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Letter to the Editor

Higher potency of the synthetic retinoid MX3350-1 compared to the natural all-*trans*-retinoic acid in modulation of cell cycle and induction of apoptosis in head and neck squamous carcinoma cells

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Dear Editor,

Retinoids, vitamin A analogs, have been shown to suppress carcinogenesis in experimental animals and to exhibit chemopreventive and therapeutic effects in clinical trials including chemoprevention of head and neck squamous cell carcinoma (HNSCC).[1] At least some of these effects could be related to the finding that all-*trans*-retinoic acid (ATRA) inhibited the growth and suppressed aberrant squamous differentiation to varying degrees in different HNSCC cell lines.[2] However, many human cancer cell lines exhibits inherent resistance to the growth inhibitory effects of ATRA.[3]

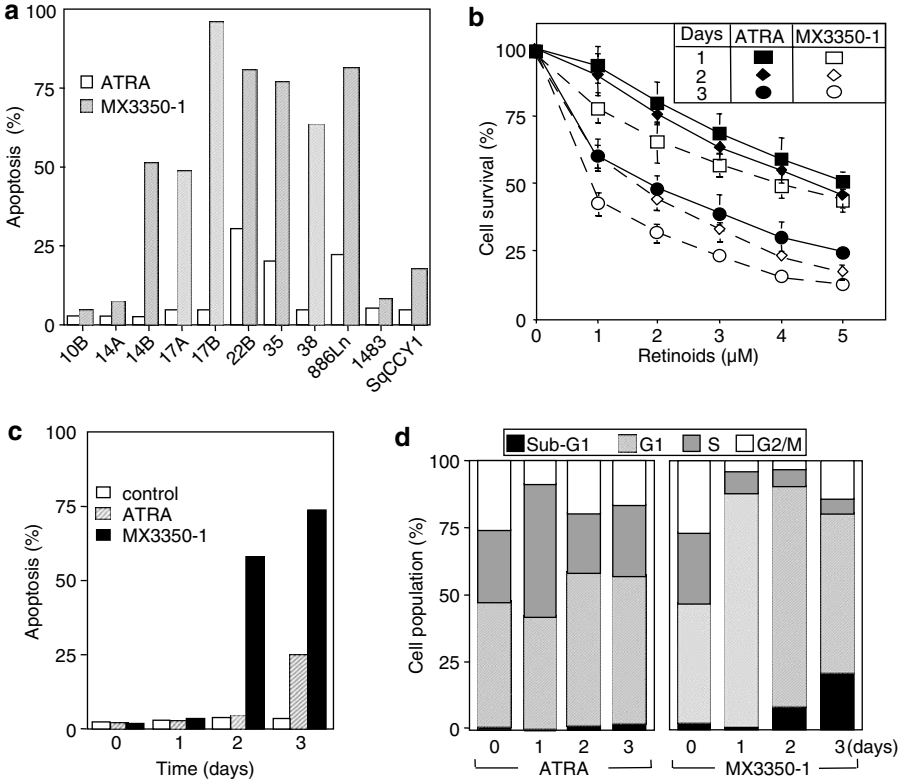

Figure 1 Effect of retinoids on cell survival, apoptosis and cell cycle in HNSCC cell lines. (**a**) HNSCC cells were seeded at a density of 1×10^6 cells per 10-cm diameter tissue culture plates. After 24 h, the cells were treated with 2 μM ATRA or MX3350-1 for 3 days. Cells were harvested, and apoptosis was detected by the TUNEL assay using the APO-DIRECT kit (Phoenix Flow Systems Inc., San Diego, CA, USA) following the manufacturer's protocol. The TUNEL apoptosis assays were performed twice independently with similar results. (**b**) UMSCC22B cells were seeded at a density of 3000 cells per well in 96-well tissue culture plates. After 24 h, cells were treated with different concentrations of ATRA or MX3350-1 for different times. Their survival was determined using the sulforhodamine B assay. Each assay was performed in triplicate and the results were calculated as the mean $\pm$ S.E.M. (**c**) UMSCC22B cells were seeded and treated with 2 μM ATRA or MX3350-1 for different days. Cells were harvested, and apoptosis was detected by the TUNEL assay as in A. (**d**) After treatment with 2 μM ATRA or MX3350-1 for different days, the cells were harvested by trypsinization, fixed with cold 70% ethanol, and stored at 4°C. The cells were stained with propidium iodide and cell cycle was analyzed by flow cytometry

In addition, some cells develop resistance during exposure to this retinoid as a result of induction of the P450 enzyme CYP26A1[4] or other mechanisms. In addition, retinoic acid has some characteristic side effects including increase in triglycerides and mucocutaneous toxicity that limited its long-term clinical use.[5] Therefore, new synthetic retinoic acid analogs have been prepared and screened for improved activity.[3,6] One of these retinoids called MX3350-1 inhibited the growth of human lung cancer cells *in vivo* in an animal model with minimal toxicity.[6] Recently, we have demonstrated that MX3350-1 induces apoptosis by activating both extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated) apoptosis pathways in UMSCC17B HNSCC cells that are resistant to ATRA.[7] However, the relative contribution of growth inhibition and apoptosis to the overall antitumor effects of MX3350-1 has not been clarified nor is it clear why MX3350-1 is so much more potent than ATRA. A comparison of the effects of ATRA and MX3350-1 (both at 2 μM) on 11 HNSCC cell lines revealed that seven of them were sensitive to MX3350-1 treatment exhibiting $\geqslant$50% growth inhibition (data not shown) and apoptosis as indicated by the TUNEL assay (Figure 1a). In contrast, ATRA was ineffective in inducing apoptosis in most cell lines and only 27% apoptosis was detected in the UMSCC22B cells. Four cell lines exhibited partial resistance to both retinoids. Although the mechanism for the unresponsiveness is not clear, it may be related to the p53 status of the cell lines because three (10B, 14A, and SqCC/Y1) of the four resistant cell lines have a mutated p53 and we found previously that the sensitivity of lung cancer cells to another retinoid-related molecule called CD437 was greater in cells with wild-type p53.[8]

We then used the UMSCC22B cell line that is sensitive to both retinoids but is more sensitive to MX3350-1 than to ATRA to compare and contrast their mechanisms of antitumor actions. Treatment of UMSCC22B cells with ATRA or MX3350-1 resulted in time- and dose-dependent cell growth inhibition and apoptosis induction (Figure 1b and c). The effect of MX3350-1 occurred faster and was more pronounced than that of ATRA at all retinoid concentrations used.

We then compared the effects of the two retinoids on cell cycle distribution (Figure 1d). Treatment with 2 μM ATRA for 1 day resulted in an increase in S-phase with concomitant decreases in G1 and G2/M phases, however, after 2 and 3 days of treatment, the proportion of cells in G1 and G2/M increased and that in S decreased. In contrast, a 1-day of treatment with 2 μM MX3350-1 resulted in a marked increase in G1 at the expense of S and G2/M, suggesting that MX3350-1 arrests cells in G1. However, subsequently (after 2 days), there was an increase in the sub-G1 cell population compatible with late stages of apoptosis. In contrast, ATRA showed only a minimal increase in the Sub-G1 population. It is noteworthy that in the UMSCC22B cells, the sub-G1 fraction consistently gives lower values compared to TUNEL (Figure 1c) or Annexin V (data not shown).

Some clues about the mechanisms underpinning the effect of MX3350-1 on the cell cycle were indicated by Western blotting analyses, which showed increased protein levels of p53, p21, and p27 and decreased levels of cyclin D1 by 6–12 h (Figure 2a and b). In contrast, ATRA had no effect or only minimal effect on these proteins at 24 h and a small effect on

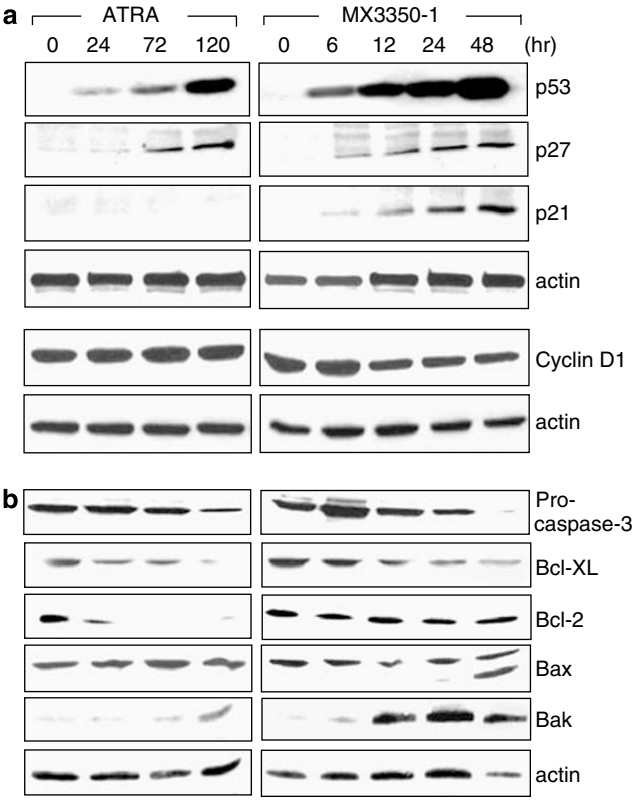

Figure 2 Effects of retinoids on protein expression level in UMSCC22B cells. Cells were cultured in the presence of 2 μM ATRA or MX3350-1 for the indicated times. At each time point, cells were harvested, extracted and subjected to Western blotting analysis using antibodies against the indicated proteins as described.[15] β-actin was used as a loading control. Each assay was performed twice independently with similar results

p53 and p27 but not on p21 after 72 h. The increase in p53 may lead to both growth arrest and apoptosis.[9] The expression of the cyclin-dependent kinase (CDK) inhibitor p21 is transcriptionally activated by p53 and can arrest cells in G1.[10] It appears that MX3350-1 was able to increase p53 levels early and this increase may have enhanced p21 induction, whereas ATRA, which has been shown to increase p21 directly in other cells, failed to do so in the UMSCC22B despite the increase in p53 (Figure 2a). This finding may explain the small effect of ATRA on G1 arrest.[11] Another reason for the greater potency of MX3350-1 is the rapid increase in p27, which shares the growth inhibitory function of p21 although it has slightly different kinase specificities.[12] The decreases in cyclin D1 may also play a role in G1 arrest by MX3350-1.[13] Therefore, the more pronounced and rapid effects of MX3350-1 on these proteins may explain why this retinoid was more potent than ATRA in inducing G1 arrest and growth inhibition.

The differential effects of MX3350-1 and ATRA on apoptosis may be partially explained by their disparate effects on members of the Bcl-2 family[14] of apoptosis regulating proteins. Recently, we reported that MX3350-1 activates the mitochondrial apoptosis pathway as evidenced by the ability of caspase-9 inhibitor to suppress this apoptosis.[7] In the present study, we found that MX3350-1 was a much more potent inducer of apoptosis than ATRA and increased the

levels of the proapoptotic Bak and Bax (including inducing a cleavage of Bax that activates it) and decreased the antiapoptotic Bcl-XL more rapidly and to a larger extent than ATRA did (Figure 2b). Although ATRA diminished the level of Bcl-2 earlier and to a much greater extent than MX3350-1 did this was apparently not sufficient to tilt the balance between proapoptotic and anti-apoptotic members of the bcl2 family towards apoptosis. We propose that the increase in Bak and Bax and the suppression of Bcl-XL induced by MX3350-1 to a much greater extent than by ATRA is more crucial for the rapid induction of apoptosis than the decline in Bcl2 and Bcl-XL induced by ATRA. As our results demonstrate that MX3350-1 is a more potent inhibitor of cell growth and a stronger inducer of apoptosis, we propose that MX3350-1 may be useful for treatment of ATRA-resistant HNSCC and possibly other cancers as well.

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