

The Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor ZD1839 Selectively Potentiates Radiation Response of Human Tumors in Nude Mice, with a Marked Improvement in Therapeutic Index¹

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ABSTRACT

Purpose: The epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 (Iressa) markedly potentiates the efficacy of many cytotoxic agents against several human cancer xenografts, irrespective of tumor EGFR expression levels. We subsequently investigated the extent to which ZD1839 might improve radiation therapy (RT) in similar animal models of human cancer within the limits of tolerance at a relevant organ site.

Experimental Design: We carried out studies of ZD1839 in *in vivo* models of human non-small cell lung (A549 and SK-LC-16) and breast (MDA-MB468) cancers and human mesothelioma (JMN). The tumors were implanted s.c. over the rib cage or on the most proximate breast and RT given ventral dorsally to the chest only, with mediastinal protection. After the tumor reached a palpable size (0.4–0.6 mm), treatment was initiated with the maximum-tolerated dose (MTD) of ZD1839 (150 mg/kg once daily × 5 for 2 successive weeks), RT (a total of 40 Gy given fractionally at 4 Gy once daily × 5 for 2 successive weeks), or both ZD1839 and RT.

Results: This level of RT induced no untoward effects in the mice and was effective (18–72%) in bringing about regression of the tumors with a few complete regressions. ZD1839 alone, given p.o. on the same schedule at its MTD (150 mg/kg), was modestly inhibitory (35–40%) to tumor growth. RT and ZD1839 could be given together at the same doses on the same schedule, resulting in marked regression

(50–99%) and a large number of complete regressions of each of the tumors studied. In these studies, the MTD of ZD1839 could be combined with the MTD of RT with no change in schedule or increase toxicity over ZD1839 or RT alone.

Conclusions: ZD1839 significantly enhanced the antitumor action of RT against the test tumors without significant adverse effects, increasing the therapeutic selectivity of ionizing radiation in these model systems. These results predict substantial benefits for this multimodality regimen of therapy in patients.

INTRODUCTION

In patients with many neoplastic disorders, RT³ given primarily or adjuvantly is an important treatment intervention (1). However, in view of the variable success rates of this therapeutic modality, attempts at improving its efficacy are warranted. Growth factor receptors and their ligands play a major role in the regulation of tumor growth, differentiation, and apoptosis (2, 3). Agents such as monoclonal antibodies (4–6) or small molecule inhibitors of the associated TK (7–10), which block the function of these receptors, particularly HER2/neu and EGFR, can have significant antitumor actions against human tumors both *in vitro* and *in vivo*. Significant therapeutic effects of these agents have also been documented in patients (11–14). These inhibitors of growth factor receptor function can also markedly potentiate the efficacy of cytotoxic agents against human tumors in animal models (15–17). ZD1839 (Iressa) is a p.o. active, selective EGFR-TK inhibitor that blocks signal transduction pathways implicated in proliferation and survival of cancer cells and other host-dependent processes promoting cancer growth. ZD1839 produced very marked enhancement of antitumor activity against various human tumor models when combined with cytotoxic drugs such as platinum, taxanes, doxorubicin, and a folate analogue (18, 19).

As some of the cytotoxic agents given with either anti-EGFR antibodies or EGFR-TK inhibitors directly damage tumor-cell DNA, it is of interest to determine the extent to which concurrent treatment with anti-EGFR agents might enhance the response of tumors to ionizing radiation. To this end, other workers have addressed this issue in the context of various experimental systems. Studies carried out with human squamous

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³The abbreviations used are: RT, radiation therapy; TK, tyrosine kinase; EGFR, epidermal growth factor receptor; EGFR-TK1, EGFR-tyrosine kinase inhibitor, qd, once daily; MTD, maximum-tolerated dose; RT-PCR, reverse transcription-PCR; NSCLC, non-small cell lung cancer.

cell carcinoma cells in culture documented (20) significant enhancement by C225 anti-EGFR monoclonal antibodies of radiation-induced apoptosis and cell kill. This same anti-EGFR antibody was also found (21, 22) to enhance the antitumor and antiangiogenesis effects of RT against these squamous cell carcinomas and the human A431 vulvar tumor xenografted in mice. Very recent studies also showed (23, 24) that ZD1839 will enhance RT-induced apoptosis and cell kill in a variety of human tumor cells in culture. This EGFR-TK inhibitor also markedly potentiated (23, 24) the antitumor and antiangiogenesis effects of RT against squamous cell and colon carcinoma xenografts in mice. Overall, these studies suggested that concurrent inhibition of EGFR function was a potentially useful approach to improving anticancer treatment with RT.

Here, we describe studies with animal models of human NSCLC and breast cancer and human mesothelioma examining the effect of ZD1839 administered daily with fractionated RT. These studies differ in respect to prior (23, 24) studies of RT with ZD1839. RT was fractionated over 10 doses given for 5 days on 2 successive weeks with and without ZD1839. Also, a comparison of the effect of RT with ZD1839 *versus* ZD1839 or RT alone was made at the MTD for each agent and the combination. More importantly, by ventrally implanting the tumors s.c. over the chest cavity, we are able to determine the effect of ventral dorsally directed RT with mediastinal protection on the lungs. This assessment of potential toxicity at a highly relevant organ site along with effects on the tumor has meaningful clinical implications in that it showed that ZD1839 could be given at its MTD with an MTD of RT with no change in schedule necessary and no increase in toxicity over ZD1839 or RT alone.

MATERIALS AND METHODS

Animal Studies. We carried out our studies of ZD1839 in *in vivo* models of human non-small cell lung (A549 and SK-LC-16) and breast (MDA-MB468) cancers and human mesothelioma (JMN). The tumors used for these studies were obtained from the American Type Culture Collection (A431, A549, and SK-LC-16) or from Dr. Brenda Gerwin (JMN) in the laboratory of Dr. Curtis Harris at the National Cancer Institute (Bethesda, MD). These human tumors were maintained by s.c. transplantation in athymic NCR nu mice. After tumor growth, a cell suspension in RPMI with 10% FCS was prepared, centrifuged, and the pellet resuspended in one-tenth the volume for implantation into animals. When the tumor reached 0.4–0.6 cm in size, the animals were randomized into control and treatment groups. Preliminary toxicology experiments determined the MTD for tolerance of ZD1839 (19) and RT (see below) on the schedule of administration used: once daily for 5 of 7 days ($qd \times 5$) in 2 successive weeks. Mice received ZD1839 via oral gavage and were irradiated, on the same schedule, ventral dorsally to the chest area only with mediastinal protection. Before irradiation, the animals were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) injected i.p. and placed in a supine position in a specialized Lucite jig. The plastic jig was radially divided into 10 compartments in which the anesthetized mice were fixed in a restraining position. A specially designed cerrobend shield was fabricated to permit

Table 1 Relative expression of EGFR and HER2/neu genes in human tumors xenografted to nude mice

Gene expression relative to β -actin gene expression was determined by quantitative real-time RT-PCR. Experimental details are provided in the text. The data shown are means of three replicate determinations done on separate days with standard error of the mean also given.

Tumor	Tumor type	Relative gene expression (SE) ^a	
		EGFR	HER2/neu
A431	Vulval	11.4 ± 1.3	0.9 ± 0.12
SK-LC-16	NSCLC	0.9 ± 0.1	0.43 ± 0.1
A549	NSCLC	0.3 ± 0.06	1.3 ± 0.3
JMN	Mesothelioma	0.22 ± 0.06	0.01 ± 0.003
MX-1	Breast	0.08 ± 0.01	3.76 ± 0.6

^a RT-PCR was initiated with total cellular RNA and amplification carried out after conversion to cDNA.

bilateral lung exposure (mediastinum protected) while protecting the rest of the body. Simulation radiographs were obtained to confirm the accuracy of this method of beam shaping to assure the inclusion of all of the targeted organs and to confirm that the mediastinum was indeed effectively shielded. Radiation was delivered using a Philips X-ray unit (MGC-20 model), operating at 320 kVp and 10 mA and filtered by 0.5 mm of Cu. The field size was 15 × 15 cm at 50 cm source to surface distance. The dose rate under these conditions was 139 cGy/min. Control (diluent alone) and ZD1839-treated mice were anesthetized for the same period of time. The doses of ZD1839 and RT chosen brought about no lethality and a weight loss of <10% during the period of treatment of the mice. Histopathological examination of the lungs was carried out on all treated mice up to 2 months after implantation of tumor (45 days after treatment) with the aid of a veterinary pathologist. The therapeutic end point selected was change in tumor volume ($\text{mm}^3 = 4/3\pi r^3$) as determined from a measurement made with a digital caliper after the treatment period. For tumors that increased in size, this measurement was made periodically during treatment and for a total of 38–41 days after treatment. For regressing tumors, the same measurements were periodically made to determine the nadir occurring after treatment cessation. Because treatment with RT or RT with ZD1839 brought about regression (see below) of all of the test tumors and complete regression of some of these tumors, an estimation of therapeutic efficacy of RT with ZD1839 *versus* RT alone was also obtained from a measure of tumor regrowth (or lack thereof) in each group during a period of 38–41 days after treatment. Statistical analysis was carried out using the Student's test. Working solutions of ZD1839 were prepared as a lactate salt (pH 5.5). This solution was held for no longer than 2 weeks at 4°C. These studies were carried out in accordance with "Principles of Laboratory Animal Care." ZD1839 was provided by AstraZeneca.

Quantitative RT-PCR. Tumor samples were harvested from mice and total RNA prepared with TRIzol reagent (Life Technologies, Inc.) from each tumor. First-strand cDNA was prepared using the Superscript system (Life Technologies, Inc.). The quantitation of EGFR and HER2/neu gene expression was carried out by real-time RT-PCR, with the aid of the ABI Prism 7700 Sequence Detection system (Taqman; PE Systems, Foster

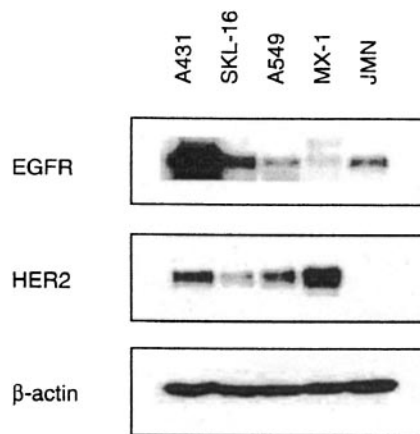


Fig. 1 Western blot analysis of EGFR and Her 2/neu in cell lysates derived from human non-small cell lung and breast tumors and human mesothelioma grown as xenografts in nude mice. The blotting procedure, source of antibodies, and other experimental details are provided in the text. The figure represents the results of several typical plots.

City, CA). The methodology has been previously described in detail (25, 26). The specific cDNAs of interest and the β -actin reference cDNA were amplified separately with the Taqman, using an oligonucleotide probe with a 5' fluorescent reporter dye (6-carboxyfluorescein) and the 3' quencher dye (6-carboxytetramethylrhodamine). The primer and probe sets were designed using Primer Express software (Applied Biosystems, Foster City, CA) to ensure that all sustained amplification with the same efficiency. Two control experiments (25, 26) were performed on these primers and probes to confirm that this was in fact the case. The sequences used are as follows: EGFR forward primer, 5'-GCGTCTCTTGCCGGAATGT-3', reverse primer, 5'-CTTGCTCACCTCCAGAAG-3', and probe: 6FAM-TGCACTTGTCACGCATTCCCTG-TAMRA; HER2/neu forward primer, 5'-AAGCCTCACAGAGATCTTGAAAGG-3', reverse primer, 5'-TCCACAAAATCGTGTCTGCTGTA-3', and probe, 6FAM-TTGATCCAGCGGAACCCCAAGC-TAMRA; and β -actin forward primer, 5'-CTGGCACCCAGACAATG-3', reverse primer, 5'-GCCGATCCACACGGAGACT-3', and probe, 6FAM-TCAAGATCATTGCTCTCCTGAGCGC-TAMRA.

Western Blotting. Western blots were performed by a standard procedure (27) using aliquots of cell lysate (60 μ g), which were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters. Protein concentration was determined with a prepared kit (Pierce, Rockford, IL). The membranes were incubated for 1 h at room temperature with antibodies against EGFR and HER2/neu (Kamiyama Biochemical, Seattle, WA) and washed before incubating with secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The protein blot was detected by enhanced chemiluminescence (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom).

RESULTS AND DISCUSSION

Expression of EGFR and HER2/neu Genes. The relative expression of EGFR and HER2/neu genes in the two

Table 2 Tolerances of NCR- ν mice exposed to various doses of radiation

The animals were irradiated ventral dorsally to the chest area with mediastinal protection. Each dose was given fractionated at 4 Gy/day \times 5 for a total of 10 days on 2 consecutive weeks. The mice were observed for a follow-up period of 2 months. Histopathology was performed on the lungs of mice dying from radiation.

Radiation dose Gy \times 10	Weight change (%)	Surviving mice (No./total)
0	-2	5/5
2	-2	5/5
4	-6	5/5
5	-14	5/5
6	-19	4/5
8	-28	3/5

NSCLC (A549 and SK-LC-16) tumors, the MX-1 breast tumors, and the JMN mesothelioma obtained from mice was determined by RT-PCR. Compared with the highly EGFR-expressing A431 cells, also obtained from mice, the relative expression of EGFR was found to be modest and variable (Table 1). Relative expression was in the order A431 \gg A549 = SK-LC-16 $>$ JMN $>$ MX-1. Because HER2/neu also appears to be targeted by ZD1839 (23, 24), we also determined the level of expression of this gene in these tumors. In this case, the relative level of expression was in the order MX-1 $>$ A549 = A431 $>$ SK-LC-16 \gg JMN. Western blotting of cell lysate from these same tumors was carried out to delineate EGFR and HER2/neu gene expression at the level of the cognate protein. These analyses (Fig. 1) document differences in the level of these proteins that was approximately commensurate with the relative levels of gene expression found by RT-PCR.

Tolerance of NCR- ν Mice to RT. Using the schedule of administration (qd \times 5 for 2 successive weeks) planned for the therapy experiments (see below), mice were treated, under anesthesia, with different doses of RT to the chest with mediastinal protection and held for a period of 2 months. Control mice and mice receiving ZD1839 were anesthetized in the same way. Tolerance was assessed using weight loss and lethality as end points. These studies identified the MTD as 40 Gy given fractionally (4 Gy qd \times 5 \times 2 weeks) on this schedule (Table 2). Mice receiving 1, 2, and 4 Gy experienced minimal weight loss, and no evidence of lung injury was manifest when histopathology examination was carried out on these mice. Higher doses resulted in significant weight loss after the first week of treatment, and at the highest doses, deaths occurred within 10 days after the end of treatment because of lung toxicity. Surviving mice in these treated groups also showed evidence of lung injuring at the termination of the experiment. The MTD of 40 Gy on this schedule of RT was used routinely in the studies described below.

Studies of RT and ZD1839 in Tumor-bearing Mice. The MTD of ZD1839 (150 mg/kg) in NCR- ν mice on this schedule was determined in a prior study from the authors' laboratory (19). This dosage and schedule were also found to have no untoward effects in the present study. The data in Table 3 show that RT at its MTD on a schedule of qd \times 5 for 2 successive weeks could be administered with an MTD of

Table 3 Antitumor activity of ZD1839 and RT given alone or together against human xenografts in nude mice

Established tumors (0.4–0.6 cm) were treated on a schedule of qd \times 5 for 2 successive weeks for both therapies (150 mg/kg ZD1839, 4 Gy of RT or both). Tumor volume was measured 1–2 days after treatment or, in the case of regression, at the nadir in tumor size. The data are the mean of three to four experiments with group size between experiments varying from 4–6 mice/group. SE is shown. Additional details are given in the text.

Tumor	Treatment		Weight change (%)	Avg. tumor diameter ^a (mm \pm SE)	Change in volume (mm ³)	Complete regressions (no./total)	No tumor regrowth ^b (no./total)	P ^c
	ZD1839	RT						
A549	–	–	–3	9.5 \pm 0.9	+450	0/19	0/19	
	+	–	–4	7.6 \pm 1.1	+230	0/19	0/19	
	–	+	–5	3.8 \pm 0.5	–17 (–31%)	0/19	0/19	
	+	+	–5	2.6 \pm 0.3	–49 (–89%)	5/19	2/19	
SK-LC-16	–	–	–2	13.6 \pm 1.5	+1246	0/16	0/16	
	+	–	–2	11.9 \pm 1.9	+837	0/16	0/16	
	–	+	–7	4.3 \pm 0.7	–11 (–18%)	0/16	0/16	
	+	+	–7	2.4 \pm 0.4	–48 (–77%)	5/16	2/16	
JMN	–	–	–3	13.2 \pm 1.3	+1207	0/22	0/22	
	+	–	–2	11.4 \pm 1.5	+758	0/22	0/22	
	–	+	–5	2.4 \pm 0.3	–21 (–50%)	4/22	1/22	
	+	+	–6	<1	–43 (–98%)	15/22	12/22	
MX-1	–	–	–1	13.5 \pm 1.8	+1183	0/12	0/12	
	+	–	–2	11.8 \pm 1.5	+790	0/12	0/12	
	–	+	–7	3.7 \pm 0.5	–74 (–72%)	2/12	0/12	
	+	+	–5	<1	–99 (–96%)	10/12	9/12	

^a Tumor diameter (volume) at the time treatment was initiated was 4.7 \pm 0.6 mm (54 mm³) for A549, 4.9 \pm 0.9 mm (61.6 mm³) for SK-LC-16, 4.3 \pm 0.8 mm (41.6 mm³) for JMN and 5.95 \pm 1.1 (103 mm³) for MX-1.

^b No regrowth of tumor 37–39 days after cessation of treatment.

^c The data was analyzed by the SAS 8.1 program using Student's test.

ZD1839 on the same schedule, without any increase in toxicity over RT or ZD1839 alone at their respective MTD values. Combined RT and ZD1839 at their individual MTDs resulted in no lethality, and weight loss was similar to that observed with RT alone (Table 3). Histopathological examination of mice up to 41 days after treatment with RT or RT with ZD1839 showed no evidence of lung toxicity.

Other data, given in Table 3 and Fig. 2, document a substantial increase in the antitumor action of combined RT and ZD1839 compared with either agent alone. RT alone at its MTD resulted (Table 3) in tumor regressions of 31, 18, 50, and 72% against A549, SK-LC-16, JMN, and MX-1 tumors, respectively, and there was a substantial delay (Fig. 2) in the regrowth of these tumors compared with controls. In addition, RT alone produced (Table 3) 4 of 22 and 2 of 14 complete regressions in mice with JMN and MX-1 tumors, respectively. Against each tumor, ZD1839 alone at its MTD resulted in modest inhibition of tumor growth (Table 3, Fig. 2) and no regression. Against these same tumors, coadministration of ZD1839 with RT significantly increased ($P = 0.0001$ – 0.00033) the regression obtained over that obtained with RT alone. The average regression of the A549, SK-LC-16, JMN, and MX-1 tumors was increased significantly, compared with that obtained with RT alone. Also, against A549, SK-LC-16, JMN, and MX-1 tumors, 5 of 19, 5 of 16, 15 of 22, and 10 of 12 mice, respectively, had complete regressions of their tumors after combination therapy, and many of these mice did not experience regrowth of these tumors for a period of at least 38–41 days after treatment. With the JMN tumor, almost four times more complete regressions were obtained with RT and ZD1839 than with RT alone. Because MX-1 was the most responsive to RT among all four tumors, a much larger initial tumor burden was implanted in mice. Still, the difference in average regression obtained against this tumor

with combined RT and ZD1839 over RT alone was not as appreciable compared with the other tumors. However, animals treated with the combination exhibited a 5-fold increase in complete regressions over RT alone. Also, with RT and ZD1839, most of the tumors that completely regressed did not regrow during a period of observation that extended for 38–41 days after treatment.

In light of the above, average regrowth of A549, SK-LC-16, and JMN tumors in the groups that received the combined treatment was substantially delayed compared with groups that received RT alone. Although the regrowth of the three tumors was measured for a total of only 38–42 days after treatment, the estimation of time of regrowth to an average tumor size at least approximating the initial pretreatment tumor size could be estimated. This time to regrowth was significantly different ($P = 0.001$ – 0.002) from that in the RT-treated groups. Also, on average, no measurable regrowth of MX-1 in the RT plus ZD1839 group was observed during this same period of time. The delay in regrowth of all four tumors in this group treated with RT and ZD1839 clearly reflects the overall degree of regression and the large fraction of complete regressions that were observed in each case. Moreover, given the very modest effect of ZD1839 on the growth of each tumor, it is also clear from the data (Fig. 2) that the time to regrowth of tumor compared with that of the RT-treated group was greater than that expected from an additive effect of ZD1839 and RT alone. Examination of mice treated with RT or RT plus ZD1839 at the end of the experiment showed no signs of lung injury.

It was of interest to further examine the effect of ZD1839 on the response to RT in these studies. An experiment was carried out in which we measured the average regression of tumor in A549 implanted mice when ZD1839 was combined with different doses of RT on the same schedule (qd \times 5 for 2

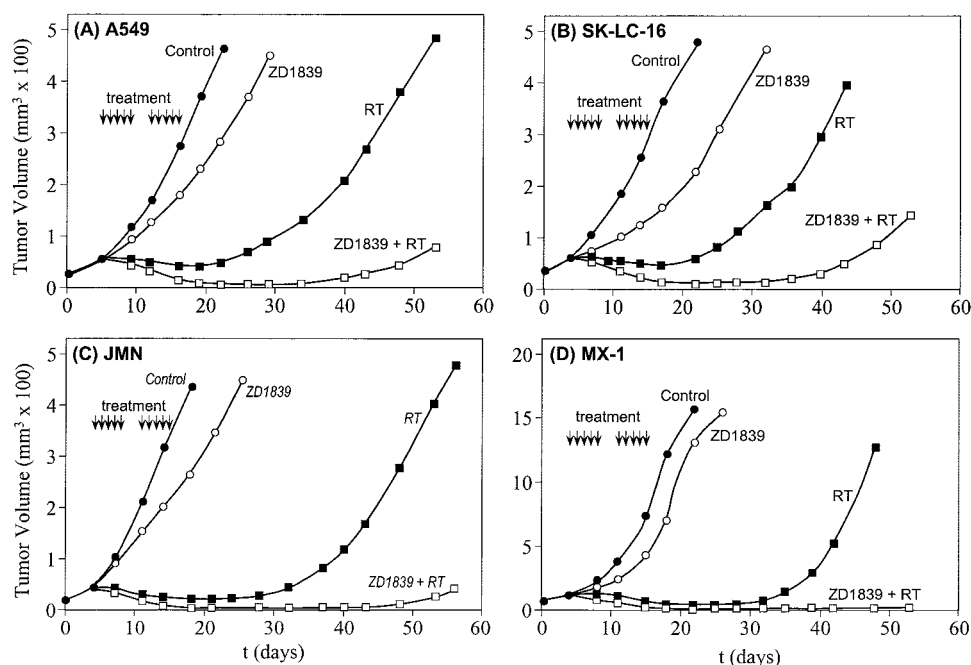


Fig. 2 Antitumor activity of ZD1839 and RT given alone or together against the A549, SL-LC-16, JMN, and MX-1 tumors xenografted to nude mice. Treatment was initiated in mice with established tumors (0.4–0.6 cm) on a schedule of qd \times 5 on 2 successive weeks. Tumor measurements were made periodically for a period of 49–52 days after initiation of treatment. The data represent the mean of three to four experiments (group size varied from 4–6 mice/group between experiments) with a SE of <14% (A549), <16% (SL-LC-16), <15% (JMN), and <14% (MX-1). A comparison of tumor volume after regrowth of A549 (A), SL-LC-16 (B), and JMN (C) to the approximate initial tumor volume at the start of treatment showed a statistically significant difference ($P = 0.001$ – 0.0015) between time of regrowth of tumor in the RT and RT plus ZD1839 groups. Assuming regrowth of MX-1 (D) to the approximate initial tumor volume at the initiation of treatment occurs no earlier than 56 days after transplant in the RT plus ZD1839 group, the difference in the estimated time of regrowth of these tumors compared with tumors in the RT group is statistically significant with a value for $P = 0.0012$.

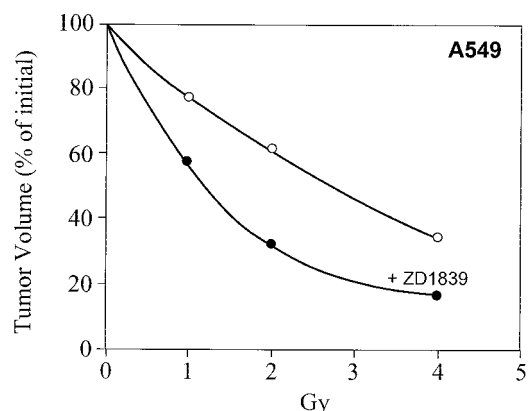


Fig. 3 Antitumor activity of RT with and without ZD1839 against the A549 tumor xenografted to nude mice. Treatment was initiated in mice (4 mice/group) with established tumor (0.5 ± 0.5 cm, 65 mm³). The regression of these tumors in each treatment group was measured at the nadir and the data in the figure expressed as average percent of initial tumor volume. The comparisons between RT and RT plus ZD1839 at each dose of RT were statistically significant ($P = 0.02$ – 0.001).

successive weeks). The results in Fig. 3 show that 150 mg/kg ZD1839 significantly increased the regression of this tumor treated with 1, 2, or 4 Gy RT on this schedule. It was also shown that RT at 2 Gy given with ZD1839 was as effective as RT alone

given at 4 Gy, thus, ZD1839 has a substantial sparing effect when combined with RT.

The model system chosen for these studies was devised to allow for a therapeutic evaluation of RT with and without ZD1839 within the context of host tolerance. RT treatment in patients with localized NSCLC, breast cancer, or mesothelioma presents great risk to the lungs. Therefore, the site of implantation of test tumors, although not orthotopic, was selected to take this potential organ-site toxicity into consideration. As a result, it seems clear that combined ZD1839 with RT was tolerated, as well as RT alone at least within these model systems. This was shown by the fact that ZD1839 could be given with RT with no modification of either dosage or schedule required to avoid toxicity.

The results also show that ZD1839 displayed a significant and consistent ability to increase responsiveness of the test tumors to RT. The consequence of this was marked enhancement in the therapeutic index for RT in this experimental setting. This was strikingly reflected in the overall increase in regression of the test tumors and the large increase in complete regressions observed with ZD1839 and RT together, compared with either agent alone. The data showing a significantly large increase in delay of tumor regrowth in the RT plus ZD1839 versus RT group are consistent with this conclusion. Of note, substantial enhancement of RT by ZD1839 did not depend on high levels of expression of EGFR in test tumors. As such, these results are similar to the results of our prior studies with some of these

same tumors, which showed that there was no requirement for high EGFR expression for ZD1839 to enhance cytotoxic therapy (19). The activation of EGFR and the consequent effect on downstream signaling can occur (28, 29) through its heterodimerization with HER2/neu. Therefore, relative expression of both EGFR and HER2/neu may impact on both proliferation and cell survival of target tumors. However, the extent to which the above results reflect the coexpression of HER2/neu in these tumors or other factors is not known (28, 29). Because, as with EGFR, the expression of this gene is also highly variable among these tumors. It is clear that additional molecular studies, similar to those already carried out by others (20–24, 28, 29) but focusing on this question, will need to be conducted in a suitable cell-culture system. In the meantime, the results of these studies appear to bode well for the combined use of ZD1839 with RT in a clinical setting.

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