalez et al., 2001; Wagner and Karnitz, 2009).

In addition, ATRA arrest in the sub-Go and decrease phase S in cell cycle. ATRA treatment from 40 nM to 1 µM causes G0/G1 cell cycle arrest through inhibit ERK1 expression, also decrease Rb phosphorylation, and increase Rb/E2F-1 association in gastrointestinal cancer stem cells (Crowe et al., 2003). In pancreatic carcinoma ATRA alone arrested cells in the G1 or G2 phase reducing the population at S-phase after 24 hours followed by apoptosis in sub-G0/G1 cells for a full inhibition of proliferation (el-Metwally et al., 2005). Previous reports have indicated that ATRA blocks cell growth, arresting cells in the pre-S phase; making cells exit the cell cycle either into a G0 quiescent state or to cell death via apoptosis (Mangiarotti et al., 1998).

Concerning apoptosis, the combination of ATRA plus CIS or PACLI induced cell death more efficiently than either drug alone. The combination with ATRA enhances the cytotoxicity of CIS in ovarian carcinoma and in squamous head and neck cancer cells (Aebi et al., 1997; Jozan et al., 2002). Induction of apoptosis has been related to the expression of RARs in human breast cancer cells (Liu et al., 1996). The combination of ATRA-PACLI is able to down-regulate hTERT and inactivate Bcl-2, promoting apoptosis in U87MG glioma cells (Karmakar et al., 2008). Also, selective retinoid α/β in combination with Taxol shown a synergistic effect through apoptosis induction in MCF7 (Vivat-Hannah et al., 2001). The cytotoxic effect of ATRA on cell growth and induction of apoptosis involves specific binding and activation of retinoic acid receptor such as RARα, β, γ (De Luca, 1991).

In lung cancer the expression patterns of RAR and RXR play an important role in prognosis, progression, survival, and lack of response to therapy (Xu et al., 1997). Our study shows that ATRA treatment participates in the induction of RARs. RARα increases at 24 hours in all groups that received ATRA treatment. RARα deregulation participates in the development of acute promyelocytic leukemia (Pandolfi, 2001) and human breast carcinoma (Farias et al., 2002). It has been suggested that ATRA mediates this antitumor activity through activation of RARα, encoded by the RARα gene (Simeone and Tari, 2004). Likewise, RARs up-regulates RARβ levels through binding to RAREs on the RARβ promoter (Khuri et al., 2000; Leroy et al., 1991).

We found increased RARβ levels in cells treated with ATRA. RARβ is the most studied tumor suppressor gene whose loss of expression and methylation is associated with the pathogenesis of lung cancer (Brabender et al., 2005; Virmani et al., 2000). In lung, breast and prostate tumors, RARβ is lost or down-regulated through inconstant promoter hyper-methylation, without gene deletion or mutation (Hayashi et al., 2001; Martinet et al., 2000; Wakelee et al., 2007). Other studies have shown that up-regulation of RARβ by ATRA correlates with growth inhibition in human breast cancer and lung cancer cells (Peng et al., 2011). Furthermore, in ATRA-sensitive lung and breast cancer cell lines RARβ is induced by ATRA (Liu et al., 1996; Zhang et al., 1996). In lung cancer, hyper-methylation of RARβ is associated with recurrence, in advanced stages of NSCLC (Kim et al., 2005; Tomizawa et al., 2004). Previously, we showed that the addition of ATRA to CT increases PFS and RR in lung cancer, and this effect was related to RARβ levels (Arrieta et al., 2011).

In a similar manner, we found that RARγ increased under ATRA treatment, and this effect was maintained up to 72 hours. RARγ plays a role in differentiation,
proliferation and apoptosis in many different cell types and cell microenvironments, also maintaining a balance. Published by Basic Research Journal of Medicine and Clinical Science between hematopoietic stem cell self-renewal and differentiation (Kumar et al., 2004; Yan et al., 2010). RARγ expression is lost in skin cancer and in some premalignant lesions (Finzi et al., 1992). Recently, high levels of RARγ have been associated with oncogenesis in hepatocellular carcinoma (Yan et al., 2010). High RARγ expression is also associated with high cytotoxicity and growth inhibition of head and neck squamous cell carcinoma cells treated with ATRA (Klaassen et al., 2001).

CONCLUSION

The combination of ATRA with CIs or PACLI enhances the antiproliferative and antiapoptotic effect in lung cancer cells and induces the expression of RARs but not RXRs. Synergistic effects are more evident when using a combining ATRA with CIs or PACLI.

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