The Antiproliferative Effects of Tyrosine Kinase Inhibitors Tyrphostins on a Human Squamous Cell Carcinoma in Vitro and in Nude Mice

Toshiyuki Yoneda, Ray M. Lyall, Maria M. Alsina, Paul E. Persons, Alfred P. Spada, Alexander Levitzki, Asher Zilberstein, and Gregory R. Mundy

Division of Endocrinology and Metabolism, Department of Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7877

ABSTRACT

Many human tumors of epithelial origin contain cells overexpressing the epidermal growth factor (EGF) receptor, and there is convincing evidence that cancer cell growth is correlated with the loss of the normal regulation of the EGF receptor signal transduction pathway. Some cancers are clearly dependent on activation of the EGF receptor for their proliferation. Recently, a class of compounds, tyrphostins, which inhibit the protein tyrosine kinase activity of the growth factor receptor, have been described. In this report, we have examined the antiproliferative effects of potent new tyrphostins on a well-characterized human squamous cell carcinoma in vitro and in vivo. We found that two of these compounds (RG-13022 and RG-14620) suppressed not only EGF-stimulated cancer cell proliferation in vitro but also tumor growth in nude mice. RG-13022 also increased the life span of these tumor-bearing nude mice. When administered to tumor-bearing nude mice together with monoclonal antibodies to the EGF receptor at a suboptimal dose which had no effect alone, inhibition of tumor growth was markedly enhanced. These data suggest that tyrphostins have potential as anticancer agents.

INTRODUCTION

Human squamous cell carcinomas are among the most difficult human malignancies to treat (1). They are notoriously resistant to chemotherapy and radiation therapy, particularly when they originate in the lungs, head, or neck (2, 3). Many human squamous cell carcinomas contain large numbers of EGF3 receptors (4) and may be dependent on the EGF receptor signaling pathway for their proliferation in vitro and growth in vivo (5, 6). Interfering with this pathway by pharmacological means may provide an effective medical treatment for these cancers.

In this paper, we describe the effects of several tyrphostins on EGF-stimulated cell proliferation in tumor cells and on EGF receptor tyrosine kinase activity in vitro. Two of these compounds, RG-13022 and RG-14620, have longer-lasting effects in cell culture than do the previously described tyrphostins (7, 8), and this property prompted us to test these compounds in vivo. We characterized the effects of these factors on cells overexpressing EGF receptors to confirm their capacity to inhibit EGF-dependent tyrosine kinase. These compounds were administered to nude mice bearing the well-characterized human squamous cell carcinoma MH-85, which is associated with three paraneoplastic syndromes, hypercalcemia, cachexia (9, 10). We found that RG-13022 and RG-14620 not only inhibited EGF-stimulated proliferation of MH-85 cells in vitro but suppressed the growth of MH-85 tumors in nude mice and the expression of the paraneoplastic syndromes. Tumor-bearing nude mice treated with these agents lived significantly longer than corresponding tumor-bearing mice treated by injections of vehicle.

MATERIALS AND METHODS

Compounds

The tyrphostins used in this study were RG-13022 and RG-14620 (Fig. 1). Forty mm stock solutions of these compounds were made in 100% DMSO and diluted with the culture medium before addition to the cells. The culture medium containing the equivalent concentrations of DMSO served as vehicle controls and had no effect on MH-85 growth in culture and in nude mice.

HER14 Cells

Cells termed HER14 were prepared by transfecting NIH 3T3 (clone 2.2) (from Charlotte Fryling, National Cancer Institute, NIH), which lack endogenous EGF receptors, with complementary DNA constructs of wild-type human EGF receptor, as described (11, 12). HER14 cells were shown to express high intrinsic tyrosine kinase activity. HER14 cells were grown in DMEM (Gibco Laboratory, Grand Island, NY) containing 10% calf serum (HyClone Laboratories, Logan, UT) and supplemented with 2 mM l-glutamine and 100 units/ml of penicillin-streptomycin (Gibco).

MH-85 Tumor and Cells

MH-85 tumor was derived from a patient with a squamous cell carcinoma of the maxilla who manifested marked leukocytosis (180,000/mm3; >90% mature granulocytes), cachexia (loss of body weight, muscle, and adipose tissue), and hypercalcemia (130 mg/dl) (9). MH-85 cells were established in culture from the tumors formed in athymic nude mice (10). The cells were grown in αMEM (Hazleton Biologics, Inc., Lenexa, KA) supplemented with 10% FCS (HyClone) and 1% penicillin-streptomycin solution.

Cell-free Autophosphorylation of EGF Receptor in Immunoprecipitates

Confluent HER14 cell layers from 15-cm tissue culture dishes were solubilized by scraping the cells in 1 ml of cell lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5-150 mM NaCl-1.5 mM MgCl2-1 mM EGTA-10% glycerol-1% Triton X-100-4 µg/ml phenylmethylsulfonyl fluoride-1 µg/ml aprotinin-1 µg/ml leupeptin). The lysates were cleared by centrifuging at maximum speed in an Eppendorf microcentrifuge for 15 min at 4°C. Fifty µl of lysate were used for each reaction. EGF (0.5 µg/ml; Toyobo Inc., New York, NY) was added to the lysate for 10 min at 4°C prior to immunoprecipitation. Lysates were incubated with protein A-Sepharose-purified mAb108 (13) complex for 90 min in the cold and then washed 3 times with buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5-150 mM NaCl-0.1% Triton X-100-10% glycerol). The washed immunoprecipitate was aliquoted and preincubated for 30 min on ice with tyrphostins in 30 µl of the same buffer containing 5 mM MnCl2 and...
200 μM sodium orthovanadate. Autophosphorylation was initiated by adding 4.5 μCi of [γ-32P]ATP (Dupont NEN, Wilmington, DE) and unlabeled ATP to give a final concentration of 2 μM ATP. After 2 min at 4°C the reaction was terminated with 55 μl of 2x sodium dodecyl sulfate sample buffer, and the samples were electrophoretically separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel (14). Quantitation was carried out by densitometry scanning of the autoradiograms.

**Autophosphorylation of EGF Receptor in HER14 Cells**

Confluent HER14 cells in 10-cm tissue culture dishes were transferred to DMEM containing 0.5% FCS and incubated with tyrphostins either overnight or for various periods of time. The cells were then stimulated for 5 min with EGF (500 ng/ml) and lysed and subjected to immunoblotting with anti-phosphotyrosine antibodies as described (15). Quantitation was carried out by densitometry scanning of autoradiograms.

**Colony Formation**

MH-85 cells (100/well; 24-well plate; Corning Glassware, Corning, NY) and HER14 cells (200/dish; 10-cm dish) were plated in complete medium, either αMEM or DMEM, respectively, supplemented with 10% FCS. After overnight culture, the culture medium was switched to αMEM supplemented with 0.2% FCS and 50 ng/ml EGF (MH-85) or DMEM supplemented with 0.5% FCS and 50 ng/ml EGF (HER14). The cells were cultured in this medium in the presence or absence of increasing concentrations of RG-13022 or RG-14620 for 10 days. At the end of culture, the cells were fixed with 4% (v/v) formaldehyde in calcium-magnesium-free phosphate-buffered saline for 15 min at room temperature and stained with hematoxylin (13). Numbers of colonies including more than 20 cells in each well were counted under the microscope.

**DNA Synthesis**

MH-85. MH-85 cells (1 × 10^4/0.1 ml) were plated onto 96-well plates (flat bottom; Corning) and cultured in αMEM supplemented with 10% FCS overnight. The cells were then fed with αMEM supplemented with 0.2% FCS and 50 ng/ml EGF and cultured in this medium in the absence or presence of increasing concentrations of RG-13022 or RG-14620 for 44 h. The cells were then incubated with 0.2 μCi/well [methyl-3H]thymidine (2.0 Ci/mmol; ICN Radiochemicals, Costa Mesa, CA) for a further 4 h. At the end of incubation, the cells were detached by treatment with trypsin-EDTA solution (Gibco) and harvested onto glass fiber strips using a multiple automated cell harvester (Cambridge Technology, Inc., Watertown, MA). The radioactivity was measured by liquid scintillation counting.

**HER14 Cells. DNA synthesis in HER14 cells was measured as described (8)**

**Inoculation of MH-85 Tumor into Nude Mice**

MH-85 tumors 5 mm in diameter were inoculated s.c. into the right dorsal portion of 4- to 6-week-old male BALB/c nu/nu mice (Harlan Sprague-Dawley, Indianapolis, IN). Animals were fed ad libitum on autoclaved diet (Tekland-LH-485; calcium, 0.83 mg/kg; phosphorus, 0.65 mg/kg; Harlan Sprague-Dawley) and tap water (acidified to pH 2.5). Tumor sizes were measured once a week under anesthesia with nembutal (0.05 mg/kg body weight, i.p.) and calculated as described (16).

**Administration of Tyrphostins in MH-85 Tumor-bearing Nude Mice**

RG-13022 or RG-14620 in 0.1 ml 100% DMSO was injected i.p. twice a day from 1 day after MH-85 tumor inoculation. Control animals were given the same vehicle.

**RESULTS**

**Inhibition of Cell-free EGF Receptor Autophosphorylation by RG-13022.** In a cell-free reaction RG-13022 inhibited the autophosphorylation reaction of the EGF receptor in immunoprecipitates with an IC_{50} of 4 μM (Fig. 2). An amount of DMSO equivalent to that used in the highest RG-13022 dose showed no inhibitory effect (data not shown).

**Inhibition of EGF Receptor Autophosphorylation in Intact HER14 Cells by RG-13022.** RG-13022 inhibited autophosphorylation of the EGF-receptor in HER14 cells after an overnight incubation (Fig. 3a). The IC_{50} of RG-13022 was 5 μM. When a maximally inhibitory concentration of RG-13022 (60 μM) was added to HER14 cells and the incubation was continued for various times, it was found that maximal inhibition of autophosphorylation persisted even up to 48 h (Fig. 3b). This was in contrast to the situation found for RG-50864 (8) for which inhibition was reversed by 48 h.

**Inhibition of HER14 Growth in Culture by RG-13022 and RG-14620.** RG-13022 and RG-14620 inhibited colony formation and DNA synthesis by HER14 cells, which were stimulated by 50 ng/ml EGF, in a dose-dependent manner (Fig. 4). The IC_{50S} for RG-13022 and RG-14620 were 1 and 3 μM for HER14 colony formation and 3 and 1 μM for HER14 DNA synthesis, respectively.

The longer-lasting actions of RG-13022 and RG-14620 on autophosphorylation and growth in HER14 cells in vivo led us to examine these compounds in MH-85 tumors in vivo as well as on MH-85 cells in vitro. MH-85 cells were shown to overexpress endogenous EGF receptors and are dependent on the
EGF receptor signal transduction pathway for their growth both in vitro and in nude mice (17).

Inhibition of MH-85 Growth in Culture by RG-13022 and RG-14620. RG-13022 and RG-14620 also suppressed colony formation and DNA synthesis by EGF-stimulated MH-85 cells (Fig. 5) in a dose-dependent manner. The IC_{50} for RG-13022 and RG-14620 on MH-85 cells were 7 and 4 μM for colony formation and 1.5 and 1.25 μM for DNA synthesis, respectively. The growth-inhibitory effect of RG-13022 and RG-14620 was reversible (data not shown). Growth of Chinese hamster ovary cells (18), which do not express EGF receptors, was not significantly decreased by RG-13022 even at concentrations as high as 40 μM (data not shown).

Inhibition of MH-85 Growth in Nude Mice by RG-13022 and RG-14620. Nude mice implanted with MH-85 tumors manifested profound hypercalcemia, cachexia, and leukocytosis as the tumor grew (Fig. 6, right; Ref. 10). RG-13022 (400 μg/mouse/day) significantly inhibited MH-85 tumor growth (Fig. 7). Because of slower tumor growth, MH-85 tumor-bearing animals receiving injections of RG-13022 showed less cachexia and hypercalcemia (Fig. 6, left), ate more food, and were more active than untreated MH-85 tumor-bearing animals. As a result of suppression of tumor growth, RG-13022 prolonged the life span of MH-85 tumor-bearing animals (Fig. 8). Administration of RG-13022 at a dose of 100 μg/mouse/day did not show any effect on MH-85 tumor growth (Fig. 9). A newer compound, RG-14620, at a dose of 200 μg/mouse/day inhibited MH-85 tumor growth in nude mice to the same degree as that caused by 400 μg RG-13022 (Table 1).

Effect of RG-13022 and mAb108 Combination on MH-85 Growth in Nude Mice. We previously showed that MH-85 growth in nude mice was markedly suppressed by anti-EGF receptor mAb108 (17). Since mAb108 and tyrphostins act at different steps in the signaling pathway, we thought it possible that the effects of RG-13022 may be enhanced if used in conjunction with mAb108. Administration of mAb108 i.p. (1 mg/mouse/day) at 1, 5, and 10 days after MH-85 inoculation of RG-13022 (400 μg/mouse/day) for 14 days profoundly inhibited MH-85 growth in nude mice (Fig. 9). Lower concentrations of mAb108 (10 μg) or RG-13022 (100 μg) failed to decrease MH-85 growth. However, combined administration of these lower concentrations of mAb108 and RG-13022 synergistically decreased MH-85 growth (Fig. 9).
TYROSINE KINASE INHIBITOR ON HUMAN SQUAMOUS CANCER GROWTH

Colony Formation

Fig. 5. Effect of RG-13022 (○) or RG-14620 (△) on colony formation (A) and DNA synthesis (B) by MH-85 cells. Cells (100/well, 24 wells in A and 1 × 10^4/well, 96 wells in B) were treated with RG-13022 or RG-14620 in the presence of 50 ng/ml EGF for 10 days (A) and 48 h (B). Colony numbers and [3H]dThd uptake by EGF-stimulated MH-85 cells which were not treated with RG-13022 and RG-14620 were 48/well and 18,322 dpm/well, respectively. Assays were carried out in duplicate. The same results were obtained in several separate experiments. Ordinate, percentage of untreated cultures.

DISCUSSION

In this paper, we have shown that the tyrosine kinase inhibitors RG-13022 and RG-14620 inhibit tumor growth both in vitro and in vivo. These compounds were found to inhibit EGF receptor autophosphorylation in immunoprecipitates and in intact living cells that overexpress the EGF receptor (HER 14). In contrast to the previously described tyrosine kinase inhibitor RG-50864 (8), these compounds continue to inhibit autophosphorylation in intact cells even after 48 h in cell culture (Fig 3B). RG-13022 in solution undergoes a light-induced trans/cis isomerization. The cis and trans forms of RG-13022 are equally inhibitory, and their biological effect is stable in cell culture for many days. In contrast, RG-14620 is chemically stable. It was this property of the prolonged duration of intracellular effect that suggested RG-13022 and RG-14620 as potential anticancer candidates in vivo. RG-13022 and RG-14620 should be effective in those tumors which contain cells which overexpress the EGF receptor and respond to inhibition of the EGF receptor signaling pathway. We assessed the effects of these agents on the growth of a human squamous cell carcinoma (MH-85) which overexpresses in EGF receptors. In other experiments, we have found that growth of this tumor in vivo can be inhibited by anti-EGF receptor mAb108 (3) and by removal of the submandibular glands which in the adult male mouse are the source of more than 95% of the circulating EGF. Injection of EGF in sialoadenectomized mice leads to enhanced MH-85 tumor growth (17). Thus, growth of the MH-85 tumor in vivo is dependent on the EGF receptor signaling pathway, and this model provides a suitable system for examining the potential effects of tyrphostins. Using this model, we have clearly demonstrated that two of the tyrphostin compounds (RG-13022 and RG-14620) suppress MH-85 tumor cell growth in culture and in nude mice and prolong the survival of tumor-bearing animals.

It is suggested that RG-13022 and RG-14620 are effective when used from the time of tumor inoculation. When the compounds were administered to animals after tumors were already established they failed to cause significant tumor regression. The same result is seen when monoclonal antibodies to human EGF receptors (19) or other chemotherapeutic agents (20) are used in established tumors. These agents all cause cytotoxicity of a fraction of the proliferating tumor cells, and thus the larger the tumor at the time of the administration, the

unpublished observations.
Tyrosine kinase inhibitor on human squamous cancer growth

TVROSINE KINASE INHIBITOR ON HUMAN SQUAMOUS CANCER GROWTH

Fig. 7. Effect of RG-13022 on MH-85 tumor growth in nude mice. Arrows, 200 µg/mouse/injection, 2 injections/day of RG-13022 were administered i.p. from 1 day after MH-85 tumor implantation for 10 days (•). Control animals (○) received equal amounts of vehicle. Points, mean of 3 separate experiments; bars, SE; numbers in parentheses, number of animals studied; *, significantly smaller than control group (P < 0.01).

Fig. 8. Survival of MH-85 tumor-bearing animals treated with (----) or without (- - - -) RG-13022 (200 µg/mouse/injection; 2 injections/day) every day for 5 weeks.

less the absolute effect. This suggests that these agents may be most useful therapeutically if administered in conjunction with a debulking treatment such as surgical ablation. Based on the demonstration that combination of suboptimal doses of mAb108 and RG-13022 synergistically decreased MH-85 growth in nude mice, it is also possible that RG-13022 and RG-14620 will have greater utility when used in conjunction with drugs such as cisplatinum and doxorubicin. These agents have been found to exert profound antitumor effects when used in conjunction with anti-EGF receptor mAb108 in several models of human cancer (13, 21, 22).

The MH-85 tumor is associated with three paraneoplastic syndromes both in the original patient (9) as well as in tumor-bearing nude mice (10). These paraneoplastic syndromes are hypercalcemia, leukocytosis, and cachexia. Each of these syndromes was readily monitored in tumor-bearing mice by following blood ionized calcium, WBC, and body weight. When tumor mass or burden was diminished by treatment with the tyrophostins, we found a simultaneous decrease in the severity of these paraneoplastic syndromes. Other experiments suggest these syndromes are mediated, at least in part, by a tumor factor which stimulates the production of cytokines such as tumor necrosis factor by host immune cells, and these tumor products and host cytokines work in concert to cause the paraneoplastic syndromes (23). However, these syndromes are entirely dependent on the mass of the transplanted tumor, and decreasing tumor mass by use of the tyrosine kinase inhibitors RG-13022 and RG-14620 also leads to abatement of the paraneoplastic syndromes.

Our data suggest that tyrophostins may be useful anticancer drug candidates if they can be shown to have limited or acceptable toxicity. Because of the ubiquitous presence of tyrosine kinases in various normal tissues, the tyrosine kinase inhibitors may be cytotoxic to these tissues as well as to MH-85 tumors. However, in our experiments, growth of Chinese hamster ovary cells which lack endogenous EGF receptors was not suppressed by tyrophostins at concentrations 20 to 30 times higher than the IC_{50} for MH-85, suggesting that tyrophostins might act preferentially on cells with high intrinsic expression of EGF receptor tyrosine kinase. Moreover, MH-85 tumor-bearing nude mice treated with 400 µg/day RG-13022 or with 200 µg/day RG-14620 for 4 weeks were more active, ate more food, and lost less weight than untreated animals. This is partly due to decreased tumor growth, but it at least suggests that RG-13022 and RG-14620 administered according to our protocol are not

Table 1 Effect of tyrophostins on MH-85 growth in nude mice
Tyrophostins (200 µg for RG-13022 or 100 µg for RG-14620/mouse/injection; 2 injections/day) were injected i.p. from the time of tumor inoculation for 4 weeks. Tumor size was measured 5 weeks after tumor inoculation and calculated as described in the text.

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<th>Treatment (µg/mouse/day)</th>
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<th>Tumor size (cm³)</th>
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<td>RG-13022 (400 µg/mouse/day)</td>
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<td>5 ± 3*</td>
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<td>2</td>
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<td>4</td>
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* Significantly different from DMSO-treated group (P < 0.01).
harmful to the host animals. Of course, extensive histological examination of the organs of animals receiving injections of tyrphostins is needed. Although the results described here have demonstrated that tyrphostin inhibits growth of breast cancer cells such as MCF-7 and T47D cells which are known to express which inhibit the tyrosine kinase activity of the EGF receptor phostins inhibit growth of breast cancer cell which is stimulated growth of MH-85, our more recent data have shown that tyrphostins inhibit growth of breast cancer cell which is stimulated by insulin, insulin-like growth factor I and II, and estrogen (25). Thus, antiproliferative effects of tyrphostins are not as specific for cells with overexpression of EGF receptors as we had initially anticipated but do seem to be effective on malignant cells with high intrinsic tyrosine kinase activity. The effects of tyrphostins on other non-EGF receptor types of tyrosine kinase have not been extensively studied yet. In our preliminary experiments, RG-10322 decreased the activity of tyrosine kinase in human osteosarcoma cell line MG-63 which shows the same mobility as that of src tyrosine kinase of the human leukemia cell line HL-60 (26) on a native gel, suggesting tyrphostins act also on non-receptor-type tyrosine kinases such as src.

In conclusion, this work demonstrates that the compounds which inhibit the tyrosine kinase activity of the EGF receptor also inhibit the growth of a human squamous cell carcinoma in nude mice. These results raise the possibility that tyrosine kinase inhibitors may prove to be useful agents for the treatment of a variety of cancers in which tyrosine phosphorylation by oncogene products plays a role in cell transformation.

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