Effects of the farnesyl transferase inhibitor R115777 (Zarnestra) on mammary carcinogenesis: prevention, therapy, and role of HaRas mutations

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Abstract
The ability of the farnesyl transferase inhibitor R115777 to act as a cancer therapeutic/preventive agent and to modulate proliferation/apoptosis markers was determined in the methylnitrosourea-induced model of mammary carcinogenesis. Female Sprague-Dawley rats were given methylnitrosourea at 50 days of age. In the prevention study, R115777 (5, 16, or 50 mg/kg body weight/d), beginning 5 days after methylnitrosourea treatment, decreased the formation of mammary cancers by 6%, 42%, and 75%, respectively. Approximately 50% of the mammary cancers that developed had HaRas mutations. Only 1 of 15 tumors that grew out in the presence of R115777 (16 or 50 mg/kg body weight/d) had a HaRas mutation. In the therapeutic study, a surgical biopsy of a mammary cancer was done to determine HaRas status, and growth of the cancer was then followed during treatment of the rat with R115777. Virtually every cancer with a HaRas mutation underwent complete regression within 3 weeks, whereas tumors without a HaRas mutation had variable responses to the inhibitor. Both of these studies implied a high sensitivity of tumors with HaRas mutations to the effects of R115777. In order to understand the preferential susceptibility of tumors with HaRas mutations, rats with a palpable cancer were treated with R115777 for a period of 36 or 96 hours prior to sacrifice, and the proliferation and apoptosis levels in the cancers were determined. The proliferative index was significantly (>85%) decreased in all mammary cancers with HaRas mutations, whereas variable responses were observed in cancers without HaRas mutations. Apoptosis was also measured and a 5-fold increase was observed in HaRas mutant tumors, again with varying responses in the HaRas wild-type cancers. Thus, R115777 was active in the prevention and therapy of these chemically induced mammary cancers, but was strikingly more effective in cancers with HaRas mutations. [Mol Cancer Ther 2006;5(4):1073–8]

Introduction
The Ras GTPase genes are involved in signaling a wide range of growth factors (1). Apparently reflecting their central role in regulating cell proliferation, Ras is one of the most commonly mutated human oncogenes (2). This family of genes is mutated in a large percentage of two major lethal cancers in humans (lung and colon), and seems to be mutated in virtually every pancreatic cancer; implying that its mutation may be mandatory for this form of cancer. The major role of Ras mutations in the field of carcinogenesis was initially observed >35 years ago and was based on the finding that two of the earliest transforming viruses (Harvey sarcoma virus and Kirsten sarcoma virus) had mutations in the Ras family of genes (3, 4). The proteins coded by the Ras oncogene must be prenylated in order to be transported to the cell membrane and then activated (4). Because of the significant role of the Ras proteins in oncogenesis and the requirement for their prenylation, inhibition of this process has become a major target for pharmacologic intervention. R115777 is an orally available farnesyl transferase inhibitor (FTI) that blocks farnesylation of a wide variety of proteins including HaRas and N-Ras (5). Like most of the FTIs, it takes higher doses of R115777 to block the farnesylation of KiRas. However, although R115777 is relatively ineffective in blocking farnesylation of KiRas, it has shown efficacy in xenografts against cells with KiRas mutations and no Ras mutations (5).

Chemically induced models of mammary carcinogenesis were initially developed by Huggins (6). Female Sprague-Dawley rats treated with methylnitrosourea developed multiple hormonally responsive mammary cancers starting within 5 weeks after giving the carcinogen at 50 days of age (7). In this model, 50% of the resulting tumors had mutations in codon 12 of HaRas (8, 9). In contrast to this high frequency in the rat models of mammary cancer, mutations in this gene are infrequently observed in human...
breast cancers (1). However, increased expression of Ras itself or of mitogen-activated protein kinase (one of its downstream effector elements) is overexpressed in 50% of breast cancers (10). Also, overexpression of upstream elements (e.g., Neu and EGFr) are relatively common in human breast cancer (11, 12).

Transgenic models overexpressing mutated Ras oncogenes have been previously employed to show the efficacy of the FTIs (13, 14). In the present studies, we have employed the methylnitrosourea-induced model of mammary carcinogenesis. Certain advantages of the methylnitrosourea-induced mammary cancer model include: (a) cancers arise in situ, (b) ∼50% of the cancers have mutations in HaRas, and (c) the cancers and mutated Ras genes are focal in nature. This model is different from studies with most transgenic mice in which virtually all the cells in the mammary gland will express the mutated Ras oncogene at high levels (13, 14). The preventive and therapeutic effects of R115777 in the methylnitrosourea model were employed as the primary end points. In addition, the effects of R115777 on proliferation and apoptosis were examined in palpable tumors following short-term treatment (36–96 hours). The use of these two biomarkers were based on several considerations: (a) alterations in proliferation and apoptosis are intimately involved in the carcinogenic process as well as the therapeutic effects of most agents, (b) the use of these specific measures has more direct clinical applicability than many other potential end points, and (c) we have previously shown that these variables are modulated by a variety of agents in this specific model system (15, 16).

In the present studies, the therapeutic and preventive effects of FTI in the methylnitrosourea mammary cancer model were evaluated to address the following questions: (a) is R115777 effective in this model system as both a preventive and therapeutic agent, (b) is the efficacy of R115777 more striking in cancers with mutations in HaRas, and (c) could one predict the long-term efficacy of the FTI on a cancer by employing cell proliferation and apoptosis as biomarkers after short-term treatment with the agent?

Materials and Methods

Supplies

Chemicals and other materials were obtained as follows: ethanol, trioctanoin, and corn oil were from Sigma Chemical Co. (St. Louis, MO); methylnitrosourea was from Ash-Stevens, Inc. (Detroit, MI); R115777 was a gift from Johnson & Johnson Pharmaceutical (Spring House, PA); Teklad mash (4%) diet and Sprague-Dawley rats were from Harlan Sprague-Dawley, Inc. (Indianapolis, IN).

Dispensing Methylnitrosourea and R115777

Female Sprague-Dawley rats were obtained at 28 days of age and housed in polycarbonate cages (five rats/cage). At 50 days of age, the rats received one i.v. injection of methylnitrosourea (50 mg/kg body weight) via the jugular vein. R115777 was given as a suspension by gavage. The vehicle for R115777 was 40% β-cyclodextrin/0.1 N HCl (50:50, v/v). The rats received 0.5 mL/d, 6×/wk.

Determination of HaRas Mutation Status in Mammary Cancers

Mutations in the mammary cancers were determined by isolating DNA from the tumors and sequencing by employing the previously described methods of Lubet et al. (8).

Chemoprevention Studies

The initiation of treatment with R115777 was begun 5 days after giving methylnitrosourea to the rats. The groups (n = 15/group) included: group 1, R115777 (50 mg/kg body weight/d); group 2, R115777 (16 mg/kg body weight/d); group 3, R115777 (5 mg/kg body weight/d); and group 4, vehicle. Rats were palpated for mammary cancers twice each week and weighed once a week. Mammary tumors were excised, weighed, and processed for histologic classification at the end of the study (150 days after giving methylnitrosourea). Statistical analyses of cancer incidence and latency were determined using log-rank analysis and differences in cancer multiplicity were determined by the Armitage test (17). At the end of the study, mammary cancers in the various groups were frozen in liquid nitrogen so that HaRas mutation status could be determined.

Treatment of Palpable Cancers

Rats were treated with methylnitrosourea and palpated for mammary cancers as described above. When a rat developed a mammary cancer measuring ∼200 mm², the tumor was surgically biopsied (collecting a 75–125 mg tissue sample). The skin above the tumor was closed with metal wound clips, and the clips were removed after 7 days. The tumor biopsy was frozen in liquid nitrogen, stored at −85°C, and used to determine HaRas mutation status. Rats bearing the biopsied cancers were then treated with R115777 (50 mg/kg body weight/d) by gavage beginning 5 days later. Tumor size was measured twice a week with calipers for a period of 28 days. The largest diameter of the cancer was measured and this value was multiplied by the perpendicular diameter (size was expressed in square millimeters). Each cancer was measured for 28 days to determine its growth pattern. The resulting growth rates for HaRas-positive tumors and HaRas-negative tumors were also compared with the Wilcoxon rank test.

Effects of R115777 on Proliferation and Apoptosis in Cancers

Animals bearing small palpable mammary cancers (∼100–150 mm²) were treated with R115777 (50 mg/kg body weight/d) for a period of 36 or 96 hours. Two time periods were evaluated because the optimal time for examining antiproliferative effects was not known. Rats were injected i.p. with bromodeoxyuridine (BrdUrd; 100 mg/kg body weight) in saline 2 hours prior to sacrifice. At necropsy, tumors were divided into two portions. Approximately one-half of the cancer was fixed overnight in 10%
neutral formalin for histologic classification and for measuring proliferation index and apoptotic index, whereas the remaining portion was snap-frozen and employed in RNA studies (presented in a separate article).5

**Cell Proliferation.** The nuclei of proliferating cells labeled with BrdUrd were identified employing an anti-BrdUrd monoclonal antibody (Becton Dickinson, Palo Alto, CA) and ABC kit (16, 17). More than 1,000 cells were randomly scored from each cancer and the percentage of BrdUrd-labeled cells was evaluated in tumor areas distant from any necrosis.

**Cell Apoptosis.** Apoptotic cells were identified by the terminal nucleotidyl transferase–mediated nick end labeling method as previously described (16), employing methods recommended by the ApoTag in situ hybridization detection kit (Oncor, Co., Gaithersburg, MD). The top sections of each slide, which were incubated without digoxigenin-dUTP, were used as a negative control. As positive controls, mammary glands of rats were used 6 days after ovariectomy (when the number of apoptotic cells was high). Tissue sections were counterstained by methyl green for visualization of tumor morphology. From each tumor, >1,500 cells were evaluated for the presence of apoptotic cells. Statistical analysis of proliferation and apoptosis were done as previously described (15, 16).

**Results**

**Efficacy of R115777 in the Prevention of Mammary Cancers**

Beginning 5 days after methylnitrosourea injection, rats received R115777 six times a week for the remainder of the study. There were no effects of FTI on body weight gain of the rats at any of the doses tested. Also, there were no clinical signs of toxicity. In this study, rats treated with methylnitrosourea only developed 3.5 mammary cancers/rat with an average tumor weight of 2.76 g. R115777 at dose levels of 50, 16, and 5 mg/kg body weight/d reduced mammary cancer multiplicity by 75% \((P < 0.01)\), 42% \((P < 0.05)\), and 6% \((P > 0.05)\), respectively (Fig. 1). Mammary cancer weights of rats receiving the high dose of R115777 were reduced by 92%; supporting the high efficacy of R115777 observed in this model system. Determination of HaRas mutations in the cancers that grew out showed that only 1 of 15 (7%) tumors at the 50 and 16 mg/kg body weight/d doses, and 6 of 15 tumors (40%) at the 5 mg/kg body weight/d dose level had a HaRas mutation. HaRas mutations were observed in 12 of 25 (48%) control mammary cancers. The latter is consistent with the 50% incidence of HaRas mutations routinely observed in mammary cancers of rats treated with methylnitrosourea only.

**Therapeutic Effects of R115777 following Biopsy of Mammary Cancers to Verify HaRas Mutation Status**

In this experiment, animals were selected with mammary cancers \(~200 \text{ mm}^2\) in size. A biopsy of the tumor was taken and 5 days later, the rat received R115777 for a period of 28 days. By monitoring tumor size, it was observed overall that 50% of the cancers displayed rapid regression, whereas the remaining tumors showed minimal growth or partial regression. After evaluating the biopsies for HaRas mutation status, it was found that six of seven cancers which were HaRas-positive (Fig. 2A) rapidly underwent complete regressions, and the remaining tumor showed a partial regression. Among the HaRas-negative tumors (i.e., no mutations), only four of eight underwent a regression of >75% (Fig. 2B). An additional two tumors regressed by 35% to 75%, and two tumors displayed an increase in tumor size. When the effects of R115777 on the growth of the HaRas-positive and -negative mammary cancers were compared, a statistically significant \((P < 0.05)\) difference was observed (Wilcoxon rank analysis).

**Effects of R115777 on Proliferation and Apoptosis in Mammary Cancers**

Animals bearing palpable mammary cancers were treated with R115777 (50 mg/kg body weight/d) for a limited length of time (36 or 96 hours). Two hours prior to sacrifice, the rats were injected with BrdUrd. Tumors were initially examined for proliferation (BrdUrd staining) and apoptosis (terminal nucleotidyl transferase–mediated nick end labeling). Subsequently, these tumors were sequenced.
for the presence of HaRas mutations. As shown in cancers from control rats (Fig. 3A), proliferation was slightly higher in cancers with HaRas mutation (proliferation index = 13.8 ± 3.9) than in tumors without a HaRas mutation (proliferation index = 11.8 ± 2.8; P > 0.05). Following treatment with R115777 (36 or 96 hours), tumors with HaRas mutations had an 85% decrease in proliferation from controls (P < 0.01). In contrast, the response in tumors without a HaRas mutation was more variable with half of the tumors showing a 66% decrease in proliferation and the remainder showing only limited decreases in proliferation (P > 0.05). However, when the results at 36 and 48 hours were combined, they were statistically significant (P < 0.05). When apoptotic effects were examined in these cancers (Fig. 3B), a 5-fold increase in apoptotic index (control, 0.61 ± 0.13; R115777, 3.07 ± 0.63; P < 0.01) was observed at 36 hours in R115777-treated rats with HaRas-mutated tumors, whereas in the cancers with no HaRas mutations, the overall increase was ~3-fold (control, 0.55 ± 0.18; R115777, 1.72 ± 0.83; P > 0.05). Similar results were seen at the 96-hour time point.

Discussion

As mentioned in the Introduction, Ras is a major component of the signaling pathway in cells and is a focal point for multiple growth factors including Neu, EGFr, etc. (1, 18). The significance of the Ras pathway was based on the fact that Ras-associated genes, HaRas and KiRas, seemed to be the primary oncogenes in two “oncogenic” retroviruses (3, 4). This was confirmed by the fact that mutations in Ras are found at high prevalence in a great variety of human cancers (2). The synthesis of molecules that might interfere with the function of Ras has been a focus for development of therapeutic agents against cancer. One major target for blocking the Ras proteins is to block their processing. Because processing of the Ras protein is required for its transport to the cell membrane and apparently its activation, it was hypothesized that blocking membrane localization by preventing farnesylation of the Ras protein would be highly effective (1, 18). Based on this rationale, various pharmaceutical companies have developed inhibitors of farnesyl transferase. One of the agents developed was R115777, an orally bioavailable competitive inhibitor of this enzyme (5). Although R115777 and a variety of other FTIs were highly effective at blocking the farnesylation of HaRas,
they were relatively ineffective at blocking the prenylation of KiRas. This is due to the fact that KiRas protein has a much higher affinity for the farnesyl transferase enzyme than the HaRas or N-Ras proteins and, therefore, cannot be readily competed off by the inhibitors (18). Additionally, the KiRas protein can be geranylated if a FTI blocks the farnesylation of KiRas, although there is some question as to whether a geranylated KiRas functions normally (18). Nevertheless, it has been shown that cell lines and xenografts containing KiRas or no Ras mutations may be susceptible to the therapeutic effects of FTIs (5), implying that one or more additional farnesylated proteins may be specific targets for FTIs (including RHO).

In the present studies, the preventive/therapeutic effects of R115777 were evaluated in the methylN-nitrosourea-induced rat mammary cancer model. In this model, the tumors that arise in situ are ductal in origin and are histologically similar to those observed in humans (19). Approximately one-half of the mammary cancers have HaRas mutations (8, 9). Irrespective of their mutational status, all the cancers are hormonally responsive and will regress following ovariectomy (6). In the chemopreventive study (Fig. 1), a dose-dependent effect of R115777 was observed at 50, 16, and 5 mg/kg body weight/d; decreasing the number of mammary cancers by 75%, 42%, and 6%, respectively. A limited number of mammary cancers in the prevention study were also assayed for HaRas mutation status. At the two highest dose levels of R115777 (50 and 16 mg/kg body weight/d), only 1 of 15 mammary cancers (7%) had a HaRas mutation, whereas 6 of 15 tumors (40%) at the lowest dose had a HaRas mutation. Our laboratories have previously reported that treatment with other preventive agents (e.g., DHEA or the aromatase inhibitor vorozole) yielded similar efficacy in preventing the development of tumors with or without mutations in the HaRas oncogene (8, 20).

To confirm the preferential sensitivity of cancers with HaRas mutations, a therapeutic study was done employing this agent. In this experiment (Fig. 2), after a rat developed a palpable cancer, a biopsy of the cancer was taken and analyzed for HaRas mutations. Beginning 5 days after the biopsy, animals were given R115777 for 28 days. Cancers with a HaRas mutation rapidly regressed, whereas tumors without a HaRas mutation had a more variable response to treatment with R115777. When the growth rate of cancers with HaRas mutations versus no mutations were compared, it was found to be significantly different ($P < 0.05$) by Wilcoxon rank analysis. Although the effects of R115777 were much greater in the HaRas mutant–bearing cancers, the compound showed some effects on all mammary tumors. These results confirmed the striking sensitivity of established tumors with a HaRas mutation to the effects of R115777 observed in the prevention study.

We also measured changes in cell proliferation and apoptosis in the cancers of rats treated short-term with R115777. Mammary cancers were evaluated in rats treated with R115777 for either 36 or 96 hours. Similar results were obtained at both time periods (Fig. 3). In HaRas-positive cancers, proliferation was consistently decreased by $>85\%$ ($P < 0.01$), whereas in tumors without HaRas mutations, the decreases were more variable; in the 30% to 65% range ($P < 0.05$). Similarly, when measuring apoptosis, cancers with HaRas mutations displayed a 5-fold increase ($P < 0.01$), whereas tumors without a HaRas mutation showed variable responses ($P > 0.05$). Thus, by employing these nonspecific indicators, it can be determined that HaRas-positive tumors (Fig. 2A), show greater short-term changes in proliferation and apoptosis than wild-type cancers which respond variably to this agent (Fig. 2B).

Our laboratory has also examined the modulation of gene expression in cancers treated for 4 days with R115777 in a separate article. It was observed that, although there were many gene expression changes that are similar in HaRas and non-HaRas cancers, the modulation of a variety of genes including various cell cycle–related genes (e.g., proliferating cell nuclear antigen, c-jun, and c-myc) were preferentially modulated in tumors with a HaRas mutation. This observation would agree with the highly significant effects on proliferation in tumors with HaRas mutations.

In summary, the following observations in an in vivo model of breast cancer have been made: (a) cancers with a HaRas mutation are highly susceptible to the therapeutic activity of R115777, which is in agreement with the agent’s reported activity in transgenic models involving a mutant HaRas oncogene. However, the profound efficacy of R115777 in this model is in contrast with a recent study showing that conditional knockout of the farnesyl transferase protein had limited effects on the induction of skin tumors induced by a chemical carcinogen which also had HaRas mutations (21). (b) R115777 had significant effects on cancers without HaRas mutations. The specific targets in the tumors without HaRas mutations are not known, although there are a variety of other farnesylated proteins that might be affected. These include RhB, the centromere–related proteins CEN E and F, and the Rab geranyltransferases (1). The efficacy in tumors without HaRas mutations agrees with human studies showing significant effectiveness of R115777 in advanced breast cancers in which virtually no HaRas mutations were present (22, 23). Interestingly, FTIs showed some preference for cancers that highly express Her-2/Neu (EGFr2; ref. 23). This finding offers a potential rationale because one would expect Ras to be a downstream effector from EGFr signaling (1, 18). (c) Alterations in biomarkers (proliferation and apoptosis) after short-term R115777 exposure parallel the efficacy of R115777 as a chemopreventive therapeutic agent for these cancers.

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References


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