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Inhibition of Epidermal Growth Factor Receptor Activity by two Pyrimidopyrimidine Derivatives

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Running Title

Pyrimidopyrimidines, a new class of EGFR Inhibitors

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Abbreviations (AEC) aminoethylcarbazole, (DMSO) dimethyl sulfoxide, (EGFR) epidermal growth factor receptor, (pEGFR) phosphorylated epidermal growth factor receptor, (HER2) human epidermal growth factor receptor 2, (HER3) human epidermal growth factor receptor 3, (HER4) human epidermal growth factor receptor 4, (HRPO) horse radish peroxidase, (InsRK) insulin receptor kinase, (IGF1R) insulin-like growth factor 1 receptor, (HGFR) hepatocyte growth factor receptor, (c-src) Rous Sarcoma virus encoded kinase, (VEGFR2) vascular endothelial growth factor receptor 2, (FCS) fetal calf serum and (PBS) phosphate buffered saline, poly(EY) polymer of glutamine(E) and tyrosine (Y), (ELISA) enzyme-linked immunosorbent assay, (MAPK) mitogen-activated protein kinase; (pMAPK) phosphorylated MAPK, (MEK) MAPK/ Extracellular signal-regulated kinase Kinase and (PY) phosphotyrosine.

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Abstract

Overexpression of the epidermal growth factor receptors EGFR and HER2 occurs frequently in human cancers and is associated with aggressive tumor behavior and poor patient prognosis. We have investigated the effects *in vitro* and *in vivo* of a new class of inhibitor molecules on the growth of several human cancer cell lines. BIBX1382 and BIBU1361are two new selective EGFR kinase inhibitors which do not block the activity of other tyrosine kinases. BIBU1361 blocked EGF induced phosphorylation of EGFR and also prevented down stream responses such as MEK (MAPK/ Extracellular signal-regulated kinase kinase) and MAPK (mitogen-activated protein kinase) activation in cells. In accordance with these observations thymidine incorporation into EGFR-expressing KB cells was selectively and potently inhibited by BIBX1382 and BIBU1361 with half-maximally effective doses in the nanomolar range. Oral administration of these compounds inhibited the growth of established human xenografts in athymic mice, including vulval and head and neck squamous cell carcinomas. Tumor growth inhibition by BIBX1382 coincided with reduced pEGFR and Ki-67 levels *in vivo* which is in accordance with the expected effect of EGFR inhibitors. Collectively, these results show that the structural class of pyrimidopyrimidines, exemplified here by BIBX1382 and BIBU1361, represents an interesting scaffold for the design of EGFR inhibitors.

Introduction

The control of proliferation, differentiation, migration and apoptosis is essential for the development and homeostasis of multicellular organisms. Receptor tyrosine kinases, located in the plasma membrane, in concert with their cognate ligands control many of these processes. These receptors share basic structural elements and exist in distinct forms and have been classified in 18 different families (Shawver, 1999;Ullrich and Schlessinger, 1990). The human EGF receptor (HER1, c-erb-B1) is a member of the class-I trans-membrane growth factor receptor family comprising HER2, for which no ligand has been described, HER3 and HER4. It is noteworthy that a large group of ligands, comprising EGF-related peptides and heregulins, differentially binds to these receptors. The ligands are encoded by more than 10 different genes and alternative splicing as well as post-translational processing adds to the complexity of this signaling system. Upon binding to the extracellular domain, the ligands induce either homo- or heterodimerization (Carraway, III and Cantley, 1994). The preferred dimerization partner is the HER2 molecule, which is thought to act as a common subunit (Karunagaran et al., 1996). The close vicinity of the intracellular domains results in the activation of the kinases through transphosphorylation of the receptor subunits. The phosphorylated tyrosine residues in the cytoplasmic tail of the respective receptor will act as competent docking sites for an array of downstream signaling molecules. The signal transduction pathways include the mitogenic Ras/Raf/MAP kinase cascade, the PI3K/Akt, the phospholipase C and the Jak/Stat pathways (Arteaga, 2001;Carpenter, 2000;Fernandes et al., 1999;van der et al., 1994). Ultimately, the nature of the cellular response to class-I receptor activation will depend on the type of the signaling cascades used, and on the amplitude of the down stream signals and the cellular signaling environment.

The observation that the class-I receptor pathway is often implicated in the development and progression of many common human epithelial cancers makes the blockade of this growth pathway an attractive target for anticancer therapy (Grunwald and Hidalgo, 2003;Gullick, 1991;Helmout Modjtahedi and Christopher Dean, 1994;Klapper et al., 2000;Mendelsohn and Baselga, 2003;Ross et al., 2003;Sainsbury et al., 1987). Several approaches have been used to block class-I receptor function in cancer cells. Among these, monoclonal antibodies directed to the EGFR (e.g. IMC-C225) or HER2 receptor (Herceptin), as well as small molecules inhibiting the tyrosine kinase activity show promising activity as anti-cancer agents in preclinical models and clinical trials (Sridhar et al., 2003). In recent years, several classes of small molecules, inhibiting the function of EGFR or HER2 molecules have been identified (Traxler, 2003). All these small molecules bind competitively to the ATP binding pocket of the receptor kinase moiety and thus inhibit class-I receptor activation. Although similar in their inhibition mode, these compounds differ with regard to potency and selectivity. The quinazolines *Iressa* and *erlotinib* which are the most advanced compounds are selective and reversible EGFR inhibitors. Irreversible inhibitors like the quinazoline *canertinib* (Slichenmyer et al., 2001;Smaill et al., 2000) and the cyanoquinoline EKB-569 (Wissner et al., 2003), covalently bind into the catalytic cleft of the EGFR molecule and display long lasting inhibition which is only overcome by new receptor synthesis (Fry et al., 1998). Some inhibitors such as *lapatinib* and *canertinib* are dual kinase inhibitors that simultaneously inhibit the EGFR and HER2 kinase activities. Here we report about two new EGFR inhibitors belonging to the structural class of pyrimido [5,4-d]-pyrimidines comprising a number of molecules displaying strong inhibitory activity against EGFR dependent processes

in vitro, and showing good anti-tumor efficacy *in vivo*. While BIBX1382 failed to achieve plasma exposure in humans due to a first path metabolism (Dittrich et al., 2002) BIBU1361 is expected to be unaffected by this metabolism.

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Materials and Methods

Materials:

BIBX1382, BIBU1361 and Iressa - BIBX1382, N8-(3-Chloro-4-fluoro-phenyl)-N2-(1-methyl-piperidin-4-yl)-pyrimido[5,4-d]pyrimidine-2,8-diamine, and BIBU1361, (3-Chloro-4-fluoro-phenyl)-[6-(4-diethylaminomethyl-piperidin-1-yl)-pyrimido[5,4-d]pyrimidin-4-yl]-amine, were synthesized at Boehringer Ingelheim (Himmelsbach, 1997) and *Iressa* (4-(3-chloro-4-fluoro-anilino-7-methoxy-6-(3-morpholinopropoxy) quinazoline) was synthesized as described (Gibson, 1996).

Cell lines - The human cancer cell lines KB (oral epidermoid carcinoma), A431 (vulval squamous cell carcinoma) and FaDu (hypopharyngeal squamous cell carcinoma) were obtained from the American Type Tissue Culture Collection. The HN5 cell line (head and neck squamous cell carcinoma) was kindly provided by Michael J. O'Hare (Ludwig Institute for Cancer Research, London). All cell lines were maintained at 37°C in a 5% $C0_2$ humidified atmosphere in Dulbecco's Modified Eagle's Medium (Life technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (Sigma, Germany), 1 mM Na-pyruvate, 1% non-essential aminoacids, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

Methods:

Kinase Assays - The cytoplasmic tyrosine kinase domains of EGFR, HER2, VEGFR2, HGFR, IGF1R, ß-InsRK and c-src were cloned as such or as fusion proteins into pFastBac (Gibco, UK). The protein was expressed in Sf9 insect cells using the baculovirus expression system according to the manufacturer's protocol.

Sf9 cells were harvested 72 hours post-infection, washed twice with PBS and extracted with HEPEX (20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM ß-glycerophosphate, 10 mM para-nitro-phenylphosphate, 30 mM NaF, 5 mM EDTA, 5% glycerol, 1% Triton X-100, 1 mM Na3VO4, 0.1% SDS, 0.5 µg/ml pepstatin A, 2.5 µg/ml 3,4-dichloroisocoumarin, 2.5 µg/ml Trans-epoxysuccinyl-L-leucyl-L-amido butane, aprotinin 20 KIU/ml, leupeptin 2 µg/ml, benzamidine 1 mM and 0.002% PMSF). The extracts were used for the determination of IC_{50} values. The dilution of enzymes was set so that incorporation of phosphate was linear with respect to time and amount of enzyme. Enzyme activities were assayed in the presence or absence of serial dilutions of the inhibitor performed in 50 % DMSO. Each microtiter plate contained internal controls such as blank, maximum reaction (no inhibitor added) and historical reference compound (substance with known IC_{50}value),.

EGFR kinase activity was assessed in a 50 µl-reaction containing 5 % DMSO, 40 mM HEPES pH 7.4, 10 mM Mg-acetate, 0.5 mg/ml poly (Glu-Tyr), 0.05% Triton X-100, 50 µM ATP, 1 µCi [γ-^{33}P] ATP and 10 µl of enzyme preparation. Assays were carried out at room temperature for 30 minutes and terminated by the addition of 10 µl of 5 % H_3PO_4. The entire reaction was trapped onto GF/B filters and the incorporated radioactivity was determined by scintillation counting using a Microbeta Counter™.

The reaction conditions for the radioactive HER2 kinase assay have been described previously (Stratowa et al., 1999). The radioactive assays for VEGFR2 and HGFR were performed as described above with the following modifications: VEGFR2 assays contained 5 % Me_2SO, 40 mM HEPES pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mg/ml poly(EY), 0.05% Triton X-100, 100 µM ATP, 1 µCi [γ-^{33}P]ATP and the reaction

was carried out at room temperature for 20 minutes. The HGFR assays were performed like the VEGFR2 assays but in the absence of $MnCl_2$.

The kinases assays for c-src, ß-InsRK and IGF1R were adapted to a non-radioactive setup. For the c-src kinase assays a 100 µl-reaction contained 10 µl of inhibitor in 50 % Me_2SO, 20 µl of substrate solution (200 mM HEPES pH 7.4, 50 mM Mg-acetate, 2.5 mg/ml poly(EY), 5 µg/ml bio-poly(EY), 1000 µM Na_3VO_4) and 20µl enzyme preparation. The enzymatic reaction was started by addition of 50µl of a 1 mM ATP solution made in 10 mM Mg-acetate. For the ß-InsR kinase assays the 20 µl substrate solution contained 250 mM Tris pH 7.4, 10mM DTT, 2.5 mg/ml poly(EY), 5 µg/ml bio-poly(EY) and the 2 mM ATP solution was made in 8 mM $MnCl_2$, 20 mM Mg-acetate. For the IGF1R kinase assays the 20 µl substrate solution contained 200 mM HEPES pH 7.4, 2.5 mg/ml poly (EY), 5 µg/ml bio-poly(EY) and the 2 mM ATP solution was made in 10 mM $MgCl_2$ and 20 mM $MnCl_2$. The non-radioactive kinase reactions were carried out at room temperature for 30 minutes (20 minutes for IGF1R) and terminated by the addition of 50 µl of stop solution (250 mM EDTA in 20 mM HEPES pH 7.4). 100 µl were transferred to a streptavidin-coated microtiter plate, after an incubation time of 60 min at room temperature the plate was washed with 200 µl of wash solution (50 mM Tris, 0,05% Tween20). 100 µl of an HRPO- labeled anti-PY antibody (PY20H Anti-PY:HRP supplied by Transduction Laboratories, USA) 250 ng/ml were added. After a 60 min incubation the plate was washed three times with a 200 µl- wash solution. The samples were then developed with a 100 µl Substrate Solution (v/v, 1:1, supplied by Bender MedSystems, Austria; catalogue BMS402 and BMS403). The reaction was stopped after 10 min with 100 µl 1M phosphoric acid. The plate was transferred to an ELISA reader and extinction was measured at OD_{450nm}.

The data generated with these assays were analyzed with the program PRISM v3.0 (GraphPad Software Inc. San Diego, CA). The inhibitor concentrations were transformed to logarithmic values and the raw data were normalized. These normalized values were used to calculate the IC_{50} by a nonlinear regression curve fit (sigmoidal dose-response, variable slope). All iteration data had a correlation coefficient above 0.9 and top and bottom values of the curves displayed a window of at least 5.

EGFR and pEGFR ELISA assays - EGF receptor phosphorylation was assessed in A431 cells using a commercially available ELISA kit (QIA95, Oncogene Research Products, San Diego, CA) according to the manufacturer's protocol with minor modifications. In brief, $1x10^4$ A431-cells were transferred into each well of a 96-well microtiter plate in 90 µl serum-free culture medium. Cells were incubated at 37 °C and 5 % CO_2 in a humidified atmosphere overnight. On the next day, 10 µl of each test compound concentration were added to duplicate wells. Each microtiter plate contained internal controls, such as blank, maximum reaction and historical reference compound, for the qualification of the results. The microtiter plates were then incubated at 37 °C and 5% CO_2 in a humidified atmosphere for one hour. EGF-stimulation was done at a final concentration of 100 ng/ml for 10 minutes at room temperature. Cells were washed two times with 200 µl/well ice cold PBS before addition of 120 µl/well receptor extraction buffer provided in the ELISA kit and shaking for one hour at room temperature. After extraction, a 100 µl of cell extract were transferred to the assay plate provided in the kit. After one hour incubation at room temperature the plate was washed three times with water before addition of the HRP-conjugate. The bound antibody was then detected with TMB substrate solution.

Coloring was stopped with 100 µl of stop solution. The extinction was measured at 450 nm. All incubation steps were performed on a microtiter plate shaker.

Expression of the EGF receptor was determined with a commercially available assay (QIA35, Oncogene Research Products, San Diego, CA) and the A431 extracts were diluted with the sample diluent included in the kit and processed as described in the manufacturer's protocol. Each dilution was measured in duplicates.

The data generated with both assays were analyzed with PRISM as described above.

Thymidine incorporation assays - Subconfluent KB cultures were used for the assays. Cells were trypsinized, washed twice in serum free culture medium and seeded in 200 µl medium at a density of 1500 cells per well in a flat bottom 96 well culture dish. A 10 µl aliquot of either 200 µM or 60 µM compound dilution made in PBS was added to the respective first well and serially transferred between the wells. All samples were prepared in triplicates. Compounds were tested between 3 and 10000 nM. EGF-stimulated cells received 30 ng/ml EGF which was determined to be the optimal dose in these assays; serum-stimulated cells received 0.5% FCS; unstimulated controls received neither EGF nor serum. After addition of the inhibitors and growth factors, the cells were incubated for 76 hours, then 3H-thymidine (0,1 µCi/well) was added. After an additional 16 hours, the cells were harvested on glass fiber filters and the incorporated radioactivity was determined by scintillation counting using a Top Count (Packard, Meriden, CT). To determine inhibition of thymidine incorporation by EGFR inhibitors, the difference between the mean counts of stimulated and unstimulated control wells was set to a 100%. Counts incorporated by unstimulated samples were set to 0%. The stimulation of compound-treated samples

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was expressed in % of the full stimulation. The inhibitor concentrations were transformed to logarithmic values. The percent-stimulation values were used to calculate the IC_{50} by iteration using a nonlinear regression curve fit program (PRISM (sigmoidal dose-response with variable slope).

In Vivo xenograft experiments - Five to six-week-old athymic NMRI-nu/nu female mice (21-31 g) were purchased from Harlan (Germany) and maintained under specific pathogen-free conditions. All experiments complied with the Declaration of Helsinki and European Policy Legislations (FELASA and GV-SOLAS) on the Care and Use of Laboratory Animals. After acclimatization mice were inoculated s.c. with 1 x 10^6 (in 100 µl) A431, FaDu or HN5 cells into the right flank of the animal. After 7-11 days tumors reached a average volume of 40-130 mm^3. The mice were randomized and treated daily p.o. with BIBX1382, BIBU1361 or vehicle control on the basis of individual weights. Tumors were measured 3 times a week with calipers, and tumor volumes were calculated by the formula π/6 x length x $(width)^2$. Experimental compounds were prepared in 25% aqueous hydroxypropyl-ß-cyclodextrin (Aldrich # 33259-3, St. Louis, MO) and administered by intragastral gavage. The administration volume was 10 ml/kg body weight.

Determination of pEGFR level in tumor samples- Excised A431 tumors were snap frozen in liquid nitrogen immediately after dissection. The tumors were lyophilized over night and extracted with HEPEX (20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM ß-glycerophosphate, 10 mM para-nitro-phenylphosphate, 30 mM NaF, 5 mM EDTA, 5% glycerol, 1% Triton X-100, 1 mM Na3VO4, 0.1% SDS, 0.5 µg/ml pepstatin A, 2.5 µg/ml 3,4-dichloroisocoumarin, 2.5 µg/ml Trans-epoxysuccinyl-L-

leucyl-L-amido butane, aprotinin 20 KIU/ml, leupeptin 2 µg/ml, benzamidine 1 mM and 0.002% PMSF). The EGFR and pEGFR levels were determined using the ELISA procedures described above (QIA95 (pEGFR) and QIA35 (EGFR), Oncogene Research Products, San Diego, CA). In order to asses the PY-EGFR ratio (PY-EGFR units/ EGFR molecule) an in-house standard of EGF-stimulated HN5 cells was used. The EGFR concentration of this standard was 8560 fmol/ml as determined by QIA35. The PY-EGFR concentration was arbitrarily assigned a concentration of $2.8x10^{15}$ PY units/ml. The PY-EGFR /EFGR ratio was calculated from the results of the PY-EGFR QIA95 ELISA (expressed in units/ml) and the EGFR QIA35 ELISA (expressed in fmol/ml) and expressed as arbitrary PY-units/EGFR molecule.

Histology and Immunohistochemistry - The proliferation of tumor cells in samples was assessed by Ki-67 immunostaining. Frozen tumor tissues were paraffin embedded, sectioned to 3-5 µm in thickness deparaffinized and subjected to immunohistochemistry protocol as previously described (Heider et al., 1993b;Heider et al., 1993a). For Ki-67 detection an affinity purified rabbit anti-human Ki-67 serum was applied (DAKO, Glostrup, Denmark). As secondary antibody either a biotinylated swine anti-rabbit or rabbit anti-mouse antibody was used, followed by incubation with the avidin-biotin-peroxidase complex (DAKO, Glostrup, Denmark). After color development in AEC the slides were counterstained with hematoxylin and mounted with aqueous mounting medium. For evaluation of histology, H&E stained serial sections were used.

Results

Potency and selectivity of BIBX1382 and BIBU1361 in molecular assays -

The data presented in Table 1 demonstrates that BIBX1382 and BIBU1361 are both potent and selective sub-micromolar inhibitors of the EGFR kinase activity. An IC_{50} value of 3 nM was determined for both compounds. The potency of these two compounds compares to the one obtained with Iressa which is a leading EGFR inhibitor in the field. Inhibition of the closest family member, HER2, was 100 to 1000-fold less potent. Furthermore, BIBX1382 and BIBU1361 did not inhibit a number of other related tyrosine kinases.

Inhibition of EGFR-autophosphorylation in cells -

The inhibitory activity of these two compounds was confirmed at the cellular level. A431 cells which highly express the EGF receptor, were used to examine the effect of BIBU1361 and BIBX1382 on EGF-induced EGFR phosphorylation. A 1-hour pre-treatment of the cells with either compound inhibited EGF-induced phosphorylation of EGFR in a dose dependent manner. For BIBU1361 the EC_{50} was 122 nM. As a control, the EGF receptor expression was monitored and showed no variation in this experimental setup (Figure 1). Under the same conditions, the drug concentrations required for half-maximal inhibition by BIBX1382 and Iressa were 141 nM and 86 nM respectively (data not shown). Thus this data demonstrates that both pyrimidopyrimidines reach their target in the cell and that the potency compares to that of Iressa.

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Effects of BIBU1361 on downstream signaling -

After showing that BIBU1361 is capable of inhibiting the activation of the EGF receptor we further evaluated whether this compound would also abrogate downstream signaling events. Several signaling pathways that control the G_1/S transition are activated in response to EGF in A431 cells. Therefore, the effect of BIBU1361 on one such pathway (activation of the MAPK signaling) was analyzed in more details by means of electrophoretic analyses. An aliquot of the extracts used for the autophosphorylation assays was loaded on SDS PAGE and blotted onto PVDF for Western blot analyses. In line with its ability to prevent EGFR activation, BIBU1361 also suppressed the activation of MEK, a central molecule in the MAPK cascade (Figure 2). Pre-treatment of A431 cells with 313 nM resulted in nearly complete inhibition of phospho-MEK signal. While MAPK is still activated in the starved A431 cells pre-treatment for 1 hour with 1250 nM BIBU1361 induced complete dephosphorylation of this molecule.

In line with the previous results, treatment of cells with either BIBX1382 or BIBU1361 suppresses EGF-induced thymidine incorporation in KB cells in a dose dependent manner. For BIBX1382 the EC_{50} value is 150 nM. Pre-treatment of KB cells with 400 nM resulted in almost complete inhibition of new DNA synthesis. Similarly, the EC_{50} value for BIBU1361 was 225 nM (data not shown). In comparison, the FCS dependent incorporation was inhibited with an EC_{50} value of 3195 nM (Figure 3). Thymidine incorporation can also be stimulated by HGF in this cell line. The EC_{50} was 1815 nM (data not shown). Taken together, the cellular data shows that BIBX1382 and BIBU1361 are potent and selective inhibitors of EGF-induced DNA synthesis.

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Anti-tumor activity of BIBX1382 and BIBU1361 -

The anti-tumor activity of BIBX1382 and BIBU1361 was evaluated *in vivo* in various xenograft models including A431, HN5 and FaDu. In nude mice, oral once daily dosing at 10 mg/kg with either BIBX1382 or BIBU1361 completely suppressed tumor growth of human A431 xenografts with respective T/C values of 15% and 6% after 2 weeks of treatment (Figure 4). The average tumor volume of the control group had reached at least 1000 mm^3. On the last treatment-day, plasma samples were obtained 4, 8 and 24 hours after application. Both compounds reached their maximum 4 hours after administration. The C_{4h} values were 2222 nM (BIBX1382) and 755 nM for BIBU1361. The C_{24h} with 244 nM and 356 nM respectively were in the range of the EC_{50} values in the different cellular assays. No weight loss was observed in treated animals over the course of the 2 week treatment period. Hence, this data demonstrates that both compounds at a daily dose of 10 mg/kg are well tolerated in mice and display anti-tumor activity as single agents.

BIBX1382 and BIBU1361 were concurrently evaluated in additional tumor models. The data in Table 2 shows that both compounds also suppress tumor growth of additional xenograft models thus confirming their anti-tumor efficacy. The effect of BIBX1382 is dose dependent in A431, FaDu and HN5 xenografts. Dose dependency was observed in the A431 model for BIBU1361.

The anti-neoplastic potential of BIBX1382 was further explored in a long-term oral administration schedule. A431-tumor bearing mice were treated once daily for 170 days at a dose of 50 mg/kg. The body weights were stable and no adverse clinical signs could be observed in the treated animals for the whole treatment period. Tumor regressions were obtained in all treated individuals (n = 7) after 90 days. While two of the animals were tumor free at the end of the experiment, and this even

after the 100-day recovery period, two of the tumors started to regrow after cessation of treatment and 3 tumors did not significantly progress in size during the experiment. Thus, this data demonstrates the feasibility of long-term treatment schedules with BIBX1382.

Effects of BIBX1382 on tumor cells in vivo -

In order to correlate the anti-tumor activity of these compounds with biomarker modulation, we investigated *in vivo,* the ability of BIBX1382 to inhibit EGFR phosphorylation in tumors derived from A431-xenograft bearing mice treated daily p.o. for 14 days, using biochemical and immunohistochemical approaches. The data in Figure 6A shows that the EGF receptor is constitutively phosphorylated in control A431 tumors. Treatment of mice, carrying established A431 tumors, at a dose of 50 mg/kg/day results in dephosphorylation of the EGF receptor (figure 6A). Thus the anti-tumor activity of BIBX1382 coincides with inhibition of the constitutive tyrosine phosphorylation of the EGF receptor molecule in A431 xenografts.

The anti-proliferative effect of BIBX1382 was confirmed *in vivo* by assessing tumor cell proliferation as determined by Ki-67 immunostaining of tumor tissue. A431 xenografts were treated daily p.o. with vehicle control or 30 mg/kg BIBX1382 for two weeks. The formalin fixed and paraffin embedded tumor tissues were then subjected to Ki-67 immunohistochemistry (see materials and methods). The counts of proliferative tumor cells in treated tissues were significantly decreased compared to vehicle-treated control tumors. The microphotographs of Ki-67 immunostaining illustrating a reduction in proliferation are shown in figure 6B and 6C. A similar reduction in Ki-67 staining was observed with BIBU1361 (data not shown). This data demonstrates that BIBX1382 and BIBU1361 inhibit tumor cell proliferation *in vivo*.

Discussion

Excess EGFR-signaling is implicated in the development of a significant proportion of human carcinomas and glioblastomas. Therefore, inhibition of EGFR-signaling has been viewed as a promising approach for the treatment of EGFR-positive human tumors. Both, anti-EGFR antibodies and small-molecule EGFR tyrosine kinase inhibitors are being actively tested in clinical trials (Grunwald and Hidalgo, 2003).

Here we report about a new structural class of small-molecule EGFR tyrosine kinase inhibitors, namely pyrimido-[5,4-d]-pyrimidines. BIBX1382 and BIBU1361 are two examples of this class and inhibit the EGFR tyrosine kinase activity *in vitro* in the low nanomolar range. This potency is comparable to other small molecule inhibitors derived from other structural classes (Table 1 and (Traxler, 2003)). Relative to 4 distinct members of the tyrosine kinase family, both compounds appear to be selective for the class-I receptors. Furthermore, at a concentration of 10 μM BIBX1382 and BIBU1361 failed to inhibit the kinase activities of a number of serine/threonine kinases comprising MEK, Phosphorylase kinase, protein kinase A and stress activated protein kinase 2α (data not shown) thus confirming the selectivity of BIBX1382 and BIBU1361 for the EGFR kinase. Since all the kinase assays were performed at saturating ATP concentrations cellular activity for these compounds could be anticipated.

Indeed, BIBX1382 and BIBU1361 in the nanomolar range, could interfere with the EGF-induced EGFR autophosphorylation in intact A431 cells (Figure 1). In accordance with this observation, BIBU1361 also blocked downstream EGFR signaling events. Many intracellular proteins get phosphorylated in response to mitogenic signals such as EGF. Some examples are PLCγ, PI3K, SHC or MEK. In some cells constitutive activation (phosphorylation) of these proteins has been linked

to the transformed phenotype. Thus, a reduction in the phosphorylation status in one of these signal transducers induced by BIBU1361 could suggest an antiproliferative effect of this compound. Therefore, we analyzed the effect of BIBU1361, 10 min after EGF addition, on the activation of MEK and MAPK. As shown in Figure 2 BIBU1361 at concentrations of 313 nM completely blocked activation of MEK. In mitogenic assays performed with KB cells (Fig. 3), BIBU1361 as well as BIBX1382 demonstrated antiproliferative activity. The EC_{50} values for the two compounds were in the nanomolar range when EGF was used as a mitogenic agent. The maintained capacity of KB cells for proliferation in response to HGF or FCS in the presence of micromolar concentrations of either BIBU1361 or BIBX1382 confirms the selectivity of these compounds. Taken together, the cellular data show that BIBX1382 and BIBU1361 are potent and selective inhibitors of the EGFR pathway.

Both, BIBX1382 and BIBU1361, in mice and rats show serum concentrations of the compounds which are sufficient to achieve therapeutic efficacy. The ability of BIBX1382 and BIBU1361 to inhibit either A431 or HN5 tumor growth is similar to that reported for other EGFR inhibitors currently in clinical trials (Fan et al., 1993;Modjtahedi et al., 1998). Treatment for 14 days, at doses ranging between 10 and 30 mg/kg/day resulted in complete tumor growth suppression in these models (Figure 4 and Table 3).

To further explore the potential of these compounds as anti-cancer agents a long-term treatment study with BIBX1382 was initiated. Mice bearing A431 tumors were treated daily for a period of 170 days. Tumor regressions were observed in all treated individuals after 90 days (Figure 5). Therefore, this data could suggest that sustained long-term inhibition of EGFR signaling in A431 tumors is required for a curative

effect. Indeed, in five out of seven individuals, tumors had either disappeared or remained growth suppressed even during the 100 day treatment-free period. It has to be acknowledged that 2 of the tumors did regrow after termination of treatment. Interestingly, one of these tumors when passaged into mice responded again to BIBX1382 treatment (data not shown). This data also clearly demonstrates that, in mice, BIBX1382 can be given for an extended period of time with good tolerability.

In the 14-day A431 xenograft experiments, determination of the plasma concentrations of BIBX1382 and BIBU1361 on the last day of treatment, 24 hours post application, revealed that the minimum plasma levels were 244 and 356 nM respectively. It is noteworthy that in the cellular assays described herein such concentrations resulted in nearly complete inhibition of EGFR dependent processes. Therefore, this observation suggests that *in vivo* the EGF dependent processes are completely and permanently inhibited by BIBX1382 and BIBU1361 over a 24-hour period. The biochemical data shown in Figure 6A is consistent with this observation. Indeed, a 2-week treatment of mice carrying established A431 tumors with BIBX1382 induced dephosphorylation of constitutively activated EGFR. Because of its ability to block early EGFR signaling *in vivo*, it was anticipated that BIBX1382 would also inhibit tumor cell proliferation in the tumor. Ki-67 immunostaining was used to visualize proliferating cells *in vivo*. In the treated samples (Figure 6C) a reduction of stained cells was evident.

In conclusion, we have shown that pyrimidopyrimidines, exemplified here by BIBX1382 and BIBU1361, are potent inhibitors of EGFR-mediated signaling in vitro. In vivo, both compounds demonstrate anti-tumor activity in xenograft models such as A431, FaDu and HN5. All these models have proven to be reliable predictors for class-I receptor targeted therapeutics such as *erlotinib* (Pollack et al., 1999), *Iressa*

(Sirotnak et al., 2000), *canertinib* (Nelson and Fry, 2001) or *lapatinib* (Rusnak et al., 2001) which all have advanced to clinical trials. The results described above also show that BIBX1382 is well tolerated in long term treatment regimens in animal models with sufficient exposure. Unfortunately, BIBX1382 which was tested in clinical phase I studies did not show acceptable plasma exposure in humans. The reason for this behavior has been attributed to a first path metabolism (Dittrich et al., 2002) and the enzymes involved have been resolved and are the subject of another publication. Unlike BIBX1382, BIBU1361 is not a substrate for this enzyme and therefore plasma exposure in humans is expected. Thus, pyrimidopyrimidines, in principle can be viewed as an attractive structural class for the development anti-cancer agents for patients whose cancers display aberrant EGFR signaling. Attention should be paid to first path metabolism effects as demonstrated for BIBX1382.

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Legends for Figures

Figure 1. - Inhibition of EGF-induced EGFR tyrosine autophosphorylation by BIBU1361. (A) A431 cells were serum starved overnight to induce EGFR dephosphorylation. On the next day cells were treated for 1 hour with BIBU1361 at indicated concentrations before stimulation with EGF (100 ng/ml). After a 10 min. stimulation the amount of tyrosine phosphorylated EGFR was determined as described in "Materials and Methods". Three experiments were performed and confirmed the nanomolar potency of BIBU1361 in this assay. (B) The data in the insert shows the concentration of EGFR and demonstrates that EGFR expression is not altered during the experiment.

Figure 2. - Inhibition of EGF-induced signaling by BIBU1361 in A431 cells.
A431 cells were incubated for 1 hour with BIBU1361 at various concentrations and subsequently stimulated with EGF for 10 minutes. After treatment the cells were extracted and aliquots were subjected to SDS-PAGE electrophoresis before blotting onto PVDF membranes. A Western blot analysis revealing MEK (middle panel), phosphorylated MEK (pMEK) and MAPK (pMAPK) is shown in the figure below. The dot in the upper left corner of the pMEK and MEK panels is due to unspecific staining.

Figure 3. - Inhibition of thymidine incorporation into KB cells. KB cells were seeded at a density of 1500 cells per well in a flat bottom 96 well culture dish, supplemented with BIBU1361 at indicated concentrations and maintained for 3 days at 37ºC. After adding 0.1 µCi [^{3}H] thymidine to each well, the cells were stimulated either with EGF (30 ng/ml) or with 0.5% FCS. Thymidine incorporation was determined by scintillation counting whereby the difference between the mean counts of stimulated and unstimulated wells was set to a 100%.

Figure 4. - Inhibition of A431 tumor growth in nude mice by BIBX1382 and BIBU1361. After establishment of tumors the animals were treated daily p.o. for 2 weeks with either 10 mg/kg BIBX1382 (○), 10 mg/kg BIBU1361 (■) or vehicle alone (●). Tumor size was measured at indicated time points using electronic calipers. In the graph, the average tumor volume ± standard deviation is plotted over time. A p value < 0.01 was determined by statistical analysis of the tumor volumes in each group using one way ANOVA (Dunnett's multiple test) (see also table 2).

Figure 5. - Inhibition of A431 tumor growth in nude mice by BIBX1382. After establishment of tumors the animals were treated daily p.o. for 170 days with 50 mg/kg BIBX1382 (○) or with vehicle alone (●). Tumor size was measured at indicated time points using electronic calipers. The relative tumor volume of each individual mouse is plotted over time. The blue curves describe tumors that regrow after cessation of treatment. The green curves show tumors that remain constant while the red curves depict tumors that disappeared.

Figure 6. - In vivo modulation of phosphorylated EGFR and Ki-67 nuclear antigen by BIBX1382 in A431 xenografts. Animals carrying established A431 tumors were treated daily p.o. with either 50 mg/kg (A) or 30 mg/kg (C) of BIBX1382 for 2 weeks. At the end of the experiment tumors were excised 6 hour after the last application and subjected to biochemical analysis for activated EGFR (panel A) or immunohistochemical analysis for Ki-67 nuclear antigen (B, control; C, treated). The PY-EGFR/EGFR ratio was determined as described in Materials and Methods. The data was gathered from 4 vehicle-treated animals (control) and 4 BIBX1382-treated animals. The reduction in pEGFR level was highly significant (p=0.0011; Unpaired T-test). Reduced pEGFR staining is paralleled by reduced Ki-67 stain on tumor cells (C, arrows). In addition, occasional Ki-67 reactivity with

host stromal cells can be observed. Original magnifications were 200x (B, C). The structural elements were revealed by hematoxylin counter stain.

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TABLES

Table 1. - Inhibitory activity of BIBU1361 and BIBX1382 on the tyrosine kinase activity of various protein kinases. The IC_{50} values were generated from 11 point dose-response curves and are given in nM. The IC_{50} values were determined as described in "Materials and Methods“ and confirmed in at least 3 independent experiments. The IC_{50} values for Iressa were determined for comparison.

Kinases	BIBU1361	BIBX1382	Iressa
EGFR	3	3	4
HER2	290	3400	1830
IGF1R	> 10'000	> 10'000	> 10'000
ß-InsRK	> 10'000	> 10'000	> 10'000
HGFR	> 10'000	> 10'000	> 10'000
c-src	> 10'000	> 10'000	> 10'000
VEGFR-2	> 10'000	> 10'000	> 10'000

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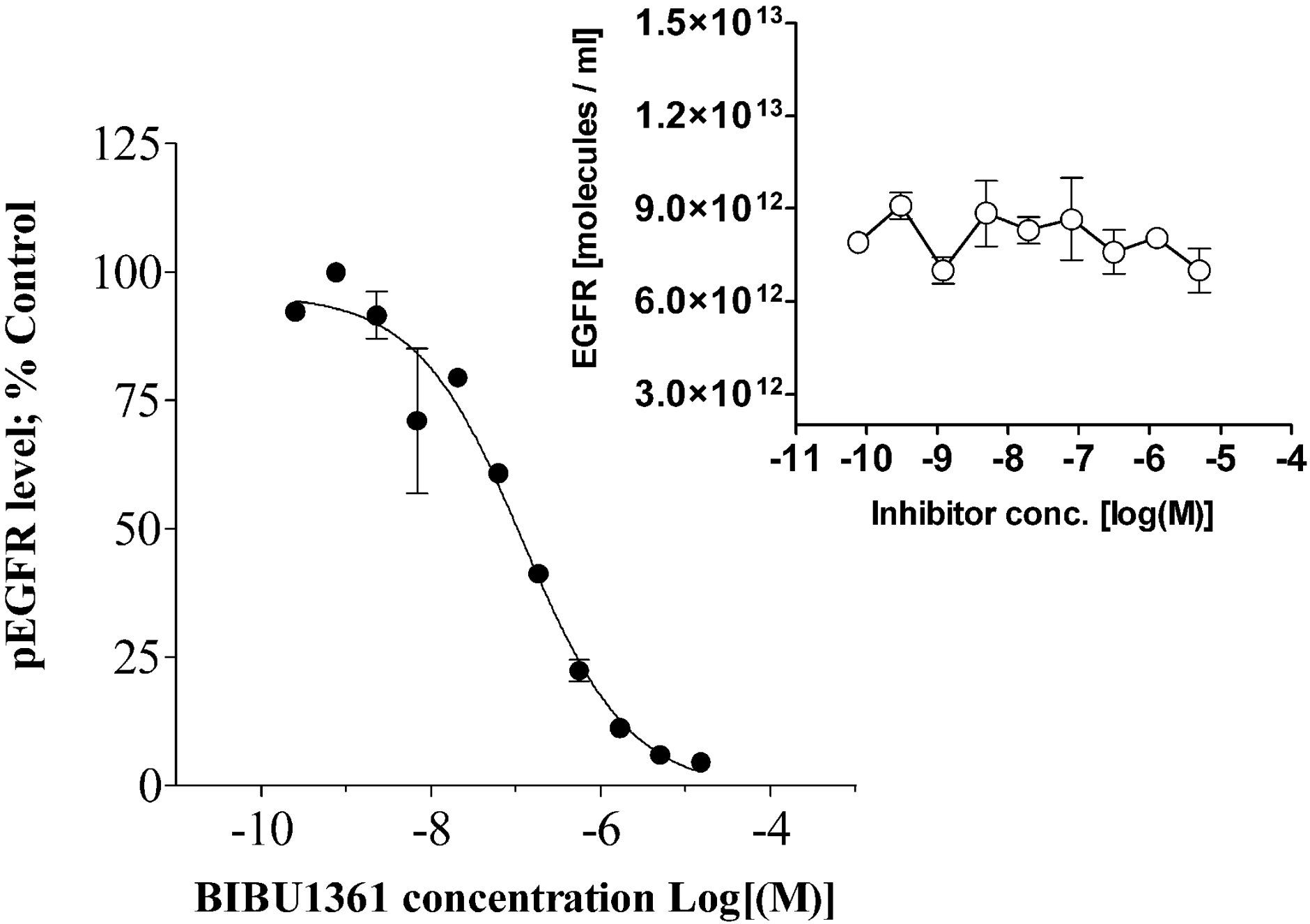
Table 2. - Anti-tumor activity of BIBX1382 and BIBU1361 in various xenografts models in nude mice. After establishment of tumors the animals were treated daily p.o. with different doses of BIBX1382 or BIBU1361. When average tumor volume of the control group had reached 1000-1500 mm^3, tumor volumes of the treated animals were compared to the controls (T/C [%]). The numbers in parentheses represent the number of animals in each group.

		A431	**FaDu**	**HN5**
Compound	**Dose [mg/kg/d]**	**T/C [%]**	**T/C [%]**	**T/C [%]**
BIBX1382	70	5** (5)	-	5 n.s. (7)
	60	-	11* (5)	-
	40	5** (5)	-	-
	10	15** (5)	100 n.s. (3)	21 n.s. (5)
BIBU1361	30	3** (5)	-	6** (10)
	10	6** (5)	-	-
	3	25** (10)	-	-

** $p<0.01$
* $p<0.05$
n.s. not significant



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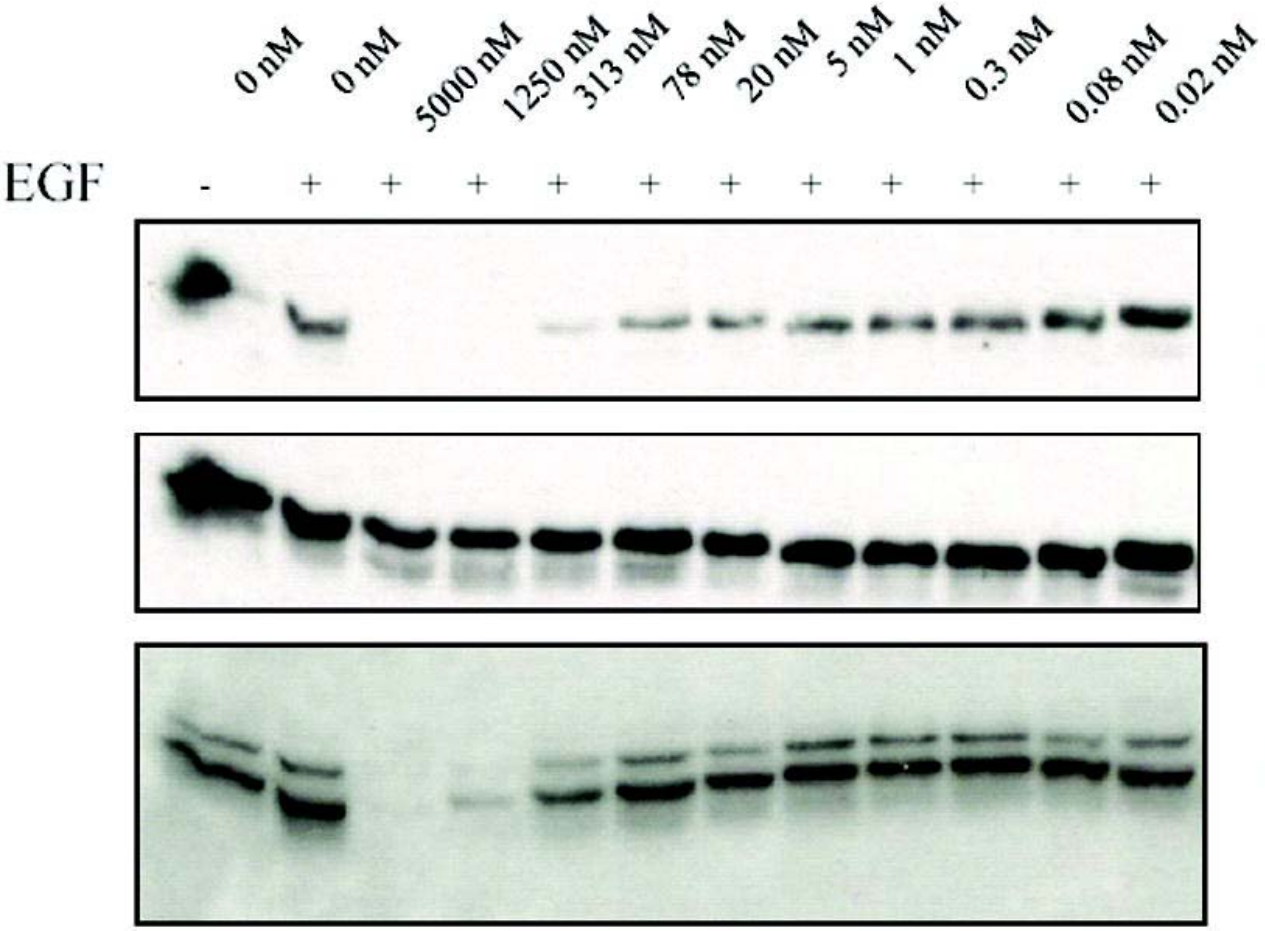

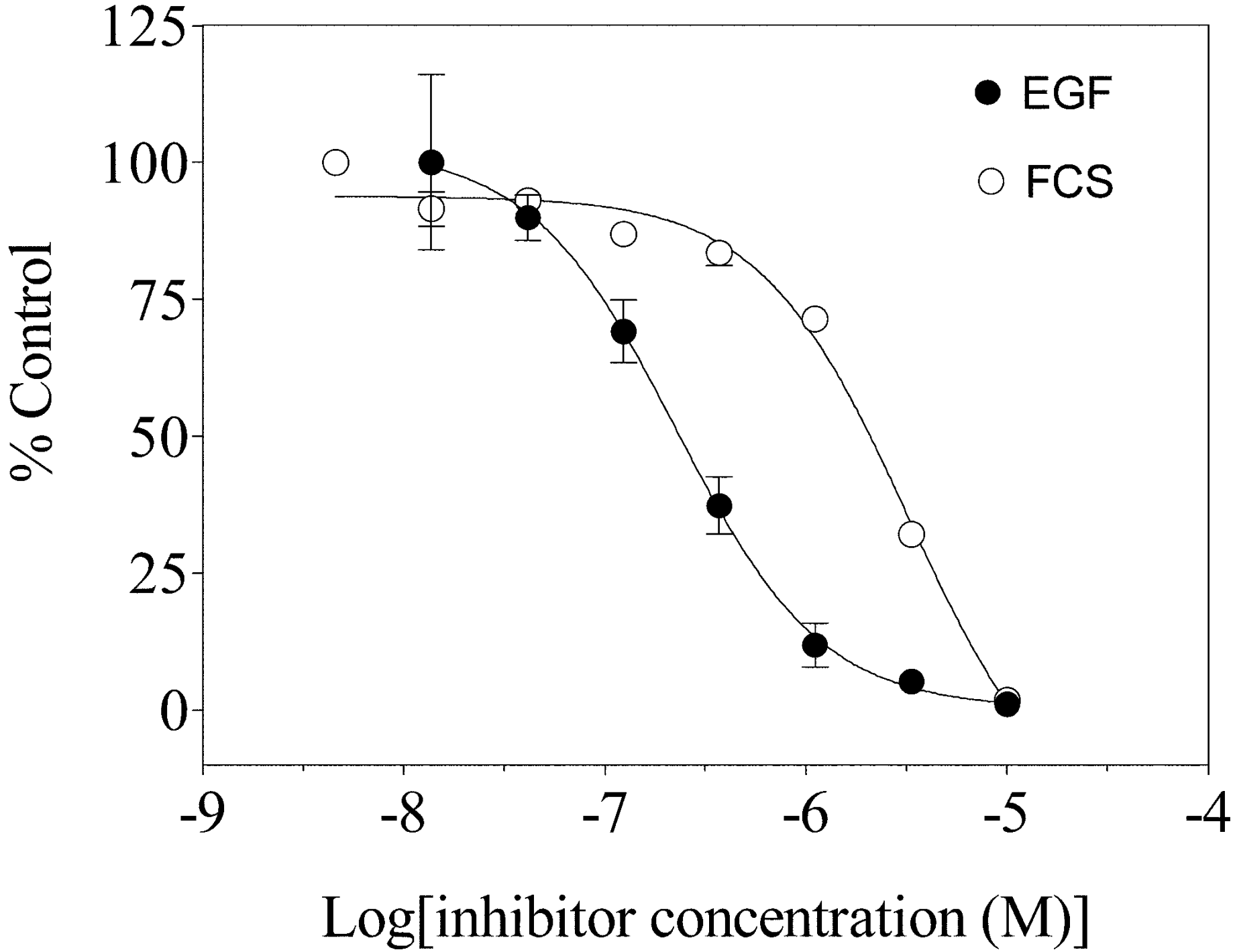

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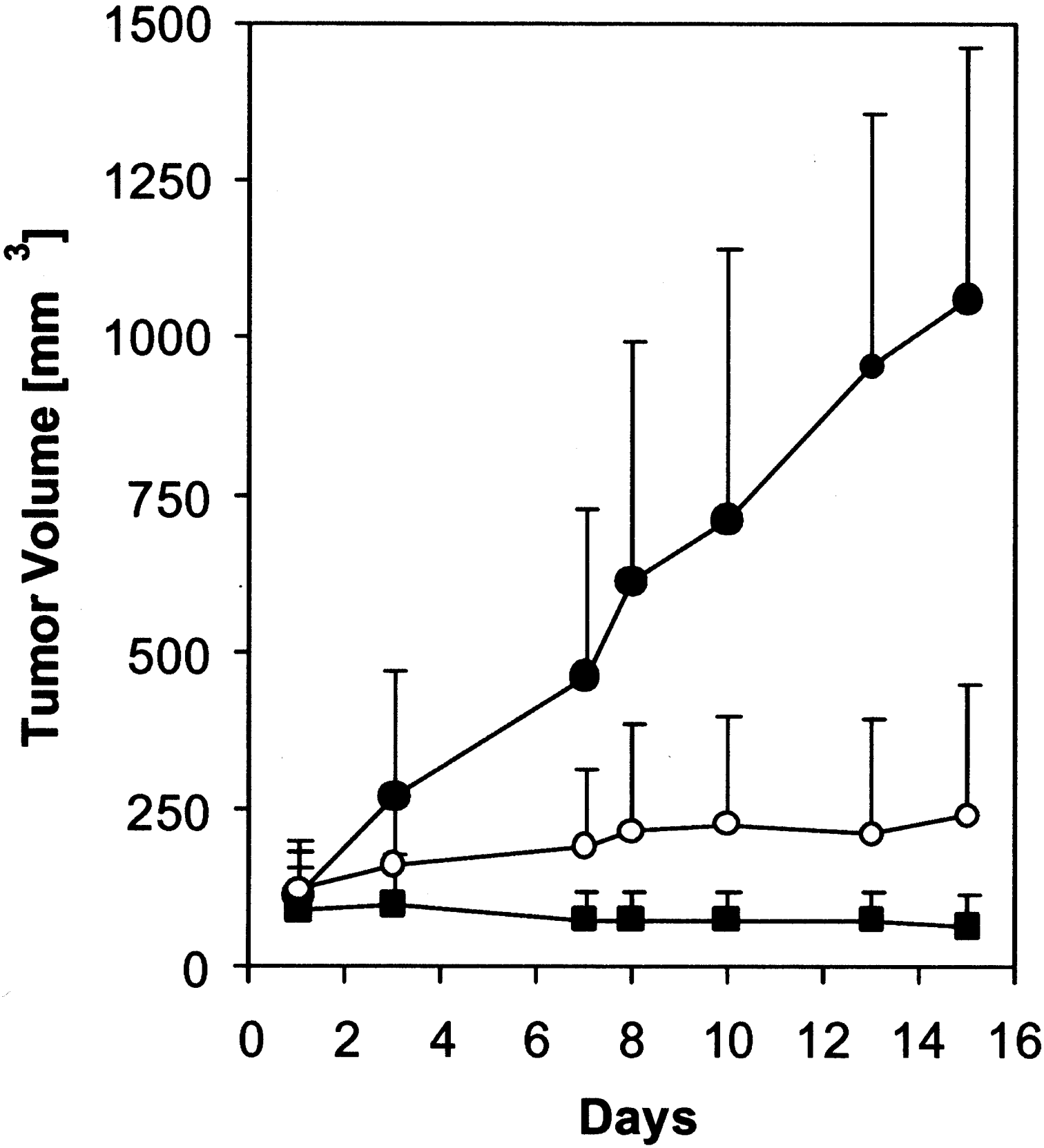

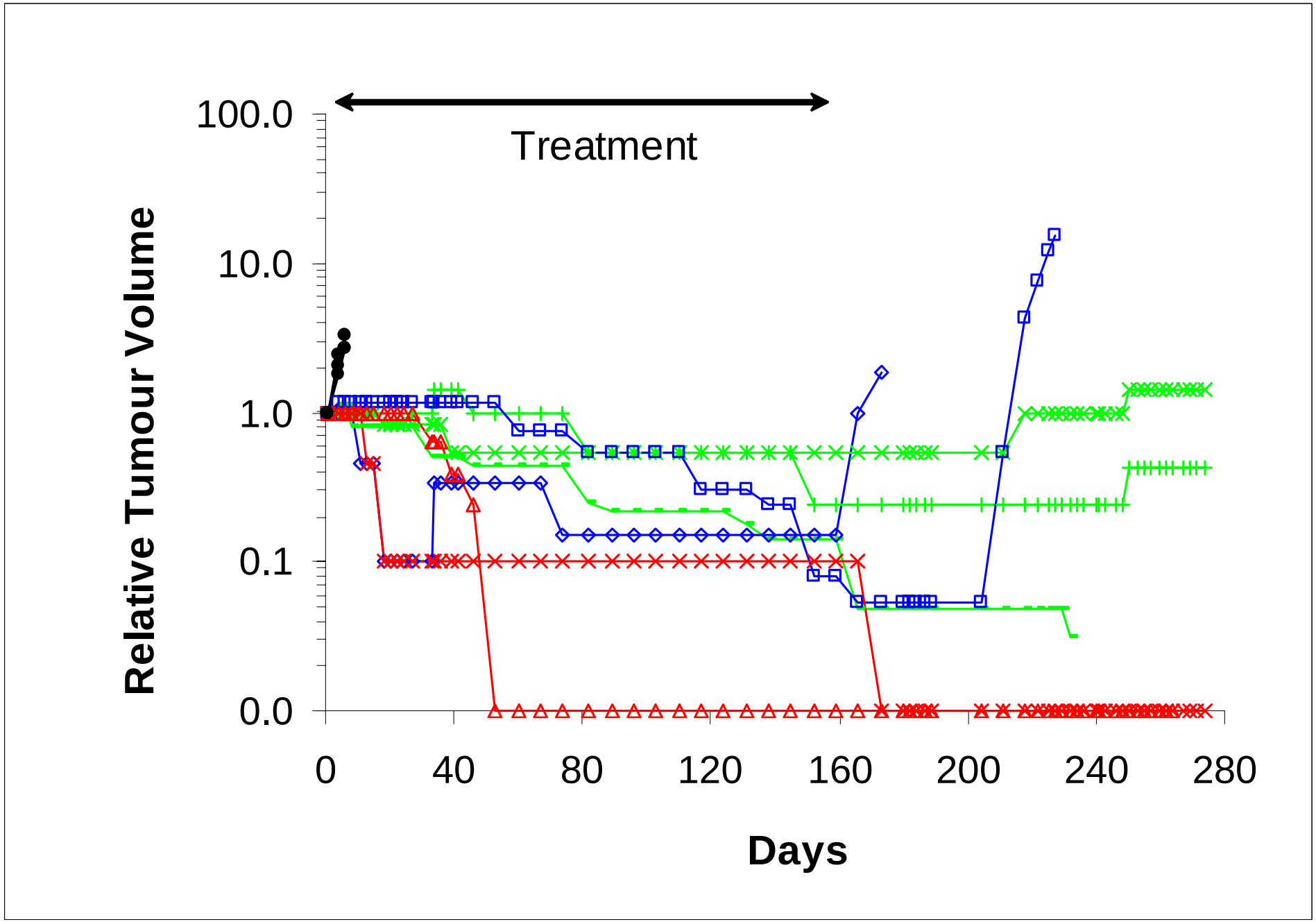

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