Successful Chemotherapy of Experimental Neuroendocrine Lung Tumors in Hamsters with an Antagonist of Ca^{2+}/Calmodulin

H. M. Schuller, E. Correa, M. Orloff, and G. K. Reznik

Experimental Oncology Laboratory, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee 37901-1071 [H. M. S., E. C., M. O. J., and Byk Gulden Pharmaceuticals, Institute for Pathology and Toxicology, Friedrich-Ebert Damm 101, 2000 Hamburg 70, Federal Republic of Germany [G. K. R.]

ABSTRACT

The chemotherapeutic effect of B859-35, the (−)-enantiomer of dihydroprynie 3-methyl-5-3-(4,4-diphenyl-1-piperidinyl)-propyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylate-hydrochloride (nimodipine) was tested in tumors induced in Syrian golden hamsters by N-nitrosodimethylamine (DEN). Peripheral pulmonary adenomas/adenocarcinomas were induced in hamsters maintained under ambient air conditions by multiple s.c. injections of DEN for 20 weeks. We have reproducibly shown that within this time interval lung adenomas develop in a significant number of the animals. The carcinogen treatment was discontinued at this point and one group of these hamsters was given B859-35 intragastrically 5 days/week for 20 weeks while the second group of such tumor-bearing hamsters were returned to ambient air conditions. One group of these tumor-bearing hamsters were kept for an identical time interval without further treatment.

Neuroendocrine lung tumors were induced in hamsters maintained in an atmosphere of 60% O₂ by multiple s.c. injections of DEN for 8 weeks. We have reproducibly shown that within this short time interval neuroendocrine lung tumors develop in a significant number of the animals. The carcinogen treatment was discontinued at this point and the animals were returned to ambient air conditions. One group of these tumor-bearing hamsters was then given B859-35 intragastrically 5 days/week for 20 weeks while a second group of these hamsters was kept untreated for an identical time interval. A control group was given s.c. injections of saline for 20 weeks under ambient air conditions.

A dramatic and selective antitumorigenic effect of B859-35 was observed in the neuroendocrine lung tumors and nasal cavity tumors induced by DEN/hyperoxia while tumors of larynx/trachea were not affected. B859-35 had no effect on peripheral adenomas/adenocarcinomas, nasal cavity tumors, papillary polyps of larynx/trachea, or liver tumors induced by DEN under ambient air conditions.

INTRODUCTION

Neuroendocrine tumors and peripheral adenocarcinomas are the most common histological lung tumor types in humans (1) and both demonstrate a strong epidemiological link with cigarette smoking. As with all other malignant lung tumor types, 12-month survival after diagnosis is rare (2), although neuroendocrine carcinomas often demonstrate an initial response to radio- and chemotherapy.

We have established in our laboratory two animal models which allow us to study the reactions of these two lung cancer types to chemicals and drugs in vivo. In both cases we are using male Syrian golden hamsters as experimental animals and carcinogenic N-nitrosamines as cancer-inducing chemicals. However, while for the reproducible induction of peripheral adenomas/adenocarcinomas within 20 weeks the hamsters are maintained under ambient room air conditions while receiving multiple s.c. injections of N-nitrosodiethylamine (3, 4), neuroendocrine lung tumors are reproducibly induced within 8 weeks at a high incidence in hamsters maintained under hyperoxia while receiving identical treatments of nitrosamine (5).

Serial sacrifice type experiments, including assessment of morphological changes by transmission electron microscopy (3, 4), and immunocytochemistry (6) have shown that the adenomas and adenocarcinomas induced by DEN in the lungs of hamsters maintained under ambient air conditions are derived from Clara cells and express features of Clara cells and alveolar type II cells. In contrast, treatment with DEN of hamsters maintained under hyperoxia initially causes focal changes in the differentiation of alveolar type II cells into bombesin-producing neuroendocrine cells, and neuroendocrine lung tumors develop from such altered foci (7). All of the neuroendocrine lung tumors demonstrate positive immunoreactivity to mammalian bombesin, calcitonin, and neuron specific enolase, while the lungs and serum of such tumor bearing animals demonstrate high levels of bombesin and calcitonin by radioimmunoassay (7). None of these neuroendocrine markers are expressed in the adenomas/adenocarcinomas, lungs, or serum (except for very low calcitonin levels in serum and lungs) of hamsters treated with nitrosamine while maintained under ambient air conditions.

In vitro experiments with human lung cancer cell lines have demonstrated that a large proportion of human neuroendocrine lung tumors produce mammalian bombesin which acts as an autocrine growth factor (2, 8, 9). It has been demonstrated that the secretion of this peptide is regulated via acetylcholine receptors of the muscarinic and nicotinic subtypes in cell lines derived from small cell carcinomas (9) (which represent the most malignant variant of neuroendocrine lung tumors). On the other hand, evidence has been provided that the secretion of calcitonin and mammalian bombesin in normal pulmonary neuroendocrine cells is regulated via acetylcholine receptors of the nicotinic subtype only (11, 18). There is evidence that both types of acetylcholine receptors as well as the receptor for mammalian bombesin operate via ion channels with a high affinity for Ca²⁺ as second messenger (12, 13). The Ca²⁺-dependent signal transduction of this second messenger pathway utilizes calmodulin for the processing of intracellular Ca²⁺ further downstream (14). In contrast, Clara cell secretion is regulated via β adrenergic receptors (15), and studies conducted in our laboratory have shown that β adrenergic agonists stimulate cell proliferation in cell lines derived from human lung adenocarcinomas and comprised of Clara cells (16). Contrary to the acetylcholine and bombesin receptors, β adrenergic receptors do not operate via ion channels as second messengers (12). In fact, they represent the prototype of enzyme-coupled type II receptors which function via cyclic AMP as second messenger (12).

Ca²⁺-channel antagonists have been suggested as potential anticarcingenic agents because many growth factors (including mammalian bombesin) which stimulate cancer cell proliferation...
are taken up by receptors which operate via ion channels with high affinity for Ca\(^{2+}\) (17).

B859-35 is one of two stereoisomers which constitute the dihydropyrrine 3-methyl-5,3-(4,4-diphenyl-1-piperidinyl)-propyl-1, 4-dihydro-2, 6-dimethyl-4-(3-nitrophenyl)-pyridine-3, 5-dicarboxylate hydrochloride. The mixture of the two isomers has been shown to have antihypertensive effects in mammals (18), an effect believed to be mediated via antagonism of Ca\(^{2+}\)-channels and \(\alpha_1\)-adrenergic receptors (18-20). Recently it has been shown that this effect rests with the (+)-isomer (niguldipine) while the (-)-isomer, B859-35, has only minimal effects on blood pressure (19). The activity of B859-35 is thought to be primarily due to a pronounced inhibition of Ca\(^{2+}\)/calmodulin dependent intracellular pathways which constitute the second messenger system of many growth factors.

It has been shown that the B859-35 has antitumorogenic effects in vitro in a variety of rodent tumor systems as well as in human lung cancer cell lines of different histological types. We therefore tested the chemotherapeutic effects of this compound on two different histological lung tumor types in hamsters: neuroendocrine lung tumors which express cholinergic and bombesin receptors which are known to operate via Ca\(^{2+}\)-channels and calmodulin as second messengers versus Clara cell adenomas which express \(\beta\) adrenergic receptors known to function via cyclic AMP as second messengers.

MATERIALS AND METHODS

Six-week-old male, outbred Syrian golden hamsters (Sendai virus free) were purchased from Charles River (Wilmington, MA). Male animals were used for this experiment to avoid a possible influence of the sexual cycle in females which might have interfered with the reproducibility of the induction of a neuroendocrine tumor. The hamsters were maintained (5/cage) under standard laboratory conditions (20°C; 40% relative humidity, standard Purina rodent chow, and water ad libitum, 12-h day, 12-h night cycle) for 2 weeks to allow them to recover from stress of shipment and to adjust to the new environment.

The animals were then randomly divided into groups of 12 hamsters/group except for group 4 which had 13 hamsters because we expected some premature animal losses in this group. Group 1 (controls) were s.c. given injections every Tuesday and Thursday of 0.1 ml/100 g body weight physiological saline (= solvent of DEN). Group 2 (DEN/ambient air) was s.c. given injections every Tuesday and Thursday for 20 weeks of 17.5 mg/kg DEN (98%, Sigma, St. Louis, MO) in physiological saline (injection volume, 0.1 ml/100 g body weight) while maintained under ambient room air conditions. Group 3 (DEN/air/B859-35) were s.c. given injections every Tuesday and Thursday of the same dosing regimen of DEN as group 2 for 20 weeks and were treated from then on for the remaining 20 weeks of the experiment with B859-35 (compound provided by Byk Gulden Pharmaceuticals, Konstanz, Federal Republic of Germany, 32.5 mg/kg body weight dissolved in polyethylene glycol, 400 and 0.01 N HCl according to instructions provided by Byk Gulden, Konstanz). Groups 4 (DEN/oxygen) and 5 (DEN/oxygen/B859-35) were placed in their own cages into a vinyl chamber (Standard Safety Equipment Co., Palatine, IL) which was equilibrated at 60% oxygen by mixing 100% oxygen with compressed air (6, 8). Both these groups remained in the chamber for 8 weeks, at which time they were returned to ambient room air conditions. Group 4 was given injections every Tuesday and Thursday of the same dosing regimen of DEN as groups 2 and 3 for a total of 8 weeks (while the animals were kept under hyperoxia). Group 5 received carcinogen treatment identical to that of group 4 but was then treated with B859-35 at a dosing regimen identical to that of group 3 for 20 weeks after having been removed from the hyperoxia chambers and being maintained under ambient room air conditions.

In all cases, B859-35 was administered by gavage every day (except week ends). All animal treatments were conducted between 9 and 11:00 a.m. to avoid uncontrollable variables due to diurnal variations in metabolic state. All hamsters were sacrificed 20 weeks after start of the treatment with B859-35, at which time also the control group and groups treated with DEN/air alone or DEN/hyperoxia alone were sacrificed.

The animals were sacrificed with a pentobarbital overdose. Complete necropsies were conducted and all major organs were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Selected lung tumors were subjected to immuno-cytochemical stains by using primary antisera for mammalian bombesin and calcitonin and the Vectastain ABC-kit (Vector, Burlingame, CA) as previously described (5).

The overall tumor incidence among groups as well as the organ distribution of tumors and histological tumor types were tested for significant differences (comparing DEN treated versus control and comparing chemotherapy versus nonchemotherapy) by the paired t test.

RESULTS

All of the control animals (group 1) demonstrated macroscopically and microscopically normal organs. In particular, none of these animals had any tumors.

In group 3 (DEN/air/B859-35) four hamsters died and were cannibalized 1 and 2 weeks before treatment with B859-35 started. A repeat of this dosing regimen was therefore conducted with four additional animals.

Similarly, three hamsters of group 5 (DEN/O2/B859-35) died 1 and 2 weeks prior to start with B859-35. A repeat of this dosing regimen was therefore conducted with 3 additional hamsters. We believe that in all these animals the sudden death was attributable to papillary polyps in larynx/trachea, a condition known to interfere with the swallowing of food that often results in food aspiration and subsequent asphyxiation.

In group 2 (DEN/air) a pronounced carcinogenic response was observed in nasal cavity, larynx/trachea, lungs, and liver, with many of the hamsters developing tumors in multiple organs (Table 1, Figs. 1–3). Most of the nasal cavity tumors were localized in the olfactory region and were diagnosed as poorly differentiated adenocarcinomas by histopathology (Fig. 2). In one hamster, a squamous cell carcinoma was found in the respiratory region of the nasal cavity. Multiple areas of focal hyperplasias and/or squamous metaplasias were also common. The histopathology of tumors in larynx and trachea was identical in all DEN-treated groups regardless of additional

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* Values statistically significant (P < 0.01) as compared with control group.

Table 1. Anticarcinogenic effects of B859-35

Animals under ambient air conditions were treated for 20 weeks with DEN (time required to induce lung adenomas/adenocarcinomas (3), then started on B859-35 for 20 weeks as anticancer therapy. Animals under hyperoxia (O2) were treated for 8 weeks with DEN (time required for induction of neuroendocrine lung tumors (5)) then started on B859-35 as anticancer therapy.

5 Byk Gulden, unpublished data.

H. M. Schuller, unpublished data.
Fig. 1. Papillary polyp in the trachea of a hamster of group 2 (DEN/air). Tumors in this organ are a typical response to DEN treatment with this dosing regimen. The tumors are multiple, large, and often obstruct the tracheal lumen. H & E, × 250.

Fig. 2. Histopathology of poorly differentiated adenocarcinoma in the nasal olfactory region of a hamster of group 2 (DEN/air). Note infiltration of bone. H & E, × 160.

Fig. 3. Lung adenoma of a hamster in group 2 (DEN/air). Note the glandular growth pattern which is characteristic of the Clara cell-derived lung adenomas induced with this dosing regimen of DEN under ambient air conditions. H & E, × 160.

Fig. 4. Neuroendocrine lung tumor in a hamster of group 4 (DEN/hyperoxia). The tumor is highly cellular with little or no stroma; glandular growth pattern is rare or absent. This morphology is characteristic of the neuroendocrine lung tumors induced by this dosing regimen of DEN in combination with hyperoxia. H & E, × 160.

Exposure to hyperoxia and/or B859-35. They were multiple papillary polyps (Fig. 1), some of which occupied considerable portions of the airway lumen. However, none of these lesions demonstrated invasive growth suggestive of malignancy. The lung tumors were peripheral adenomas (Fig. 3) which have been reproducibly shown to be of Clara cell origin (3, 4, 6). The liver tumors were neoplastic nodules of hepatocytes which compressed the surrounding liver parenchyma. Eight of the 12 animals constituting this group had multiple focal bile duct proliferations. Two hamsters were diagnosed with a hemangiosarcoma of the liver.

In group 3 (DEN/air/B859-35) the overall tumor incidence was not decreased as compared with group 2 (DEN/air). Moreover, the histological types of tumors and organ distribution of tumors was not significantly different among these two groups (Table 1).

In group 4 (DEN/hyperoxia) the organ distribution and tumor incidence in nasal cavity, larynx/trachea, and lung was not significantly different from groups 2 (DEN/air) and 3 (DEN/air/B859-35). However, only one of the animals demonstrated neoplastic lesions in the liver (Table 1). As has been previously reported (5) the lung tumors induced under these conditions, were neuroendocrine tumors (Figs. 4, 5) which produced mammalian bombesin (Fig. 5) and calcitonin.

In group 5 (DEN/hyperoxia/B859-35) none of the animals had tumors in the nasal cavity, lung, or liver, while the tumor incidence in larynx/trachea was only slightly reduced when compared with groups 2, 3, and 5 (Table 1).

DISCUSSION

Our data demonstrate that B859-35 has a striking and highly selective anticarcinogenic effect only on nasal cavity tumors and lung tumors induced by the combined exposure to hyperoxia and DEN. In contrast, tumors in these organs were not reduced in incidence by B859-35 when they had been induced.
by DEN/air. Moreover, the compound did not reduce the number of tumors in the larynx/trachea under any of the tested conditions.

To fully appreciate the implications of our findings, it is important to note that at the time when therapy with B859-35 started, the DEN-treated hamsters had already established lung tumors. We have reproducibly shown that DEN administration under ambient air conditions induces a high incidence of peripheral adenomas/adenocarcinomas in hamsters within 20 weeks (3). On the other hand, we have demonstrated that when administered under hyperoxic conditions DEN induces a high incidence of locally invasive neuroendocrine lung tumors within 8 weeks of treatment in the animals (5). Our experimental design is hence compatible with treatment of human lung cancer patients at stage 2 of the disease.

We have reproducibly shown that the lung tumors induced in hamsters by simultaneous exposure to DEN (5) or the tobacco-specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone\(^1\) and hyperoxia are neuroendocrine tumors which actively secrete mammalian bombesin and calcitonin while exposure to hyperoxia alone did not cause any lung tumors (5, 7). As far as the nasal cavity tumors are concerned, we do not know at this point if they are neuroendocrine in nature because the decalcification of skulls precludes the application of immunocytochemical stains. However, electron microscopic studies have provided evidence that neuroendocrine cells may be involved in the pathogenesis of some nitrosamine-induced nasal cavity tumors (20, 21). It would hence be possible that the hyperoxia, as in the lungs, selectively promoted neuroendocrine differentiation of tumors in the nasal cavity. It is therefore likely that the antitumor effect of B859-35 is selective for neuroendocrine neoplasms in the respiratory tract.

It has been amply documented that neuroendocrine lung tumors including its most malignant variant, small cell lung cancer produce a variety of polypeptides, many of which act as autocrine growth factors, thus continuously stimulating tumor growth (2, 8). Among these, mammalian bombesin is the most prominent neuroendocrine marker of this tumor category and is expressed in the majority of human neuroendocrine lung cancers (8). Mammalian bombesin is also expressed in the neuroendocrine lung tumors induced in our hamster model by nitrosamine/hyperoxia (5).\(^3\) It has been shown that the secretion of this polypeptide by small cell lung cancer is regulated by nicotinic and muscarinic acetylcholine receptors (9), while normal (nontumorous) neuroendocrine cells (10, 11) and a cell line derived from a human lung carcinoid (22) express only nicotinic receptors. It is well established that the acetylcholine receptors are type I (ion channel with high affinity to Ca\(^{2+}\)) and dependent on Ca\(^{2+}/\)calmodulin (12, 23). Moreover, there is evidence that the receptors for mammalian bombesin, an autocrine growth factor propagating neuroendocrine tumor growth (8, 24) also functions via ion channels with high affinity to Ca\(^{2+}\) and calmodulin as second messengers (13, 25). With respect to this, it would therefore seem possible that in our model B859-35 inhibits the secretion and uptake of the growth factor bombesin by blocking the Ca\(^{2+}/\)calmodulin dependent second messenger pathways of acetylcholine and bombesin receptors, respectively, thus interrupting the signal transduction necessary for the stimulation of polypeptide secretion and cell proliferation. This in turn could result in an arrest of tumor growth and ultimately the complete disappearance of neoplasia as in our experiment. In both these outlined possibilities, the key element for a chemotherapeutic effect would be the interruption of signal-regulated cell functions: production of autocrine growth factor leads to stimulation of tumor growth, leading to increase in growth factor production, etc.

It is important to note that elevated production of mammalian bombesin appears to be an intrinsic factor associated with any condition resulting in an impaired (e.g., chronic obstructive lung disease including smoking-induced disease, pulmonary fibrosis, pulmonary asbestosis) (26), or excessive (like our hyperoxia hamster model)\(^5\) (5, 7) ventilation of the lung with oxygen. Recent epidemiological evidence suggests that the development of neuroendocrine lung cancer is closely linked with the development of preceding chronic obstructive lung disease and that this condition poses a much higher risk factor for the development of this cancer type than cigarette smoking per se (27). In this context, it seems possible that a drug like B859-35, if it would selectively inhibit the secretion and/or uptake of mammalian bombesin (as suggested by the results of our experiment), could even be of value as a cancer preventive agent. Its chemotherapeutic effects on neuroendocrine tumors would be especially likely in patients at a relatively early stage of the disease and with documented production of mammalian bombesin, because the secretion of this polypeptide is regulated via Ca\(^{2+}\)-channel type acetylcholine receptors, while its uptake as autocrine growth factor by bombesin receptors appears to depend, at least in part, on the same second messenger system (13).

Our data show, for the first time, that based on an understanding of regulatory mechanisms for normal and neoplastic cell growth, a logical and "target oriented" cancer therapy selective for a well-defined tumor subcategory (in our case, bombesin-producing neuroendocrine respiratory tract tumors) can be developed. Although substantial work is required to elucidate the molecular mechanisms of action of B-859-35, the results of our experiment are encouraging because treatment of tumor-bearing hamsters with this experimental drug resulted in complete cure of a well-defined tumor subcategory without detectable adverse side effects on other tissues and organs.

Based on our findings in conjunction with favorable results from extensive toxicity and mutagenicity testing, phase I clinical trials with B859-35 have now been started.

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