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Anticancer efficacy of the irreversible EGFr tyrosine kinase inhibitor PD 0169414 against human tumor xenografts

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Abstract *Purpose*: The involvement of the EGF receptor (EGFr) family of receptors in cancers suggests that a selective inhibitor of the tyrosine kinase activity of the EGFr family could have a therapeutic effect. PD 0169414, an anilinoquinazoline, is a potent irreversible inhibitor of the EGFr family tyrosine kinase activity with IC_{50} values of 0.42 n*M* against the isolated EGF receptor, and 4.7 n*M* and 22 n*M* against EGF- and heregulin-mediated receptor phosphorylation in A431 and MDA-MB-453 cells, respectively. *Methods and Results*: Oral administration of 260 mg/kg per day PD 0169414 for 15 days to animals bearing advanced-stage A431 epidermoid carcinoma produced a 28.2-day delay in tumor growth and resulted in three complete and three partial tumor regressions in six animals. Toxicity at this dose level was limited to <6% loss of initial body weight. Doses of 160 and 100 mg/kg per day produced tumor growth delays of 29.5 and 20.9 days and two and one complete regressions in six animals, respectively. Subcutaneous, intraperitoneal, and oral routes of administration have also shown in vivo antitumor activity of PD 0169414 in a panel of human tumor xenografts. Responsive tumor lines include A431 (human epidermoid carcinoma), H125 (NSCL carcinoma), MCF-7 and UISO-BCA1 (human breast carcinoma), and SK-OV-03 (human ovarian carcinoma). The therapeutic effect ranged from delayed tumor growth (6.4 days delayed tumor growth for 14 days of treatment) to tumor regressions (32.2 days delayed tumor growth and five partial regressions in six animals) in these model systems. *Conclusion*: PD 0169414 is a specific, irreversible inhibitor of EGFr family tyrosine kinases with significant in vivo activity against a variety of relevant human tumor xenografts.

Key words Epidermal growth factor receptor · In vivo · Tyrosine kinase inhibitor · Human tumor xenograft

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Introduction

The members of the epidermal growth factor receptor (EGFr) family are receptor protein tyrosine kinases [6, 30]. The EGFr family is activated by ligand binding to the extracellular domain with subsequent conformational alteration of the extracellular domain leading to receptor family hetero- and homodimerization, resulting in intracellular autophosphorylation of tyrosine residues at the carboxyterminal tail and activation of the receptor tyrosine kinase activity [5, 7]. Autophosphorylation results in cascades of multiple intracellular signaling resulting in cell proliferation. The development and growth of many types of cancers, including breast, lung, stomach, and ovarian cancers, is associated with overexpression of the EGFr family [2, 17, 19]. Additionally, overexpression of these receptors is associated with a poor clinical prognosis, nonresponsiveness to chemotherapy, and decreased survival [1, 15, 26]. The involvement of the whole EGFr family of tyrosine kinases in cancer proliferation suggests that an inhibitor which blocks the tyrosine kinase activity of the entire EGFr family, and hence the signaling of both hetero- and homoreceptor dimers, could have a significant therapeutic effect. Compounds of this type could provide specific advantages over treatments intended to inhibit specific individual family members. Indeed, Herceptin and EGFr monoclonal antibodies that block ligand binding have previously been shown to inhibit the growth of human xenografts overexpressing EGFr [3, 14, 25].

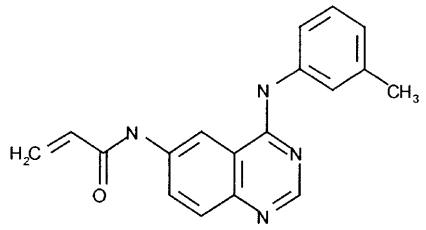

Fig. 1 Structure of PD 0169414

PD 0169414 (Fig. 1), an anilinoquinazoline analog of PD 0153035 [11], is a potent irreversible inhibitor of EGFr in vitro with IC_{50} values of 0.42 n*M*, 4.7 n*M*, and 22 n*M* against the purified EGFr enzyme, EGF-stimulated A431 cells, and heregulin-stimulated MDA-MB-453 cells, respectively [13, 27]. PD 0169414 is a selective inhibitor of the tyrosine kinase activity of the EGFr family through competitive binding at the ATP binding site which results in covalent modification of cystine 773 [4, 12, 28]. PD 0169414 exhibits extremely high specificity for the EGFr family with little or no activity against PDGF- or FGF-mediated tyrosine phosphorylation in tissue culture. The unique irreversible and highly selective properties of PD 0169414 for the EGFr kinase family results in sustained suppression of receptor tyrosine kinase activity [4, 22]. The long-lasting inhibition of receptor phosphorylation reduces concern over potentially short plasma half-lives. Furthermore, the low nanomolar potency and irreversibility reduce the need for high peak plasma levels, which in turn could minimize nonspecific toxicities.

In the studies reported here, PD 0169414 was characterized in terms of in vivo anticancer activity, target modulation, and pharmacokinetics. In addition, in vivo studies were designed to investigate the schedule and route dependence, as well as the spectrum of activity, of this novel irreversible EGFr family tyrosine kinase inhibitor.

Methods

Tumors and animals

Solid tumor models of A431 (human epidermoid carcinoma), H125 (human NSCL carcinoma), MCF-7 (estrogen-dependent human breast carcinoma), UISO-BCA-1 (human breast carcinoma), CAKI-1 (human renal carcinoma), SK-OV-03 (human ovarian adenocarcinoma) and NIH 3T3 cells transfected with human EGFr (NIH 3T3/hEGFr) were developed from cell lines and were maintained and tested in either athymic NCr-nu/nu mice or ICR-SCID mice. All tumors were serially passed as subcutaneous (s.c.) implants of tumor fragments (approximately 30 mg) from tumors weighing approximately 1 g. The tumor panel was selected because of the overexpression of at least two EGFr family members as determined by western blot analysis (Table 1). Mice used for all experiments were obtained from Charles River Breeding Laboratories and Taconic Farms (National Cancer Institute Colonies). Animals were examined prior to the initiation of experiments to ensure that they were healthy and acclimated to the laboratory environment. Animals were housed in barrier facilities with food and water provided ad libitum on a 12-h light/dark cycle. All treatment protocols were reviewed and approved by the Parke-Davis Institutional Animal Care and Use Committee and complied with NIH publication No. 85-23, revised 1985.

Table 1 Expression of EGFr family members in various in vivo tumors. The expression of EGFr, erbB-2, erbB-3, and erbB-4 was determined using immunoblotting procedures described in Materials and methods. Tumors were harvested and processed as indicated for ELISA analysis. The detected bands were compared to the band obtained for A431 tumors, which was considered a ++++ expresser of EGFr. Expression ranged from none (*ND* not detected) to overexpression (++++) (*NA* not assayed)

Tumor model	Relative receptor expression level			
	EGFr	erbB-2	erbB-3	erbB-4
A431 (epidermoid)	++++	++	ND	++++
MCF-7 (breast)	+	+++	++	+
CAKI-1 (renal)	++	+++	NA	NA
H125 (NSCLC)	+++	+++	++++	+++
UISO-BCA-1 (breast)	++	++++	++++	+++
SK-OV-03 (ovarian)	+	++++	+++	++++
NIH 3T3/hEGFR (hEGFR transfected 3T3)	++	ND	ND	+

Drug

PD 0169414 was suspended in 6% dimethylacetamide in 50 m*M* sodium lactate buffer pH 4.0 for intraperitoneal (i.p.) injection. For oral gavage administration PD 0169414 was suspended in 0.5% methyl cellulose in water. For the continuous s.c. infusion trials, the drug was formulated as an emulsion in polyvinylpyrolidine/polyethylene glycol 3350 (1:1) and then diluted 1:10 in phosphate-buffered saline (PBS), pH 7. Vehicle control groups were included in each study to confirm that the vehicles used to deliver the drugs had no significant effects on tumor growth. The treatment volumes were 0.5 ml for the i.p. and oral routes of administration and 1 µl/h for the continuous infusion experiments.

In vivo experiments

The general methods used in these studies have been described previously [10, 20, 24]. For all tests, the mice (18–22 g) were randomized and then implanted with tumor fragments (~30 mg) s.c. into the right axilla. Treatment was initiated when tumors were 100–300 mg in size (advanced stage), or early stage (day 1 following implant). Test substances were administered on the basis of group average weight. Miniosmotic pumps were obtained from Alza, Palo Alto, Calif. (Alzet model 2001). The pumps were surgically implanted s.c. according to the manufacturer's instructions. The pumps were exchanged for fresh pumps after 7 days of administration to complete 14 days of therapy. Following the 14-day administration period all pumps were removed. The animals were weighed every 3–4 days during the treatment period. Thereafter, weights were recorded weekly for 3–4 weeks. Mean body weight change is reported as the maximum treatment-related weight loss for each group. If animals gained weight during the treatment period, the weight change is reported as a "+". Tumors were measured 2 days per week and tumor mass $[(L \times W^2)/2]$ was calculated from caliper measurements as described previously [10, 20, 24]. A gross visual necropsy of each animal was performed at death or terminal sacrifice.

The following end-points were used to assess tumor response to treatment: complete regression (CR), partial regression (PR), and growth delay (T–C). The T–C data were calculated as the time (days) for the median treated tumor volume (T) to reach 750 mg minus the time (days) for the median control tumor volume (C) to reach 750 mg. A T–C of greater than 50% of the duration of therapy was considered indicative of antitumor efficacy. A response

was considered CR if no palpable tumor mass remained (<60 mg) and PR if the tumor mass was reduced to less than 50% of the initial tumor mass. In each experiment, PD 0169414 was tested over a range of doses in groups of six mice. An additional negative control group of 12 mice were given control vehicle on the same treatment schedule. Data from selected dose levels (optimal or maximum tolerated doses) in single experiments are reported.

ELISA

Tumors, three per time-point, were harvested at various time-points following single injections, snap-frozen in liquid nitrogen and crushed to a fine powder. Powders were extracted at 40 °C using a lysing reagent containing 50 m*M* HEPES (pH 7.5), 150 m*M* NaCl, 10% glycerol, 1% Triton X-100, 1% sodium orthovanadate, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 1 m*M* PMSF. An enzyme-linked immunosorbent assay (ELISA) was used for the analysis of EGFr tyrosine phosphorylation modulation. The assay was a modification of the EGFr ELISA assay (Q1A08; Calbiochem, La Jolla, Calif.). Briefly, the ELISA was a sandwich enzyme immunoassay employing mouse monoclonal human EGFr antibody immobilized onto the surface of a 96-well plate. The sample to be assayed was added and allowed to incubate for 4 h, during which any EGFr present bound to the capture antibody. Unbound material was washed away, and phosphorylated EGFr was detected with biotin-conjugated anti-phosphotyrosine (Clone 4G10, UBI). Total EGFr expression was determined according to the assay instructions. The assays were linear for total protein concentrations between 12 μg/well and 0.75 μg/well. A standard curve with EGFr obtained from control tumors was run in each assay.

Immunoblotting

Relative receptor levels were determined in each tissue sample by western blotting. Cell extracts were prepared from solid tissue samples. Tumors were harvested from untreated animals and processed as described for ELISA analysis, and the protein content quantitated (BCA Protein Assay Kit; Pierce, Rockford, Ill.). Laemmli buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, 0.001% bromophenyl blue and 50 m*M* Tris, pH 6.8) was added to each sample to make a 2 mg/ml solution and this was heated to 100 °C for 5 min. Samples were loaded onto a polyacrylamide gel (4–20%) at 50 μg per lane, and separated and transferred to a nitrocellulose membrane. The membrane was washed once in PBS/0.05% Tween 20 and then blocked for 4 h in Blocker Blotto in PBS (Pierce). The membrane was blotted overnight with anti-EGFr (F4) antibody (1 μg/ml in Blotto; Sigma, St. Louis, Mo.), anti-C-neu (Ab-3) antibody (Oncogene Science, 1 μg/ml in Blotto), anti-erbB-3 antibody (1 μg/ml in Blotto; Upstate Biotechnology, Lake Placid, N.Y.) or anti-erbB-4 (C-18) antibody (1 μg/ml in Blotto; Santa Cruz Biotech, Santa Cruz, Calif.). The blots were then washed five times in PBS/0.05% Tween 20 and incubated for 2 h in Blotto containing goat anti-mouse IgG (H + L)-HRP (1:10,000 in Blotto; Biorad, Hercules, Calif.) or anti-rabbit IgG-HRP (Sigma, 1:10,000 in Blotto). The membranes were then washed five times in PBS/0.05% Tween 20. Enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) was used to detect the protein bands. The resulting bands were compared with the band obtained for EGFr in A431 tumors, which was considered a ++++ expresser of EGFr. If no band was detected, then the tumor line was considered not to express the receptor assayed.

Pharmacokinetic analysis

At set time intervals, heparinized blood specimens for pharmacokinetic analyses were obtained from non-tumor-bearing or tumor-bearing mice following compound administration, three animals per time-point. Plasma concentrations were determined by a high-pressure liquid chromatography assay. The assay consisted of extraction of the plasma sample with ethylether buffered with 1 *M* KOH/1 *M* K_2CO_3, evaporated to dryness, and reconstituted with 200 μl 15% acetonitrile (ACN), 85% 35 m*M* phosphoric acid with 0.5% triethylamine (TEA), pH 3.0. The range of detection was 25 to 5000 ng/ml, and the standard curves were linear through the calibration range. The stationary phase consisted of a C18 column (Zorbax SB 5 μm, 4.6 × 150 mm) with C18 guard column (Zorbax SB 5 μm). The mobile phase was a mixture of 26% ACN and 74% 35 m*M* phosphoric acid with 0.5% TEA (pH 3.0) and pumped at a rate of 0.8 ml/min (Model 510 pump, Waters Associates). UV detection was accomplished at a wavelength of 355 nm (Waters Associates, Model 486). Concentrations were calculated by comparing peak height ratios of spiked internal standards to unknowns with a standard curve.

Statistics

Statistical analysis was conducted using JMP statistical software version 3 (SAS Institute, Cary. N.C.). Testing for the significance of differences between tumor time to evaluation size (750 mg) for the PD 0169414-treated group and that for the control group was carried out using the Tukey-Kramer analysis of all pairs.

Results

Pharmacokinetic and pharmacodynamic determination

The ability of PD 0169414 to inhibit EGFr phosphorylation in A431 tumors was shown by the overexpression of EGFr (Table 1). Figure 2 shows the modulation of EGFr phosphorylation in A431 xenografts and plasma pharmacokinetics following a single oral dose of PD 0169414. PD 0169414 inhibited EGFr tyrosine phosphorylation by at least 70% (91% maximum) for more than 24 h. The plasma-concentration time profile presented in Fig. 2 indicates rapid clearance from the plasma with a plasma $t_{1/2}$ (half-life) of 4.5 h. Apparently, the sustained suppression of EGFr tyrosine phosphorylation can be attributed to the irreversible inhibitory properties of PD 0169414 and correlates with the in vitro activity (IC_{50} values of 0.42 n*M*, 4.7 n*M*, and 22 n*M* against the purified EGFr enzyme, EGF-stimulated A431 cells, and heregulin-stimulated MDA-MB-453 cells, respectively) [13, 27]. The residual tyrosine phosphorylation seen in the ELISA may have been due to tyrosine phosphorylation at sites on EGFr other than those associated with activation, such as receptor internalization [21, 29], the net result of which would have been that some phosphorylation was detected by the ELISA despite complete inhibition of the kinase activity by PD 0169414.

We further investigated the pharmacokinetic profile of PD 0169414. Figures 2 and 3 show the pharmacokinetic data from athymic nude mice following i.p., oral or s.c. (continuous infusion) administration of PD 0169414. Oral administration of PD 0169414 resulted in an apparent relative bioavailability of 25% based on area under the curve (AUC) of the i.p. dose. The AUCs (0–12 h) were 12 μ*M* · h and 22.3 μ*M* · h following 100 mg/kg oral and 50 mg/kg i.p. treatment, respectively. The use of the s.c. Alzet pump to deliver 20 mg/kg per day PD 0169414 resulted in a maximal concentration (Cmax) of 0.21 μ*M*, an AUC (0–12 h) of

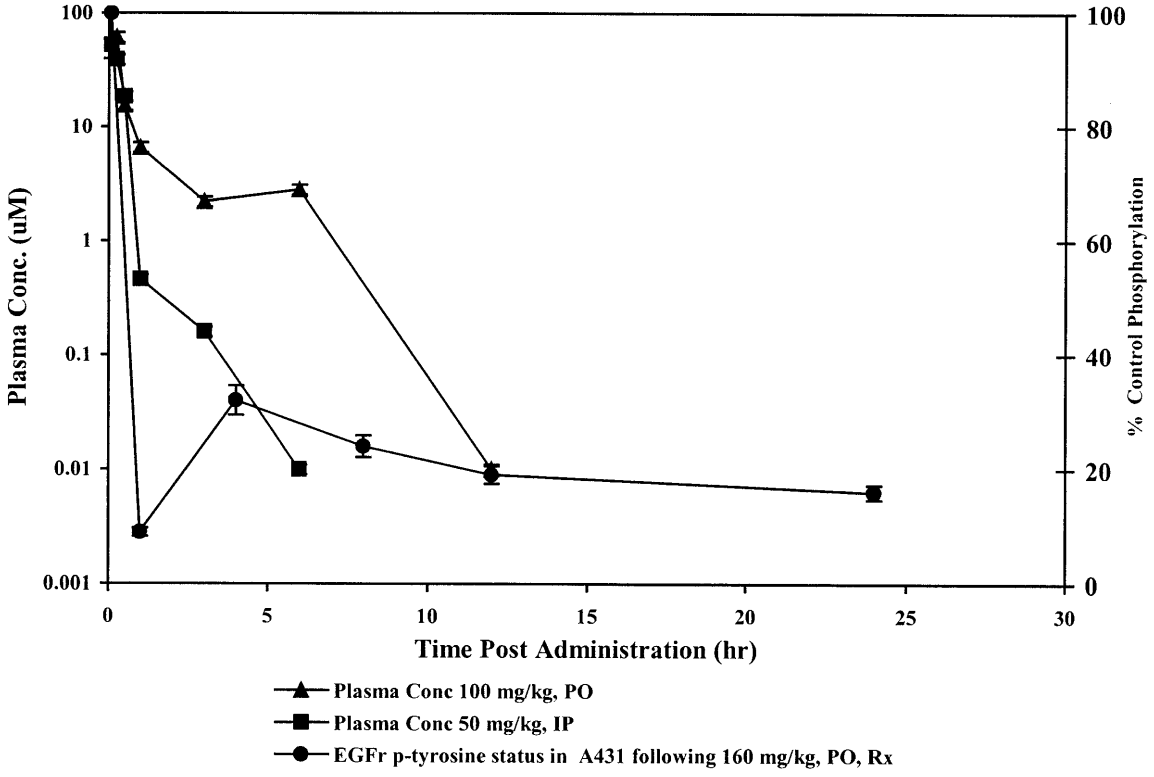

Fig. 2 PD 0169414 modulation of EGFr tyrosine phosphorylation in A431 carcinoma and plasma pharmacokinetics. Heparinized blood specimens for pharmacokinetic analyses were obtained from tumor-bearing mice following compound administration, three animals per time-point. Plasma concentrations were determined by HPLC as described in Materials and methods. Tumors were harvested at various time-points following single injections. An ELISA was used for the analysis of EGFr tyrosine phosphorylation modulation and total EGFr expression. The total amount of EGFr expression remained unchanged

7.2 $\mu M \cdot$ h, and an overall AUC (0–168 h) of 15.8 $\mu M \cdot$ h. The continuous infusion maintained plasma levels of PD 0169414 above the A431 cellular IC_{50} of 4.7 nM throughout the infusion period. The i.p. and oral dosing routes also achieved plasma levels above the in vitro cellular IC_{50} value for at least 6 and 12 h, respectively.

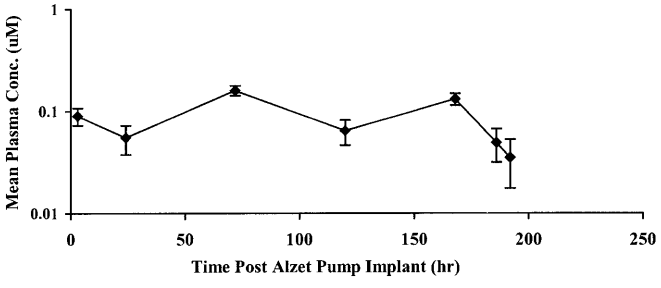

Fig. 3 Alzet pumps were removed at 168 h postimplant. Heparinized blood specimens for pharmacokinetic analyses were obtained from non-tumor-bearing mice following compound administration, three animals per time-point. Plasma concentrations were determined by HPLC as described in Materials and methods

Anticancer activity

PD 0169414 had significant in vivo activity against a variety of human tumor xenografts. In responsive tumor systems, the therapeutic activity ranged from complete regression to delayed progression (Tables 2 and 3). The inhibitory effects on tumor growth generally lasted throughout compound administration in responsive systems.

Previous in vivo data with reversible EGFr tyrosine kinase inhibitors have indicated that the plasma concentration is required to be above the cellular IC_{50} for more than 12 h. Twice-daily administration was initially chosen based on the pharmacokinetic data indicating that the plasma drug concentration levels were below the limit of detection within 6 to 12 h.

Activity obtained with daily oral administration of PD 0169414 against the A431 xenograft was equivalent to twice-daily oral or twice-daily i.p. administration. Growth delays of more than 27 days were observed over the entire dose range regardless of the treatment schedule (Table 2, Fig. 4). Tumor growth was completely suppressed for the entire dosing period. Partial tumor regressions were observed in 66% of the treated animals in these groups. Tumor suppression lasted for approximately 1 week following the cessation of dosing. The data suggest that antitumor activity was independent of the administration schedule used (Table 2). The high degree of antitumor activity regardless of schedule was consistent with the extended inhibition of EGFr tyrosine phosphorylation status seen in the A431 xenografts by ELISA (Fig. 2). Overall, oral treatment was more

Table 2 Activity of PD 0169414 against advanced-stage A431 human epidermoid carcinoma. Tumor growth delay experiments were performed as described in Materials and methods. Tumor fragments (30 mg) were implanted s.c. in the right axilla on day 0. The highest active doses for a complete dose response are shown (dose ranges: oral 260–50 mg/kg per injection, i.p. 75–29 mg/kg per injection, s.c. 22.5–10.4 mg/kg per injection) (*T–C* the time for the median treated tumor volume to reach 750 mg minus the time for the median control tumor volume to reach 750 mg)

Dose (mg/kg/injection)	Treatment schedule	Treatment route	Weight change (g)	T–C (days)	Control time to 750 mg (days)	Partial regression[a]	Complete response
75	Days 14–28, twice daily[b]	I.p.	−2.2	32.3*	19.5	5/6	0/6
10.4	Days 9–23[c]	S.c.	+	16.5*	27.1	2/6	1/6
160	Days 10–23[d]	Oral	−0.9	29.5*	17.8	4/6	2/6
80	Days 10–23, twice daily[d]	Oral	−0.1	27.3*	17.8	4/6	2/6

*$P \leq 0.05$ vs control time to 750 mg
[a] Partial regression counts include complete regressions
[b] Dosing performed twice daily with 12-h separation between doses. Tumor mass was approximately 300 mg at time of initial treatment
[c] Tumor mass was approximately 100–150 mg at time of initial treatment by continuous s.c. infusion
[d] Tumor mass was approximately 100–150 mg on the indicated schedule by gavage

Table 3 Activity of PD 0169414 against various tumor systems. Tumor growth delay experiments were performed as described in Materials and methods. Tumor fragments (30 mg) were implanted s.c. in the right axilla on day 0. Treatment was initiated when the mean tumor mass was approximately 100–150 mg on the indicated schedule, except for the early-stage studies with MCF-7 and EGFr. The highest active doses for a complete dose response are shown (*T–C* the time for the median treated tumor volume to reach 750 mg minus the time for the median control tumor volume to reach 750 mg)

Tumor	Dose (mg/kg/injection)	Treatment schedule	Treatment route	Weight change (g)	T–C (days)	Control median time to 750 mg (days)	Partial regression[a]	Complete response
MCF-7	46	Days 1–15, twice daily[b]	I.p.	+	23.1*	25.0	–	–
NIH 3T3/hEGFr	48	Days 1–14, twice daily	I.p.	−0.4	6.4*	16.6	–	–
CAKI-1	80	Days 16–20, 23–27, 30–34, twice daily	I.p.	−4.0	0	28.7	0/6	0/6
H125	50	Days 11–15, 18–22, 25–29, twice daily	I.p.	+	11.9*	28.3	0/6	0/6
H125	75	Days 21–35	Oral	+	12.8*	31.5	0/6	0/6
UISO-BCA-1	50	Days 19–23, 26–30, 33–37, twice daily	I.p.	+	9.8	40.3	0/6	0/6
SK-OV-03	46	Days 10–14, 17–21, 24–28, twice daily	I.p.	−0.4	1.3	28.4	0/6	0/6
SK-OV-03	75	Days 17–31	Oral	+	1.6	27.6	0/6	0/6
SK-OV-03	20.7	Days 9–23	S.c.[c]	+	16.4**	29.4	2/6	0/6

*$P \leq 0.05$, **$P \leq 0.1$, vs control time to 750 mg
[a] Partial regression counts include complete regressions
[b] Dosing performed twice daily with 12-h separation between doses
[c] Treatment was conducted on the indicated schedule by continuous s.c. infusion

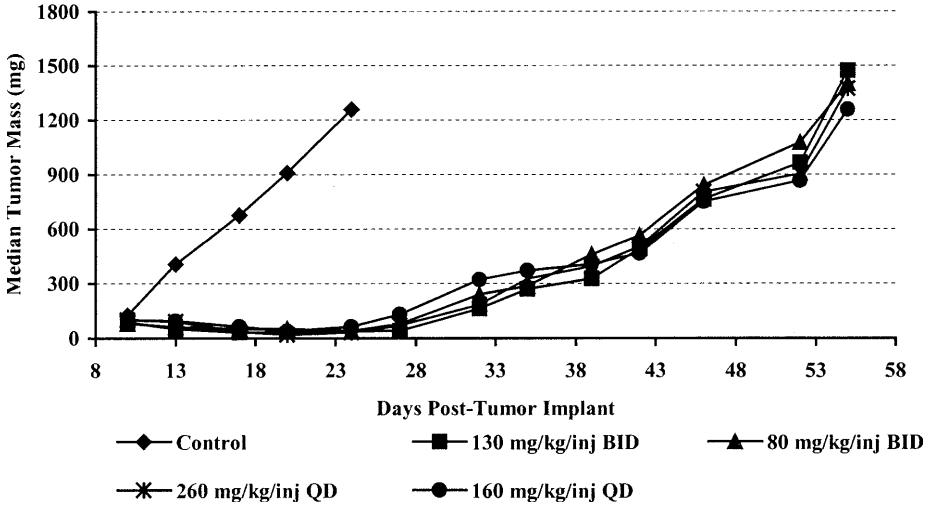

Fig. 4 A431 tumor fragments were implanted s.c., as described in Material and methods, on day 0. Therapy was initiated on day 10. Treatments were administered orally as indicated on days 10–23

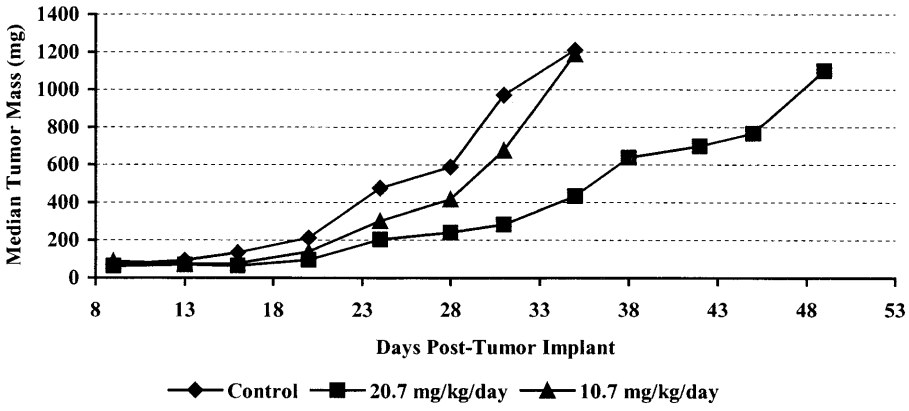

Fig. 5 SK-OV-03 tumor fragments were implanted s.c., as described in Material and methods, on day 0. Therapy was initiated on day 9. Treatments were administered orally as indicated on days 10–23

efficacious than i.p. dosing based on a broader range of effective doses and complete lack of host toxicity. An oral maximum tolerated dose was not reached (>260 mg/kg per day).

PD 0169414 was concurrently evaluated in six additional xenograft tumor model systems (Table 3). Twice-daily i.p. administration of PD 0169414 to animals bearing early-stage breast carcinoma MCF-7 resulted in excellent activity reflected by a 23.1-day delay in tumor growth for a 15-day treatment period. PD 0169414 significantly suppressed tumor growth following both oral and i.p. administration in animals with advanced-stage NSCLC H125. This is evidenced by the 12-day delay in tumor growth compared to 15 days of treatment (Table 2). Tumor growth was moderately suppressed (T–C >50% of the duration of therapy) in advanced-stage human breast carcinoma UISO-BCA-1 and early-stage NIH 3T3 cells transfected with human EGFr in animals following i.p. administration of PD 0169414 (Table 2). PD 0169414 was ineffective against CAKI-1 even though expressing two of the EGFr members (Tables 1 and 3).

The lack of sustained inhibition of tumor growth in the SK-OV-03 xenografts following either daily or twice-daily administration led to the exploration of alternative administration schedules (Table 3). We examined whether sustained exposure from frequent or continuous dosing would be required to produce a substantial therapeutic effect against SK-OV-03 in vivo. Continuous s.c. administration of 20.7 mg/kg per day PD 0169414 to animals bearing SK-OV-03 resulted in 16.4-day delay in tumor growth and partial regressions in two of six animals (Table 3, Fig. 5). This represents an increase of more than tenfold in T–C compared with that obtained with the other administration schedules evaluated against this model (Table 3).

Toxicity

Twice-daily i.p. administration of 75 mg/kg PD 0169414 to animals bearing advanced-stage A431 carcinoma resulted in one drug-related death. Findings at necropsy were limited to slight morphological changes in the liver. Necropsy findings at terminal sacrifice for all animals dosed with PD 0196414 also included slight morphological changes in the liver, with the severity greatest with the highest dose. Treatment-associated weight loss of between 5% and 13% of initial body weight was observed in groups dosed i.p. with PD 0169414. There was no significant body weight loss, gross necropsy findings or clinical signs in animals dosed orally or by continuous s.c. infusion. Overall, there were no significant observations or toxicities associated with the inhibition of epithelial proliferation (e.g. hair loss, ulceration).

Discussion

These studies suggest that PD 0169414 could have significant anticancer activity in the clinic based on multiple factors, which include antitumor efficacy, a potentially large therapeutic index, broad spectrum of antitumor effectiveness, and near complete inhibition of EGFr tyrosine phosphorylation in vivo. Additionally, if tumor growth is entirely dependent upon the EGFr family signal cascade then tumor progression should be completely inhibited by suppression of EGFr family receptor autophosphorylation. The activity seen with PD 0169414 in animals bearing A431, SK-OV-03, and MCF-7 xenografts is consistent with the hypothesis that the EGFr family is important in tumor progression in these models. This is exemplified by the complete and partial tumor mass regressions observed in animals bearing A431 and SK-OV-03 (Tables 2 and 3).

The single bolus dose pharmacokinetic data did not appear to correlate with the effects seen in the ELISA (Fig. 2). Inhibition of EGFr tyrosine phosphorylation in A431 xenografts displayed a half-life significantly greater than the pharmacokinetic half-life ($t_{1/2}$) of 4.5 h. The suppression of EGFr tyrosine phosphorylation with a $t_{1/2}$ of greater than 24 h is consistent with an irreversible inhibition of receptor phosphorylation and correlates with previous in vitro data [13, 27]. The extended pharmacodynamic effect observed in A431 xenografts with PD 0169414 may be related to the EGFr half-life of approximately 20 h in A431 cells and also to the irreversible nature of the compounds [8].

Continuous s.c. infusion of PD 0169414 resulted in a reduced A431 tumor response compared to that obtained with either oral or i.p. administration (Table 2). The likely cause of the decreased therapeutic effect in this instance was the relative insolubility of PD 0169414 in the emulsion formulation, which prevented the use of higher concentrations in the osmotic pump. Although plasma pharmacokinetics for the s.c. infusion indicated that the plasma levels were above the in vitro IC_{50} (Fig. 3), the actual levels of PD 0169414 in the tumor were not determined. Thus, the high Cmax values obtained with the i.p. and oral administration schedules may have resulted in a net increase in cumulative inhibition resulting in increased cytotoxicity. This is evidenced by the increase in the number of partial regressions, with >66% of the animals' tumors regressing in the oral and i.p. treatment groups in comparison with 33% in the continuous infusion study.

In less-responsive systems such as CAKI-1, the cells are either circumventing the inhibitory effects of drug administration or do not depend on the EGFr family for growth. This would limit the EGFr family as a survival factor for the cell line even though the cell line overexpresses one or more of these proteins (Table 1). For example, high levels of constitutively active Ras protein result in maximal stimulation of downstream targets, so that any additional input due to the EGFr family could be functionally insignificant [9]. The disruption of EGFr function in these tumors would not be expected to impair the ability of constitutively active Ras to induce cell proliferation [22]. Consequently, inhibition of EGFr would not result in any inhibition of tumor growth. Additional factors contributing to the lack of tumor response in less-responsive systems may be differences in tissue penetration, increased receptor expression/turnover by tumors in vivo, or expression of other mitogenic signaling pathways. The assumption that a protein's effect on proliferation can be inferred from its expression level may be likely to be misleading [23]. Hence, tumor response to drug administration cannot be inferred from EGFr expression levels.

Our studies with SK-OV-03 xenografts indicated increased antitumor effectiveness with continuous s.c. dosing over that obtained with either i.p. or oral administration. SK-OV-03 ovarian xenografts express erbB-2 at very high levels due to gene amplification [18]. Previous studies have shown that erbB-2 expression is rate-limiting for SK-OV-03 tumor growth in a dose-dependent manner [16]. Continuous dosing may have allowed cumulative tumor levels to reach a critical threshold for inhibition, whereas oral and i.p. dosing regimens did not. Another hypothesis could be that the turnover of erbB-2 in this xenograft line is unregulated as well as vastly overexpressed. This may lead to competent mitogenic signaling from newly synthesized receptor within several hours of the elimination of PD 169414 from the body. Under such circumstances, the inhibitor would have to be present all of the time. Continuous dosing sustained PD 0169414 plasma levels for the entire dosing period above the IC_{50} of 22 n*M* for heregulin-induced proliferation in MDA-MB 453 cells (Fig. 3). Continuous drug exposure may also have caused alterations in target receptor kinetics, including, internalization, degradation, half-life, and downregulation [16].

In summary, PD 0169414 is a specific, irreversible inhibitor of EGFr family tyrosine kinases with significant in vivo activity against a variety of relevant human tumor xenografts. In responsive tumor systems, the tumor responses ranged from complete regression to growth inhibition and delayed progression. The inhibitory effects on tumor growth generally lasted throughout the treatment period. Oral administration of PD 0169414 was well tolerated and efficacious with none of the toxicities associated with i.p. dosing. An oral maximum tolerated dose was not reached (>260 mg/kg per day). The data suggest that antitumor activity is independent of frequency of treatment over the range of schedules studied. The broad range of active doses and relative lack of toxicities indicate that PD 0169414 may serve as a prototype compound for the development of irreversible EGFr tyrosine kinase inhibitor clinical candidates.

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