Eur J Cancer Clin Oncol, Vol. 23, No. 6, pp. 861–865, 1987.
Printed in Great Britain

0277-5379/87$3.00+0.00

Prevention and Reversal by a Non-polar Arotinoid (Ro 15-0778) of 3,4-Benzpyrene- and Cigarette Smoke Condensate-induced Hyperplasia and Metaplasia of Rodent Respiratory Epithelia Grown *In Vitro*

I. LASNITZKI* and W. BOLLAG†‡

**Strangeways Research Laboratory, Cambridge CB1 4RN, U.K. and †Pharmaceutical Research, F. Hoffmann-La Roche & Co. Ltd., Basle, CH-4002, Switzerland*

Abstract—*A non-polar arotinoid, Ro* 15-0778, *has been investigated for its effect on carcinogen-induced changes in rodent respiratory epithelia in organ culture. In neonatal rat tracheas and fetal mouse lungs grown* in vitro, 3,4-*benzpyrene and cigarette smoke condensate induce an increased proliferation of epithelial cells associated with a loss of secretory activity and of ciliary function. These changes persist in the absence of carcinogens in explants transferred to control medium. Ro* 15-0778 *alone has no influence on the normal epithelial growth rate or normal differentiation. However, if combined with either benzpyrene or smoke condensate, the arotinoid antagonizes the carcinogen-induced hyperplasia and metaplasia. During simultaneous treatment, it prevents the increase in mitosis and the loss of secretory activity or ciliary function. In explants pretreated with benzpyrene or cigarette smoke condensate, Ro* 15-0778 *reverses the high proliferation rate and restores secretory differentiation and ciliary function. The compound is of experimental and clinical interest, since—in contrast to most retinoids—it lacks the signs and symptoms of the classical hypervitaminosis A syndrome. It may be justified to consider it for the treatment of early precancerous changes of the bronchial tree.*

INTRODUCTION

IT IS well established that, in addition to its physiological functions, vitamin A also possesses anticarcinogenic properties. This was first shown in mouse prostate glands in organ culture in which retinol counteracted the effects of the carcinogenic hydrocarbon methylcholanthrene [1, 2]. Several authors demonstrated *in vivo* a preventive effect of vitamin A (= retinol) and its metabolite vitamin A acid (= all-*trans*-retinoic acid) on chemically induced precancerous and cancerous conditions of various organs [3–7]. The clinical use of retinol, its esters and all-*trans*-retinoic acid is limited by their toxicity at pharmacologically active doses. In order to find compounds combining the highest possible activity with the lowest possible side effects, a great number of structural analogs have been synthesized [8–11]. Some of these retinoids showed indeed a more favorable ratio between efficacy and toxicity, such as 13-*cis*-retinoic acid, aromatic retinoids—etretinate and etretin, for example, a series of arotinoids and fenretinid. Etretinate proved to be a potent compound in preventing and reversing the effects of 3,4-benzpyrene and cigarette smoke condensate in respiratory tissues grown *in vitro* [12]. Furthermore, these retinoids display *in vivo* a preventive or even a therapeutic effect on chemically induced tumors in animals [13–20].

However, these compounds still produce toxic side-effects which may restrict their clinical use. In the therapeutic use of retinoids, a certain degree of side-effects may be accepted by the patients. However, in the field of prevention, the tolerance has to be very good to excellent. Recently a non-polar arotinoid (Ro 15-0778) was found to exert none of the typical signs and symptoms of hypervitaminosis A, manifest in skin, mucous membranes, bone, cartilage, central nervous system, liver function and lipids [9]. In preliminary experiments,

Accepted 9 January 1987.
‡To whom requests for reprints should be addressed.

this compound inhibited the carcinogen-induced hyperplasia of mouse prostate gland and rat trachea in organ culture. Furthermore, the development of chemically induced rat mammary adenocarcinomas was inhibited. The percentage of tumor-bearing rats, the mean number of tumors per rat, as well as the mean total volume of tumors per rat were dose-dependently reduced by Ro 15-0778 [21]. Since prevention of lung cancer is one of the major problems in clinical oncology, it seemed important to investigate in detail the interaction of this non-toxic arotinoid with carcinogenic agents, most probably responsible for lung cancer, on respiratory epithelia. In the present experiments, the influence of Ro 15-0778 on the effects of 3,4-benzpyrene and cigarette smoke condensate were examined in mouse lungs and rat tracheas grown in organ cultures. In this system, the tissue components and their anatomical relationship and function are preserved *in vitro*, so that the explanted tissue resembles the parent tissue. The easy replication of the experiments makes it possible to obtain both quantitative and qualitative data and in addition the changes induced by carcinogenic agents occur after considerably shorter periods of treatment than in animal experiments.

MATERIALS AND METHODS

Organ cultures

The tissues used for the experiments were tracheas from neonatal rats obtained from a strain of hooded Lister rats and 17–18-day fetal mouse lungs from a laboratory inbred strain of R mice. They were grown in culture by a modified Trowell technique [22, 23]. The tracheas were halved and the lungs divided into fragments measuring approx. 2 × 2 × 4 mm. Four halved tracheas and six to eight lung fragments were explanted on strips of Millipore filters which were placed on grids of stainless steel. The grids rested in small plastic culture chambers filled with medium up to the level of the rafts. The culture chambers were placed in Petri dishes carpeted with moist filter paper to provide a humid atmosphere. The medium used was Parker's 199 [24] supplemented with 15% newborn calf serum. Before incubation at 37.5°C, the explants were perfused with a mixture of 5% CO_2 and 95% oxygen for 20 min at a flow rate of 125 ml/min.

Compounds

3,4-Benzpyrene (BP) purchased from Fluka A.G., Buchs, Switzerland was added to the medium at a concentration of 5 μg/ml.

Cigarette smoke condensate was kindly provided by Professor Schmähl, Deutsches Krebsforschungszentrum, Heidelberg, F.R.G. A hydrocarbon-enriched neutral fraction prepared from raw condensate was used. 100 mg were homogenized under sterile conditions in calf serum with a Braun-Sonic 300 apparatus. The resulting suspension contained 10 mg/ml serum. This was added to the medium at a final concentration of 130 μg/ml.

The arotinoid Ro 15-0778 = 1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-6-[(*E*)-α-methylstyryl]naphthalene (= A), an arotinoid without a polar end group, was supplied by F. Hoffmann-La Roche & Co.Ltd., Basle, Switzerland. Its synthesis has been described [9]. Ro 15-0778 was used at a concentration of 3 μg/ml (10^{-5} M).

Experiments and evaluation

In a first series of experiments, tracheal or fetal lung explants were grown for 14 days either with 3,4-benzpyrene or with cigarette smoke condensate. These compounds were either applied alone or combined with the arotinoid (simultaneous treatment).

In a second series of experiments, both tissues were exposed to 3,4-benzpyrene or cigarette smoke condensate for 14 days and then transferred to control medium or medium containing the arotinoid for a further 4 days (successive treatment). To assess the effect of the arotinoid alone, additional sets of explants were treated with Ro 15-0778 for 14 days or explants kept previously in control medium were treated with the arotinoid for 4 days.

At the end of the culture period, the explants were incubated with colcemid, a metaphase arresting agent, for 5 hr and then fixed in Bouin's solution, dehydrated, embedded in paraffin, serially sectioned and stained with haematoxylin–eosin or by the Azan substitute method.

The criteria of effect used were histological changes in cellular differentiation and cell proliferation. The latter was quantitated and expressed as the percentage of epithelial mitoses and its standard deviation.

RESULTS

Rat trachea

In neonatal rats, the trachea is lined with one row of cuboidal cells which are occasionally ciliated and separated from the surrounding rings of cartilage by a thin layer of connective tissue fibers. After 14 days in control medium, much of the epithelium has become columnar due to an enlargement of the supranuclear cytoplasmic area, has developed cilia and formed a number of mucin producing goblets cells. Occasionally, basal cells and mitotic figures can be seen underneath the superficial epithelium. Treatment with the arotinoid alone does not alter this pattern of growth, and the epithelium is indistinguishable from that of the control explants.

In contrast, 3,4-benzpyrene profoundly affects

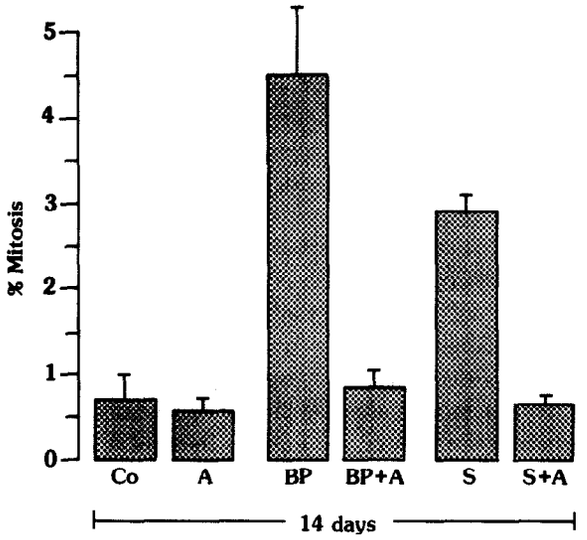

Fig. 1. Prevention of benzpyrene (BP)- and cigarette smoke condensate (S)-induced mitotic stimulation of tracheal epithelium by the arotinoid Ro 15-0778 *(A). Co = control medium.*

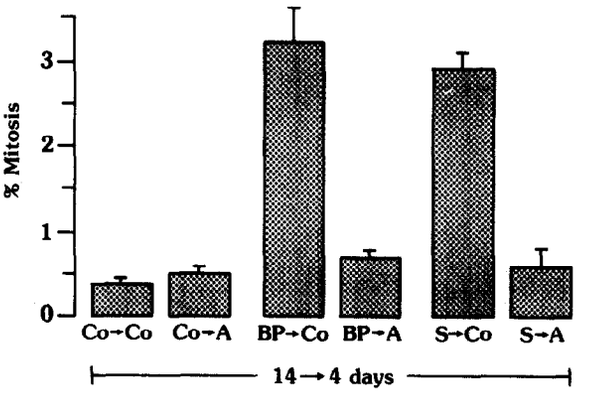

Fig. 2. Reversal of benzpyrene (BP)- and cigarette smoke condensate (S)-induced mitotic stimulation of tracheal epithelium by the arotinoid Ro 15-0778 *(A). Co = control medium.*

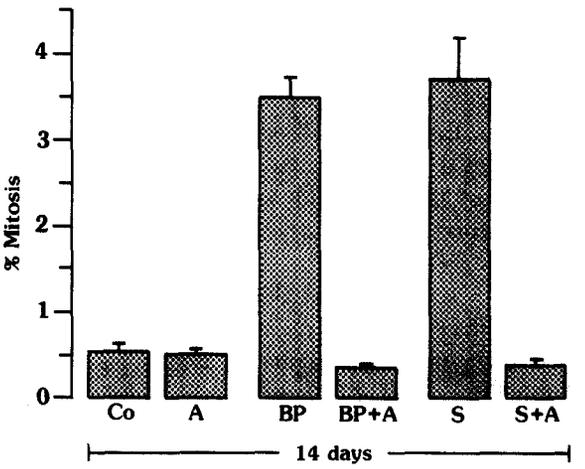

Fig. 3. Prevention of benzpyrene (BP)- and cigarette smoke condensate (S)-induced mitotic stimulation of bronchial epithelium by the arotinoid Ro 15-0778 *(A). Co = control medium.*

this basic growth pattern. Most of the superficial columnar cells lose their polarity and become flattened due to a shrinkage of the supranuclear zone. The number of goblet cells is greatly reduced and most of the cilia are damaged and shed. Cell proliferation is increased over that of the controls and many mitotic figures appear among the basal cells. The superficial cells are shed and replaced by a multilayered epithelium consisting of small crowded cells. Treatment with cigarette smoke condensate induces similar changes, but the degree of de-differentiation of the cells is less pronounced. Although there is a loss of goblet cells and clumping of cilia some of the epithelium remains columnar and polarized. In explants, pretreated with either benzpyrene or smoke condensate and transferred to control medium, the changes persist or even progress in the absence of the carcinogens.

Figure 1 shows that after 2 weeks' treatment with either benzpyrene or smoke condensate, the number of cell divisions is increased to 6.4 and 4.1 times, respectively, the control value. The arotinoid alone does not affect the normal mitotic rate significantly, but if combined with either benzpyrene or smoke condensate, it reduces cell multiplication to near control levels.

In explants pretreated with either 3,4-benzpyrene or smoke condensate and transferred to control medium, the rate of cell division amounts to 8.9 and 8.1 times, respectively, the control value. In both sets, administration of the arotinoid for 4 days reverses the increase and reduces the rate of mitosis to near control values (Fig. 2).

Fetal mouse lung

Before explantation, the lungs of 17–18-day mouse embryos consist of thin-walled alveoli lined with one row of flattened cells and some primitive bronchi lines with cuboidal epithelium, and separated from each other and from the alveoli by connective tissue fibers. After 14 days growth in control medium, a network of branching bronchi has developed. They are lined with cuboidal or columnar secretory cells, which show a small number of cell divisions. Treatment with 3,4-benzpyrene or cigarette smoke condensate increases mitosis of the bronchial cells leading to a multilayered epithelium, composed of proliferating basal cells surmounted by the original secretory cells. As in the trachea, the changes persist after transfer to control medium. Addition of the arotinoid inhibits the induction of hyperplasia, if combined with the carcinogens; if administered following the carcinogens, the arotinoid reverses the hyperplasia.

The mitotic counts (Fig. 3) show that mitosis is increased to over six-fold the control value by both benzpyrene and smoke condensate. The arotinoid alone does not affect the mitotic rate, but if combined with benzpyrene or smoke condensate, it reduces it to values below those counted in the controls.

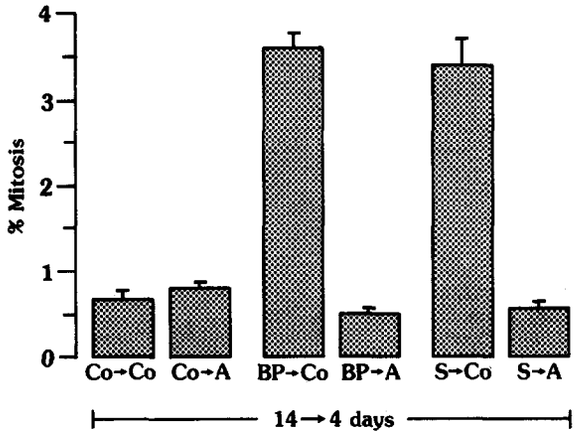

Fig. 4. Reversal of benzpyrene (BP)- and cigarette smoke condensate (S)-induced mitotic stimulation of bronchial epithelium by the arotinoid Ro 15-0778 (A). Co= control medium.

In explants pretreated with either carcinogen or smoke condensate and transferred to control medium, the rate of cell division amounts to over five times the control value. In both sets, administration of the arotinoid for 4 days reverses the increase and reduces it to near control levels (Fig. 4).

DISCUSSION

In both neonatal rat trachea and fetal mouse lung, 3,4-benzpyrene and cigarette smoke condensate stimulate epithelial cell proliferation and increase its mitotic rate. The increase in cell replication is followed by a loss of secretory activity, particularly in the trachea. The number of goblet cells is reduced and the cilia damaged. The superficial secretory cells are shed and replaced by a hyperplastic epithelium consisting of small crowded cells. Similar changes have been described for mouse tracheal epithelium *in vitro* [25] for hamster tracheal cultures exposed to 3,4-benzpyrene [26, 27] and for benzpyrene and smoke condensate treated human fetal lung [28]. Administration of the arotinoid Ro 15-0778 modifies these effects profoundly. If combined with benzpyrene or smoke condensate, it inhibits the increase in cell proliferation and prevents the metaplastic changes; if added following exposure to the carcinogens, it reverses epithelial hyperplasia and restores the secretory activity of the epithelium. Similar effects of vitamin A compounds have been reported for the same system. In hamster trachea grown in organ culture, retinol inhibited or reversed benzpyrene-induced hyperplasia and metaplasia [26] and in vitamin A-deficient tracheal cultures retinoids prevented squamous metaplasia [29]. More recent work [12] has shown that in rat trachea and fetal mouse lung *in vitro* exposed to benzpyrene and smoke condensate administration of etretinate inhibited or reversed the carcinogen-induced changes to an almost identical degree to the arotinoid Ro 15-0778.

The effectiveness of the arotinoid in the present experiments *in vitro* and its inhibition of mammary carcinogenesis *in vivo* [21] indicates that the anticarcinogenic properties of retinoids are not necessarily linked to their hypervitaminotic activity.

The arotinoid Ro 15-0778 is, like etretinate, a potent inhibitor of the carcinogen-induced early changes in both lung and trachea in organ culture. The superiority of the arotinoid Ro 15-0778 over etretinate lies in the lack of toxic side effects. Since it has been demonstrated in clinical trials that etretinate is able to induce regression of bronchial metaplasia and dysplasia in heavy smokers [30, 31] and this could implicate a prevention of lung cancer, our results suggest that the arotinoid Ro 15-0778 may be a promising candidate for the therapy of early precancerous changes of the respiratory tissues.

REFERENCES

1. Lasnitzki I. The influence of A-hypervitaminosis on the effect of 20-methylcholanthrene on mouse prostate glands grown *in vitro*. *Br J Cancer* 1955, **9**, 434–441.
2. Lasnitzki I. Growth pattern of the mouse prostate gland in organ culture and its response to sex hormones, vitamin A and 3-methylcholanthrene. *Natn Cancer Inst Monogr* 1963, **12**, 381–391.
3. Chu EW, Malmgren RA. An inhibitory effect of vitamin A on the induction of tumors of forestomach and cervix in the Syrian hamster by carcinogenic polycyclic hydrocarbons. *Cancer Res* 1965, **25**, 884–895.
4. Davies RE. Effect of vitamin A on 7,12-dimethylbenz(a)anthracene-induced papillomas in Rhino mouse skin. *Cancer Res* 1967, **27**, 237–241.
5. Saffiotti U, Montesano R, Sellakumar AR, Borg SA. Experimental cancer of the lung. Inhibition by vitamin A of the induction of tracheo-bronchial squamous metaplasia and squamous cell tumors. *Cancer* 1967, **20**, 857–864.
6. Bollag W. Prophylaxis of chemically induced benign and malignant epithelial tumors by vitamin A acid (retinoic acid). *Eur J Cancer* 1972, **8**, 689–693.
7. Cone MV, Nettesheim P. Effects of vitamin A on 3-methylcholanthrene-induced squamous metaplasia and early tumors in the respiratory tract of rats. *J Natn Cancer Inst* 1973, **50**, 1599–1606.
8. Mayer H, Bollag W, Hänni R, Ruegg R. Retinoids, a new class of compounds with prophylactic and therapeutic activities in oncology and dermatology. *Experientia* 1978, **34**, 1105–1119.

9. Loeliger P, Bollag W, Mayer H. Arotinoids, a new class of highly active retinoids. *Eur J Med Chem-Chim Ther* 1980, **15**, 9–15.
10. Klaus M, Bollag W, Huber P, Küng W. Sulfur-containing arotinoids, a new class of retinoids. *Eur J Med Chem-Chim Ther* 1983, **18**, 425–429.
11. Frickel F. Chemistry and physical properties of retinoids. In: Sporn MB, Roberts AB, Goodman DS, eds. *The Retinoids*. Orlando, Academic Press, 1984, Vol. 1, 8–145.
12. Lasnitzki I, Bollag W. Prevention and reversal by a retinoid of 3,4-benzpyrene- and cigarette smoke condensate-induced hyperplasia and metaplasia of rodent respiratory epithelia in organ culture. *Cancer Treat Rep* 1982, **66**, 1375–1380.
13. Bollag W. Therapeutic effects of an aromatic retinoic acid analog on chemically induced skin papillomas and carcinomas of mice. *Eur J Cancer* 1974, **10**, 731–747.
14. Bollag W. Prophylaxis of chemically induced epithelial tumors with an aromatic retinoic acid analog (Ro 10-9359). *Eur J Cancer* 1975, **11**, 721–724.
15. Bollag W. Arotinoids, a new class of retinoids with activities in oncology and dermatology. *Cancer Chemother Pharmacol* 1981, **7**, 27–29.
16. Bollag W, Hartmann HR. Prevention and therapy of cancer with retinoids in animals and man. *Cancer Surveys* 1983, **2**, 293–314.
17. Hartmann HR, Bollag W. The effect of arotinoids on rat mammary carcinogenesis. *Cancer Chemother Pharmacol* 1985, **15**, 141–143.
18. Bollag W. New retinoids with potential use in humans. In: Saurat JH, ed. *Retinoids: New Trends in Research and Therapy*. Basel, Karger, 1985, 274–288.
19. Sporn MB, Roberts AB. Biological methods of analysis and assay of retinoids—relationship between structure and activity. In: Sporn MB, Roberts AB, Goodman DS, eds. *The Retinoids*. Orlando, Academic Press, 1984, Vol. 1, 235–279.
20. Moon RC, Itri LM. Retinoids and cancer. In: Sporn MB, Roberts AB, Goodman DS, eds. *The Retinoids*. Orlando, Academic Press, 1984, Vol. 2, 327–371.
21. Bollag W, Hartmann HR. Inhibition of rat mammary carcinogenesis by an arotinoid without a polar end group (Ro 15-0778). *Eur J Cancer Clin Oncol* 1987, **23**, 131–135.
22. Trowell OA. The culture of mature organs in a synthetic medium. *Exp Cell Res* 1959, **16**, 111–147.
23. Lasnitzki I. The effect of actinomycin D on the cytology and RNA and protein synthesis in prostatic epithelium grown *in vitro*. *Cancer Res* 1969, **28**, 318–326.
24. Morgan JF, Morton HJ, Parker RC. Nutrition of animal cells in tissue culture. *Proc Soc Exp Biol Med* 1950, **73**, 1–8.
25. Lasnitzki I. The effect of a hydrocarbon enriched fraction from cigarette smoke condensate on mouse tracheas grown *in vitro*. *Br J Cancer* 1968, **12**, 105–109.
26. Lane BP, Miller SL. Effects of vitamin A on carcinogen induced changes in tracheal epithelial cultures. *Proc Am Assoc Cancer Res ASCO* 1976, **17**, 211.
27. Crocker TT, Sander LL. Influence of vitamin A and citral on the effects of benzo(a)pyrene on hamster trachea in organ culture. *Cancer Res* 1970, **30**, 1312–1318.
28. Lasnitzki I. The effect of a hydrocarbon enriched fraction of cigarette smoke condensate on human fetal lung grown *in vitro*. *Cancer Res* 1968, **28**, 510–516.
29. Sporn HB, Clamon GH, Dunlop NM, Newton DL, Smith JM, Saffiotti U. Activity of vitamin A analogues in cell culture of mouse epidermis and organ cultures of hamster trachea. *Nature* 1975, **253**, 47–50.
30. Gouveia J, Mathé G, Hercend T *et al.* Degree of bronchial metaplasia in heavy smokers and its regression after treatment with a retinoid. *Lancet* 1982, **i**, 710–712.
31. Brändli O, Pedio G, Sulser H, Baumann HR. Preliminary results of a six months treatment of bronchial dysplasia in heavy smokers with etretinate. Abstract, III Eur Conf Clin Oncol (ECCO), Stockholm, 16–20 June 1985.