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Chronic pharmacologic inhibition of EGFR leads to cardiac dysfunction in C57BL/6J mice

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Abstract

Molecule-targeted therapies like those against the epidermal growth factor receptor (EGFR) are becoming widely used in the oncology clinic. With improvements in treatment efficacy, many cancers are being treated as chronic diseases, with patients having prolonged exposure to several therapies that were previously only given acutely. The consequence of chronic suppression of EGFR activity may lead to unexpected toxicities like altered cardiac physiology, a common organ site for adverse drug effects. To explore this possibility, we treated C57BL/6J (B6) mice with two EGFR small molecule tyrosine kinase inhibitors (TKIs), irreversible EKB-569 and reversible AG-1478, orally for three months. In B6 female mice, chronic exposure to both TKIs depressed body weight gain and caused significant changes in left ventricular (LV) wall thickness and cardiac function. No significant differences were observed in heart weight or cardiomyocyte size but histological analysis revealed an increase in fibrosis and in the numbers of TUNEL-positive cells in the hearts from treated female mice. Consistent with histological results, LV apoptotic gene expression was altered, with significant downregulation of the anti-apoptotic gene *Bcl2l1*. Although there were no significant differences in any of these endpoints in treated male mice, suggesting sex may influence susceptibility to TKI mediated toxicity, the LVs of treated male mice had significant upregulation of *Egf*, *Erbb2* and *Nppb* over controls. Taken together, these data suggest that chronic dietary exposure to TKIs may result in pathological and physiological changes in the heart.

Keywords

Animal model; growth factors; EGFR; cardiotoxicity

Introduction

The epidermal growth factor receptor (EGFR) is the prototypical member of the ERBB family of receptor tyrosine kinases, which also includes ERBB2, ERBB3 and ERBB4 (Wells, 1999).

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Ligand-binding induces receptor homo- or heterodimerization with subsequent phosphorylation of tyrosine residues in the carboxy-terminal tail creating docking sites that initiates intracellular signaling cascades (Schlessinger, 2000). It has been estimated that increased or constitutive signaling through EGFR occurs in approximately one third of all human neoplasms; moreover, aberrant signaling is associated with poor prognosis including non-responsiveness to traditional chemotherapy and decreased survival (Salomon *et al*., 1995; Woodburn, 1999; Nicholson *et al*., 2001).

Since the EGFR was first proposed as a cancer drug target almost twenty years ago (Sato *et al*., 1983; Gill *et al*., 1984; Masui *et al*., 1984), advances in drug discovery have produced a plethora of inhibitors targeting the receptor. In particular, tyrosine kinase inhibitors (TKIs), which block EGFR activity by competing with adenosine triphosphate (ATP) for binding to the receptor's kinase pocket, have shown efficacy for several cancer types (Jimeno and Hidalgo, 2006). Two EGFR TKIs, Gefitinib/Iressa, (AstraZeneca) and Erlotinib/Tarceva, (OSI Pharmaceuticals), have received regulatory approval for use in cancer patients while several others are being evaluated in ongoing clinical trials as mono or combinatorial therapies (Marshall, 2006; Steeghs *et al*., 2007). With the enormous strides that have been made in cancer therapy, and the resultant increases in life expectancy after diagnoses, certain cancers are now perceived and treated as chronic, rather than terminal, diseases (Disis and Rivkin, 2003; Michener and Belinson, 2005; Markman, 2006; Burton *et al*., 2007; Snyderman, 2007). Although the side effects of targeted therapies like TKIs are considered mild compared to traditional chemotherapeutics, patients may now be exposed to these drugs for years rather than months. However, the long-term physiological consequences of suppressed EGFR activity are unknown.

A wealth of evidence has established that all four ERBB family members are essential to normal cardiovascular development (Gassmann *et al*., 1995; Lee *et al*., 1995; Erickson *et al*., 1997; Chen *et al*., 2000; Goishi *et al*., 2003; Iwamoto *et al*., 2003; Jackson *et al*., 2003; Negro *et al*., 2004). A role for ERBB signaling in adult cardiac homeostasis is also emerging. Three of the four receptors, EGFR, ERBB2, and ERBB4, are detected in the adult human and mouse heart; among these ERBB4 appears to be the most abundant (Srinivasan *et al*., 1998; Zhao *et al*., 1998; Zhao *et al*., 1999; Fuchs *et al*., 2003). The expression and activity of ERBB2 and ERBB4 receptors are depressed in clinical and experimentally-induced heart failure (Rohrbach *et al*., 1999; Uray *et al*., 2002; Rohrbach *et al*., 2005) and signaling via NRG1 to ERBB2/ERBB4 heterodimers is critical for adult cardiomyocyte survival (Zhao *et al*., 1998; Schneider *et al*., 2001; Fukazawa *et al*., 2003; Liu *et al*., 2006). The importance of this signaling pathway in normal cardiac physiology was not fully recognized until the unexpected and lethal cardiomyopathy reported in breast cancer clinical trials using trastuzmab (Herceptin, Genetech), a humanized monoclonal antibody targeting ERBB2 (Ewer *et al*., 1999; Schaller *et al*., 1999; Schneider *et al*., 2001; Schneider *et al*., 2002). Subsequently, mouse models with ventricular specific deletion of ERBB2 or ERBB4 were found to recapitulate the cardiac phenotype observed in clinical trials (Crone *et al*., 2002; Ozcelik *et al*., 2002; Garcia-Rivello *et al*., 2005). More recently, signaling through EGFR was shown to provide cardioprotection against stress-induced injury, and reduction in EGFR activity impacts cardiomyocyte hypertrophy and survival (Pareja *et al*., 2003; Chan *et al*., 2006a; Chan *et al*., 2006b; Howes *et al*., 2006; Zhai *et al*., 2006). To date, no *in vivo* studies have specifically assessed the effects of chronically reduced EGFR activity on adult cardiac function, as might be expected with continuous drug exposure to TKIs, despite the fact that mutant mouse models have shown considerable similarities to drug-induced toxicities in the oncology clinic (Roberts *et al*., 2004). To address this question, we used EKB-569 (6,7 disubstituted 4-anilinoquinoline-3-carbonitrile) an EGFR-selective irreversible TKI (Wissner *et al*., 2003; Erlichman *et al*., 2006), and AG-1478 (4-3-chloroamlino-6,7, dimethoxyquinazoline) a reversible TKI also selective for EGFR (Partik *et al*., 1999; Lenferink *et al*., 2000; Zhu *et al*., 2001; Ellis *et al*.,

2006), to assess the effects of chronic oral exposure to these drugs on cardiac function and pathology in wild-type mice.

Materials and methods

1) Animals and pharmacologic treatment

All mice were bred in house or obtained from The Jackson Laboratory. Male and female wild-type C57BL/6J (B6) mice (6-8 weeks of age) were randomly assigned to either AIN-93G control chow (Bioserve) (n=4 males, n=11 females) or AIN-93G chow containing the EGFR small molecule inhibitors EKB-569 (150 mg/kg of food; Wyeth) (n=8 females) or AG-1478 (144 mg/kg of food; LC Laboratories) (n=6 males, n=8 females) equivalent to 20 or 19.2 mg/kg body weight/day, respectively. Mice were weighed and provided diet *ad libitum* for 90 days. Body weights were measured at baseline and 15, 30, 60 and 90 days of treatment. Due to limited availability of EKB-569, studies were only performed in female mice to verify that results obtained with AG-1478 were not specific to one class of inhibitor. Similarly, practical issues imposed by a chronic dietary exposure regimen and the limited supply (EKB-569) or high cost (AG-1478) prohibited studies employing a range of doses via oral delivery. The dose chosen for the present studies was based on those commonly used for cancer inhibitory studies and that required to achieve a 50% reduction in the mean number of polyps using the Apc^{Min} model, a common measure for EGFR inhibitors. In a separate experiment to evaluate efficacy of AG-1478 oral delivery, B6-$Apc^{Min/+}$ weanlings of both sexes were randomly assigned to either AIN-93G control chow (Bioserve) (n=3 males, n=3 females) or AIN-93G chow containing the EGFR small molecule inhibitor AG-1478 (144 mg/kg of food; LC Laboratories) (n=3 males, n=3 females) equivalent to 20 or 19.2 mg/kg body weight/day *ad libitum* until 90 days of age. Mice were genotyped for the Apc^{Min} allele as reported (Dietrich *et al*., 1993). All protocols were approved by the UNC Institutional Animal Care and Use Committee.

Intestinal tumor analysis

At three months of age, B6-$Apc^{Min/+}$ mice were euthanized and gastrointestinal (GI) tracts from pylorus to rectum were removed. The small intestine was cut into thirds, and the caecum and colon were separated. Segments were gently flushed with PBS to remove fecal material, cut longitudinally, splayed flat on Whatmann 3MM paper and fixed overnight at 4°C in 4% paraformaldeyhyde. Polyps were counted and their diameters measured using a dissection microscope with an in-scope micrometer, allowing detection of polyps greater than 0.3 mm in diameter.

Echocardiography

Transthoracic echocardiography (TTE) was performed at baseline and prior to sacrifice using a 30 mHz probe on a Vevo 660 Ultrasonograph (VisualSonics). B6 wild-type mice were lightly anaesthetized with 1-1.5% isofluorane and a topical depilatory agent applied before placing in the left lateral decubitus position under a heat lamp to maintain body temperature at 37°C. Heart rate was maintained between 450 to 500 beats per minute. Two dimensional short and long axis views of the left ventricle were obtained. M-mode tracings were recorded and used to determine left ventricle (LV) end-diastolic diameter (LVED,d), LV end systolic diameter (LVED,s), LV posterior wall thickness diastole (LVPWTh,d) and LV posterior wall thickness systole (LVPWTh,s) over three cardiac cycles. LV fractional shortening was calculated using the formula % FS= (LVED,d-LVED,s)/(LVED,s). All measurements were performed by two independent observers blinded to the treatment group.

Histology

At necropsy, hearts, lungs, liver and kidneys were dissected from treated and control B6 wild-type mice, rinsed in PBS and weighed. Hearts were cut in cross-section just below the level of the papillary muscle. For assessment of cardiomyocyte size, cardiac cell apoptosis and fibrosis, the top half of the heart was formalin-fixed and embedded in paraffin. Sections (5 μm) were prepared at 200 μm intervals. The sections were stained with hematoxylin and eosin (H&E) for examination of gross appearance, aortic valve size and cardiomyocyte size, while Masson's Trichrome was used to facilitate visualization of fibrosis. Sections were included for measurement of aortic valves only when the aortic outflow tract was clearly visible and parallel to the plane of sectioning and the entire cross-section of two valve leaflets was visible and could be clearly traced to the attachment point. Cardiomyocyte hypertrophy was assessed by measuring cross-sectional area of 100 cardiomyocytes per periodic acid-Schiff hematoxylin (PAS-H) stained section in ten randomly selected fields having nearly circular capillary profiles and centered nuclei in the left ventricular free wall. Histological images were analyzed using Nova Prime 6.75.10 software (BioQuant Image Analysis). Apoptotic cells were identified in serial cardiac cross-sections (5-μm) with the ApopTag Fluorescein *In Situ* apoptosis detection kit (Chemicon) according to the manufacturer's protocol. Images were acquired on a Nikon E800 microscope with a Hammamatsu ORCA-ER charge-coupled device camera with Metamorph software (Molecular Devices Corp.) and processed with Photoshop (Adobe). For measurement of cardiac valve size and calcification, serial sagittal sections (5 μm) were collected from each treatment group. Von Kossa's stain was used as a marker to visualize calcification (Carlson, 1990).

Gene expression

Total RNA was extracted from the lower half of the LV from B6 wild-type mice using TRIzol (Invitrogen). After DNAse treatment, 500 ng of total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems). The expression of hypertrophy markers atrial natriuretic peptide (*Nppa*) and brain natriuretic peptide (*Nppb*), pro and anti-apoptotic markers (*Bcl2l1*, *Bax* and *Bad*) and ERBB receptors and ligands (*Egf*, *Nrg1*, *Dt*, *Erbb1* and *Erbb2*) was determined by real-time quantitative PCR (qPCR) using Taqman Univeral Master Mix and Assays-on Demand primers and probes (Applied Biosystems). Results are represented as mean fold changes relative to control groups. Reactions were run on a Stratagene MX3000P machine with analysis software. Threshold cycles (C_T) were determined by an in-program algorithm assigning a fluorescence baseline based on readings prior to exponential amplification. Fold change in expression was calculated using the $2^{-\Delta\Delta cT}$ method (Livak and Schmittgen, 2001), with *Actb* or *Gusb* as endogenous controls.

In vivo phosphorylation assays

B6 wild-type male mice (n=2) maintained on control or experimental diet for 90 days were injected subcutaneously with 5 μg/g body weight of EGF (R&D Systems) in PBS. After 10 minutes, mice were euthanized and livers and hearts removed, frozen in liquid nitrogen and stored at -80°C. The frozen tissues were sonicated in 5–10 ml/g tissue of lysis buffer consisting of 20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, 1 mM sodium vanadate and 10 mM β-glycerophosphate at 4°C. The tissue lysates were cleared by centrifugation for 10 min at 4°C and protein concentrations were determined by the Bradford assay (Bio-Rad). Protein lysates (15 μg for liver and 30 μg for heart) were separated by denaturing 7.5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad). Protein blots were incubated overnight at 4°C with rabbit polyclonal antibodies recognizing EGFR (RB-1417-P1, Labvision), phospho-EGFR (Tyr1086) (36-9700, Zymed) or phospho-p44/42 MAP Kinase (Thr202/Tyr204) (Cell Signaling) followed by incubation

with goat anti-rabbit horseradish peroxidase conjugated antibody (1858413, Pierce) and detection with an enhanced chemiluminesence system (Amersham Pharmacia/GE Healthcare).

Statistical analysis

Data is presented as mean +/- standard error of the mean (SEM). Data from control groups was pooled when there was no significant difference between parameters. The non-parametric Wilcoxon rank-sum test was used to compare tumor number and size between treatment groups. The Mann-Whitney or unpaired Student's t-test was used to compare data between respective treatment and control groups. The Kruskal-Wallis test or analysis of variance (ANOVA) was used to detect significance by treatment. All analyses were performed using StatView software (SAS). A $p < 0.05$ is considered statistically significant.

Results

Orally delivered AG-1478 is biologically active

Although the reversible EGFR inhibitor AG-1478 has been used extensively in numerous *in vitro* and *in vivo* studies, to our knowledge it has not previously been shown to have activity when delivered orally. Pharmacokinetic studies in wild-type mice using ^{3}H AG-1478 showed that tissue distribution is highest in liver (Ellis *et al*., 2006), which also has the highest total and phospho-EGFR protein content. To determine whether chronic dietary exposure of AG-1478 suppresses EGFR activity, we examined total and phosphorylated protein levels of EGFR and ERK1/2 in liver lysates from wild-type B6 mice fed either control or AG-1478 containing diets for 90 days. Liver samples from mice on AG-1478 injected with 5 μg/g body weight EGF prior to sacrifice to enhance phospho-EGFR levels had reduced phospho-EGFR and phospho-ERK1/2 protein levels compared to controls (the relative band densities were reduced by 48% and 78%, respectively), although total EGFR protein levels were similar (Fig. 1).

Previous reports demonstrated that dietary exposure to irreversible EGFR small molecule inhibitors like EKB-569 dramatically inhibit intestinal polyp formation in the $Apc^{Min/+}$ mouse model of familial colorectal cancer (Torrance *et al*., 2000; Roberts *et al*., 2002). Therefore, to biologically and quantitatively test oral delivery of AG-1478, B6-$Apc^{Min/+}$ littermates of both sexes were weaned onto chow containing AG-1478 (144 mg/kg) or control chow with *ad libitum* feeding until 90 days of age after which their intestinal tracts were removed and the number of intestinal tumors counted. AG-1478 reduced polyp number by 45% compared to controls (Table 1), almost identical to that reported for another reversible EGFR inhibitor EKI-785 (40 mg/kg/day) under similar experimental conditions (Torrance *et al*., 2000), but less than the 87% reduction in tumor number reported for EKB-569 (20 mg/kg/day). This establishes the anti-tumor efficacy of AG-1478 in $Apc^{Min/+}$ mice and demonstrates that oral delivery in the diet is an effective route.

Chronic exposure to EGFR inhibitors results in mild physiological changes

Female wild-type B6 mice chronically exposed to small molecule EGFR inhibitors exhibited depressed weight gain over the course of exposure compared to controls (Fig. 2). After 90 days of treatment, EKB-569 treated mice had lost almost 6% of their starting body weight while their respective controls gained approximately 14% over baseline body weights. Although AG-1478 treated mice and their respective control groups gained weight over the course of the experiment, drug treatment greatly retarded weight gain. Alterations in body weight suggested that EGFR inhibitors may have affected feeding behaviors or energy expenditure, or caused mild toxicity at the drug concentrations used; however, there were no signs of dehydration, lethargy or ataxia in any treatment groups.

There were no significant differences in wet heart, liver or kidney weight by treatment group. (Table 2). However, EKB-569 treated female mice had increased wet lung weights, which remained significant when normalized for body weight. Since interstitial lung disease has been reported in a subset of patients treated with the EGFR small molecule inhibitor gefitinib (Rabinowits *et al*., 2003), we used Masson's Trichrome stain for collagen production and found that EKB-569 treated female mice were indistinguishable from the control group. Similarly, there was no difference in lung inflammation. However, the lungs from EGFR inhibitor treated mice did have a slightly higher level of proteinosis than that observed in the lungs from control mice (data not shown).

EGFR inhibition results in altered cardiovascular function due to increased LV apoptosis

Chronic dietary exposure to EGFR small molecule inhibitors led to significantly altered cardiac function as assessed by TTE only in female mice, although the severity varied by drug (Table 3). Both EGFR inhibitors caused increased left ventricular end diastolic and systolic dimensions and reduced contractility, as measured by percent fractional shortening (%FS), compared to baseline values or controls. EKB-569 had the greatest effect on LV wall thickness. Consistent with echocardiographic data, H&E stained cross sections taken at the level of the papillary muscle also showed morphological evidence of LV and septal wall thinning (Fig. 3A).

Because significant alterations were seen in cardiac function with drug treatment, we conducted a histological analysis to investigate pathological endpoints such as cardiomyocyte hypertrophy, fibrosis, and apoptosis. Consistent with heart weight data, there were no significant differences in mean cardiomyocyte area or in gene expression of classic hypertrophy markers (*Nppa* and *Nppb)* in the LV by treatment in female mice (data not shown). There were also no significant differences in LV gene expression of selected *Erbb* family members and ligands (*Erbb1, Erbb2, Egf, Nrg1,* and *Dtr*; data not shown). Mild- to moderate interstitial and perivascular fibrosis, as demonstrated by Masson's Trichrome stain, was observed in the LV walls of 25% (2/8) of EKB-569 (data not shown) and greater than 50% (3/5) of AG-1478 treated female mice (Fig. 3B). Milder interstitial fibrosis was also observed in <20% (2/11) control animals (Fig. 3B). Less frequent pathological observations included the presence of thrombi and proteinaceous material in the right ventricle and neointimal hyperplasia in the coronary arteries of EGFR inhibitor-treated female mice. Interestingly, both inhibitors increased the number of TUNEL-positive cardiac cells with apoptotic cells located in the LV walls, LV papillary muscle, and left atria of female mice (Fig. 3D; Fisher's PSLD: AG-1478 vs. control, $p < 0.06$, EKB-569 vs. control: $p < 0.05$). Consistent with TUNEL staining, altered expression of apoptotic genes was observed in the LV of inhibitor-treated female mice relative to controls (Fig. 3E). Expression of the anti-apoptotic gene *Bcl2l1* was suppressed by approximately 50%, and the pro-apoptotic genes *Bad* and *Bax* were also altered, albeit not reaching statistical significance.

Since earlier evidence demonstrated that EGFR activity is required for normal semilunar valve development (Chen *et al*., 2000), we investigated the effects of chronic exposure to EGFR inhibitors on morphological and histological changes in cardiac valves. Initial results using EKB-569 suggested that reduced EGFR activity might trigger excessive extracellular matrix (ECM) production and calcification in adult valves. All EKB-569 treated female mice, but less than half of the control mice, had evidence of aortic valve calcification by von Kossa staining (Fig. 4A). However, all B6 female mice from respective control and AG-1478 groups had some evidence of calcification, suggesting that EGFR inhibitors may exacerbate preexisting susceptibilities to valvular calcification. Both sexes showed signs of increased valve thickness (Fig. 4B and C) and interestingly, there were also a significant dietary effect on mean valve thickness (Fig. 4B). Since the synthetic AIN-93G diet has higher fat content than regular chow

(11% AIN-93G versus 5% normal chow) and B6 mice are known to be prone to valvulopathy induced by high fat diet (Drolet *et al*., 2006), the EGFR inhibitors likely enhance diet-induced valvular pathologies.

EGFR inhibitors show gender specific effects

It is well established that gender dramatically influences physiological and pathological responses to xenobiotics. To determine if chronic EGFR inhibition affected males similarly to females, a cohort of 6-8 week old male B6 mice were fed AG-1478 or control diets under identical conditions. Male mice had no significant differences in body weight gain (Fig. 2C), organ weights (Table 2) or cardiovascular function (Table 3) after 90 days of treatment, nor significant differences in cardiac pathology (no evidence of fibrosis, cellular necrosis or increased apoptosis; Fig. 3D). Aortic valves tended to be larger with AG-1478 treatment, but this did not reach significance (Fig. 4C). There were also no significant changes in cardiac expression of apoptotic genes (*Bcl2l1, Bax* or *Bad*) by treatment groups (Fig. 3E). However, the hypertrophy marker *Nppb* was upregulated in the hearts of AG-1478 treated male mice, despite the fact that mean cardiomyocyte area was unchanged. Unlike females, *Erbb2* and *Egf* transcripts were upregulated compared to controls (Fig. 5), suggestive of compensatory changes.

Discussion

Consistent with previous reports using TKIs EKB-569 or EKI-785 (Torrance *et al*., 2000), we demonstrated that dietary delivery of the EGFR small molecule inhibitor AG-1478 effectively represses EGFR-kinase activity and tumorigenesis *in vivo*. Employing chronic oral exposure of AG-1478 and EKB-569, TKIs from different chemical classes, we found marked changes in weight gain and cardiac function in B6 female mice. Drug exposure also resulted in pathological changes indicative of cardiotoxicity. Most notably, the number of TUNEL positive cells was increased by nearly threefold in the hearts of AG-1478 treated female B6 mice compared to controls, which was supported molecularly by significantly decreased expression of the anti-apoptotic gene *Bcl2l1* in cardiac tissue. Drug treatment also exacerbated diet-induced pathological changes in cardiac valves. To our knowledge, this is the first study to extensively evaluate cardiac function and pathology after chronic oral exposure to EGFR TKIs in adult mice, modeling exposure of patients to EGFR TKIs in the oncology clinic.

Interestingly, gender may influence response to TKIs, as unlike females, we saw no differences in physiological and pathological parameters by treatment in male B6 mice. Although we detected no significant differences by gender or treatment in cardiac EGFR expression, sexual dimorphism in basal EGF levels has been reported with male mice having higher protein levels in salivary glands (Stern *et al*., 2000) and higher transcript levels in pituitary glands (Lazar and Blum, 1992) compared to females. Since we found that *Egf, Erbb2* and *Nppb* transcripts were upregulated in the LV of male but not female AG-1478 exposed mice relative to their respective controls, it is possible that increased expression of these genes in the male heart, coupled with higher circulating ligand levels in males, may compensate for reduced EGFR activity and contribute to the observed male-specific protection from cardiotoxicity.

Results of our studies suggest that EKB-569 may be more toxic than AG-1478. EKB-569 exposure resulted in body weight loss, compared to suppression of body weight gain with AG-1478 treatment. Interestingly, reports from Phase I clinical trials reported anorexia in approximately 20% of patients receiving intermittent doses of EKB-569 (Erlichman *et al*., 2006). Similarly, hearts from EKB-569 treated mice had thinner LV walls and significantly more TUNEL-positive cells compared to controls, although AG-1478 caused greater depression in systolic function. Despite milder changes in cardiac contractility, wet lung weights were significantly increased with EKB-569 exposure. It is important to note that

interstitial lung disease has been reported in a subset of patients receiving gefinitib in non-small cell lung cancer clinical trials (Cersosimo, 2006; Yoneda *et al*., 2006; Yamamoto *et al*., 2007). Although we did not observe increased pulmonary fibrosis, indirect evidence of pulmonary damage was supported by increased pulmonary proteinosis and thrombi with proteinaceous material in the RV of EGFR inhibitor treated mice.

Differences between mode of inhibition, potency and selectivity between the two TKIs used in our experimental regimen may account for the discrepancy in toxicity. EKB-569 is an irreversible inhibitor, forming a covalent bond with the Cys 773 residue within the EGFR catalytic domain, while AG-1478 is a competitive inhibitor of ATP binding (Wissner *et al*., 2003). With irreversible inhibition, normal levels of EGFR activity are only recovered after gene transcription and translation. Recent findings suggest irreversible inhibitors may prevent the acquired resistance seen in non-small cell lung cancer patients treated with competitive inhibitors such as gefitinib and erlontinib (Kwak *et al*., 2005; Baselga, 2006). While these properties are promising for cancer therapy, irreversible TKIs may adversely affect cardiomyocyte function and survival, since EGFR transcript levels are normally very low in the adult mouse and human heart. The AG-1478 diet resulted in an approximately 45% reduction in polyp number, while at approximately the same concentration in identical base chow, EKB-569 caused about 87% reduction in polyp number in the Apc^{Min} mouse model (Torrance *et al*., 2000). A single oral dose of EKB-569 (10 mg/kg) was previously reported to rapidly inhibit EGFR kinase activity by 90% while multiple intraperitoneal doses of AG-1478 (126 μM/kg) decreased phosphorylation of EGFR and ERK1/2 by nearly 60% and over 70%, respectively, in xenograft studies (Ellis *et al*., 2006; Torrance *et al*., 2000). This data suggests that EKB-569 is more potent than AG-1478, and the greater toxicity observed with EKB-569 may reflect more potent EGFR TKI activity.

Although the current data suggests that the observed cardiotoxities are not off-target effects, but rather caused by perturbed cardiac homeostasis in the absence of normal EGFR activity, collateral inhibition of ERBB2 may contribute to the cardiotoxicity of EGFR TKIs. Since EGFR and ERBB2 have a high sequence homology in their catalytic domains (Coussens *et al*., 1985; Yamamoto *et al*., 1986; Arteaga, 2001), it is not surprising that many TKIs suppress activity of both receptors. In cell free systems, AG-1478 showed higher selectivity for EGFR over ERBB2 (IC_{50}, 3 nM EGFR versus >100 μM ERBB2) than EKB-569 (IC_{50}, 0.08 μM EGFR versus 1.23 μM for ERBB2) (Levitzki and Gazit, 1995; Jimeno and Hidalgo, 2006). In cell based assays using human carcinoma cell lines which overexpress EGFR (A431) or ERBB2 (SKB3), the IC_{50} for EKB-569 was 0.03 μg/mL and 0.007 μg/mL, respectively, consistent with effective inhibition of both receptors. Mice with myocardium-specific deletion of *Erbb2* resulted in a 70% decrease in myocardial *Erbb2* expression and a significant increase in cardiomyocyte apoptosis with anthracycline exposure (Crone *et al*., 2002). Moreover, gene therapy with over-expression of *Bcl2l1* partially rescued the dilated cardiomyopathy in these mice. Recent data also demonstrated similarly depressed *Bcl2l1* expression, cardiomyocyte apoptosis, and mitochondrial dysfunction in isolated cardiomyocytes with exposure to the anti-ERBB2 drug Herceptin (Trastuzamab) (Grazette *et al*., 2004). Given the well documented roles of ERBB2 and ERBB4 signaling in cardiomyocyte survival, it is possible that greater cardiac cell death and LV dilatation observed with EKB-569 exposure reflects greater off-target inhibition of ERBB2 and/or ERBB4.

Consistent with the growing literature underscoring the cardioprotective roles of ERBB signaling *in vitro* and *in vivo*, our studies suggest that prolonged exposure to TKIs targeting EGFR may compromise cardiac function in susceptible individuals. Recent analysis documents a major increase in the 10 year survivorship for many common cancers in the US compared to the late 1980's, thus more individuals may be exposed to TKIs and other molecule-targeted therapeutics for longer durations (Brenner and Arndt, 2004). Although overall, the side-effects

of targeted therapies such as the TKIs are well-tolerated compared to older chemotherapeutic drugs, our results indicate that, as with Herceptin therapy, cardiovascular function should be closely monitored with chronic exposure to EGFR TKIs.

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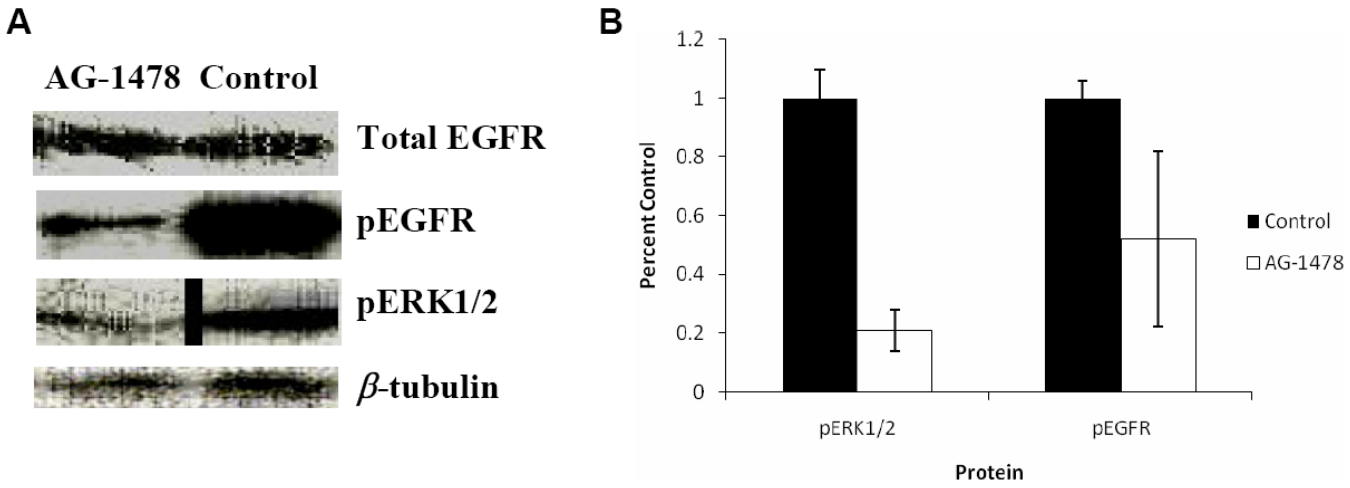

Fig. 1.

Immunoblot analysis of liver lysates. Wild-type B6 male mice were exposed to the EGFR small molecule inhibitor AG-1478 in AIN 93G diet (AG-1478) or AIN 93G diet alone (control) for three months (n=2). (A) Western blots and (B) quantification of EGFR activation.

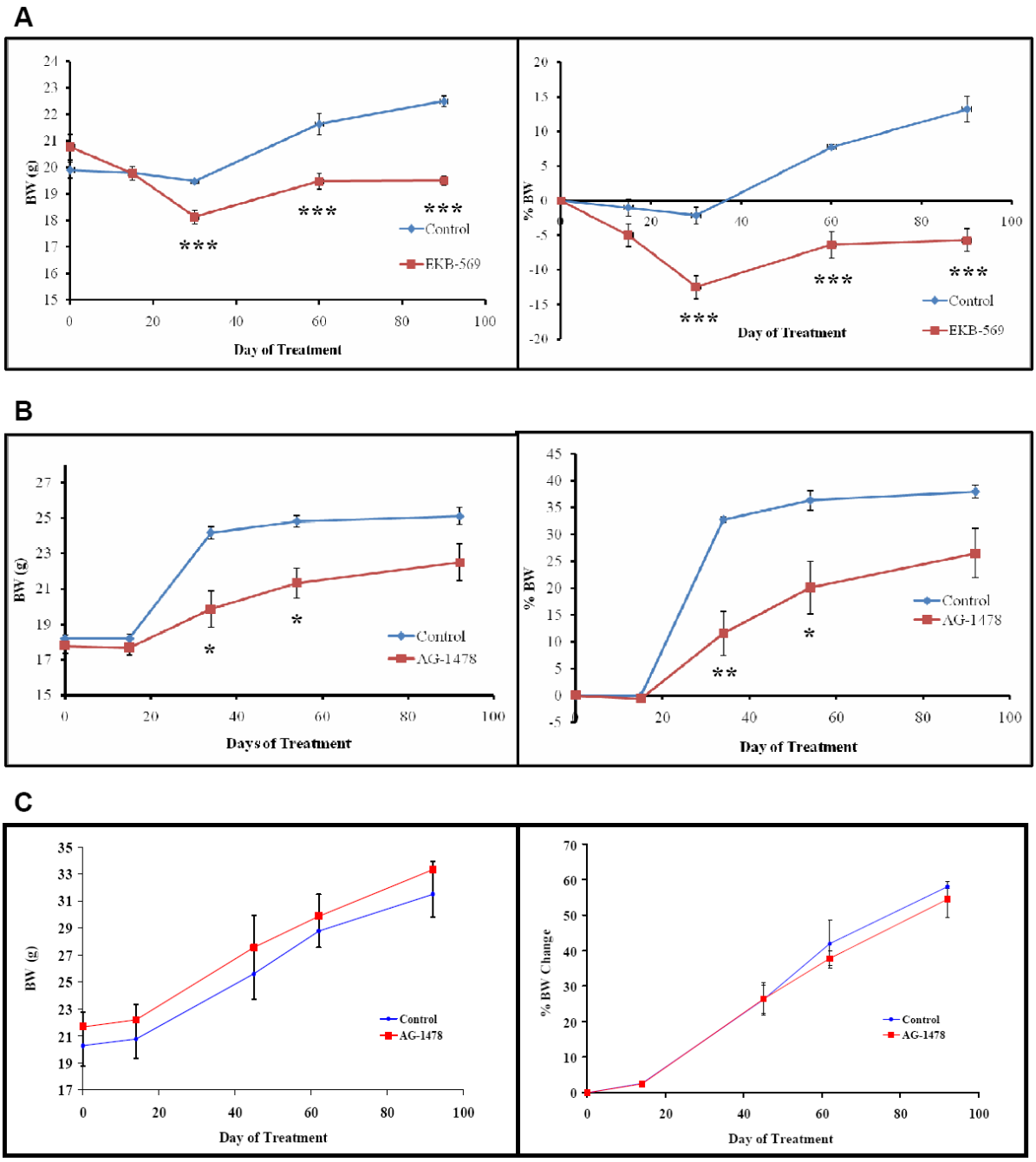

Fig. 2.

Effects of EGFR inhibitors on weight gain in B6 mice. Female mice were treated with EKB-569 (A) or AG-1478 (B) or male mice with (C) AG-1478. Left plots, body weight; right plots, percent change in body weight from start of diet. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

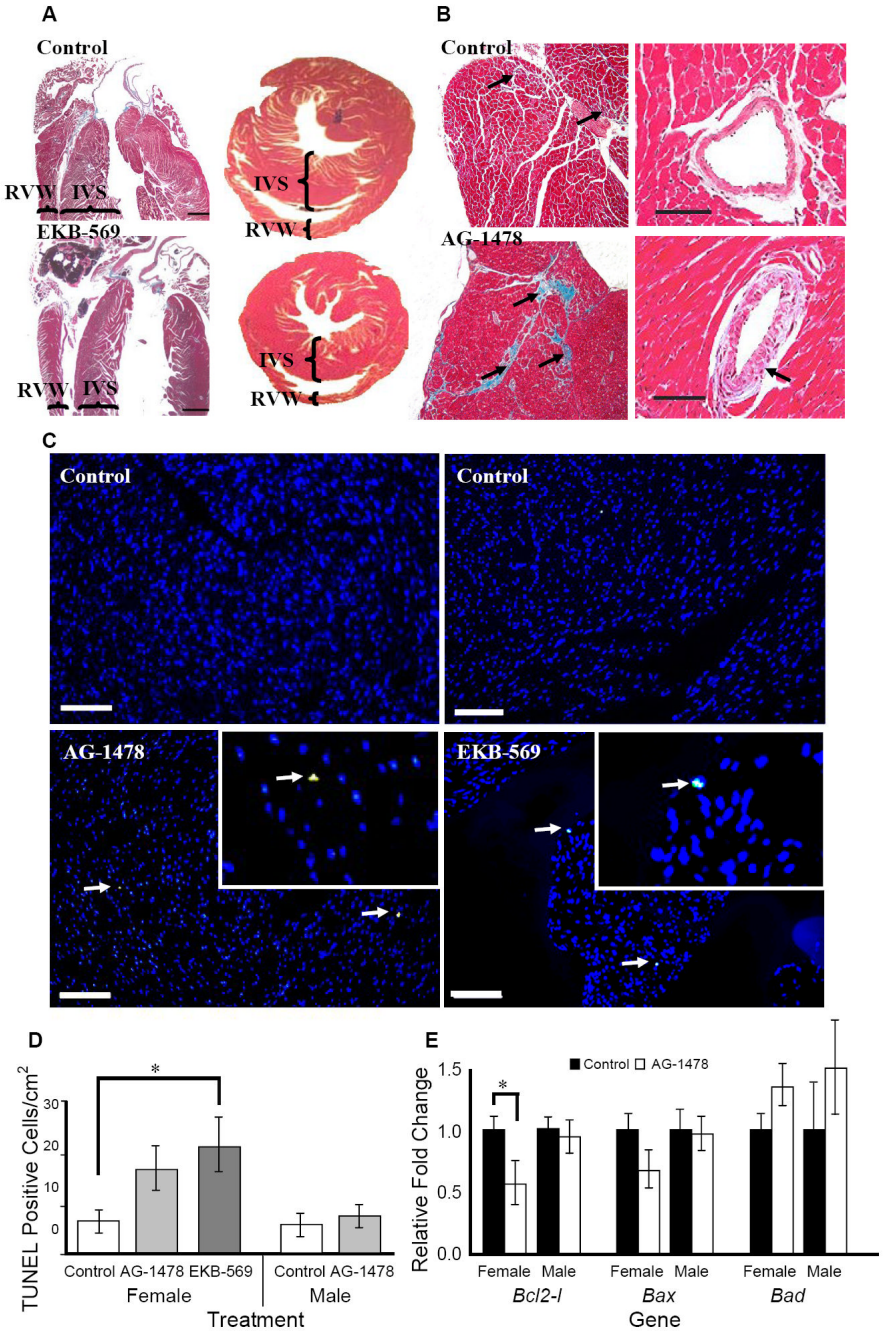

Fig. 3.

Pathological changes in hearts from B6 mice chronically exposed to EGFR inhibitors. Representative images depicting LV wall thinning (A) interstitial fibrosis (B) and increased numbers of apoptotic cardiac cells as detected by TUNEL staining (C) in the hearts of EKB-569 and AG-1478 exposed female mice. Arrows point to fibrosis (B) or TUNEL positive nuclei (C). Insets in (C) are higher magnification of TUNEL positive cells. D) Quantification of TUNEL-positive cardiac cells in female and male mice chronically exposed to EGFR inhibitors compared to controls. E) Relative fold changes in gene expression of proapoptotic and anti-apoptotic genes in the LV of B6 female and male mice chronically exposed to AG-1478

compared to controls. *Bcl2l1*= B-cell leukemia/lymphoma 2, *Bax*= *Bcl2*-associated X protein and *Bad*= *Bcl2*-associated death promoter. *$p < 0.05$.

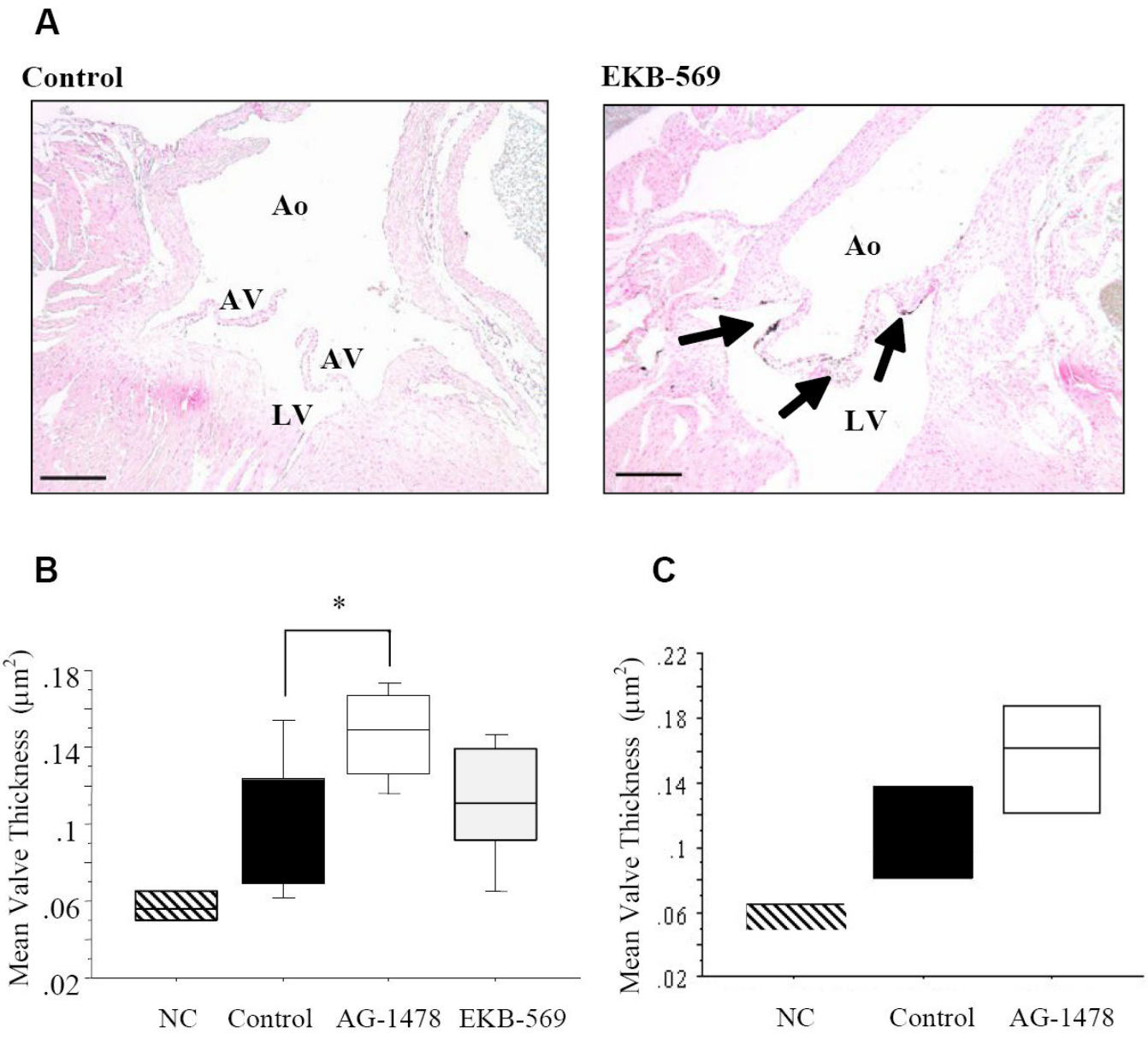

Fig. 4.

Pathological changes in the aortic valves of B6 mice chronically exposed to EGFR inhibitors. Calcification was detected in the aortic valve leaflets of male and female mice exposed to EGFR inhibitors by von Kossa stain (A). Aortic valve leaflets were thickened in EGFR inhibitor-exposed B6 mice of both sexes (female, B and male, C) compared to controls fed normal chow (NC) or AIN-93G chow (Control). Arrows in A indicate positive staining (black) for calcium deposition. Ao = aortic outflow tract, AV= aortic valve, LV=left ventricle. *$p < 0.05$. Bar = 100 µm.

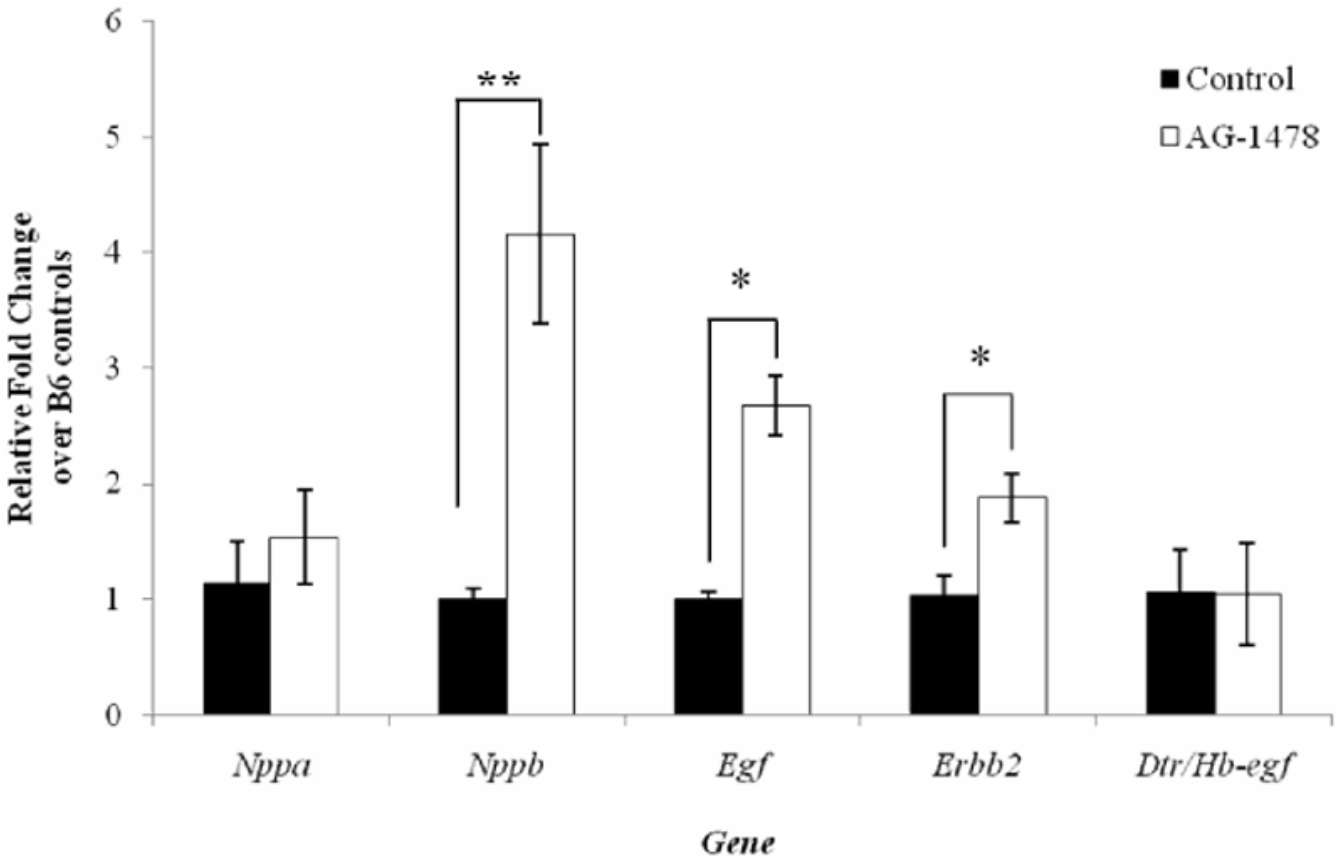

Fig. 5.

Relative fold changes in gene expression in the LV of B6 male mice chronically exposed to AG-1478 compared to controls. *Nppa* = nautriuretic peptide precursor A; *Nppb* = natriuretic peptide precursor B; *Egf* = epidermal growth factor; *Erbb2* = erythroblastic leukemia viral oncogene homolog 2 neuro/glioblastoma derived oncogene homolog (avian); *Dtr* = Diptheria toxin receptor/heparin-binding epidermal growth factor. $*p < 0.05$, $**p < 0.01$.

Table 1

Tumor development in *Apc*^{Min} mice

Treatment Group	N[a]	Poly Count[b]	Percent Control
Control	6	53.1 +/- 14.8	100.0
AG-1478	6	23.7 +/- 5.8	55.4

[a] three males and three females in each group;

[b] $p = 0.004$

Table 2

Organ and body weights

Treatment	N	Sex	BW (g)	HW (g)	LuW (g)	H:BW (mg:g)	Lu:BW (mg:g)
Control	8	females	22.04±0.40	121.93±6.0	147.85±10.80	5.74±0.31	6.94±0.48
EKB-569	8	female	19.65±0.21**	112.61±5.73	184.25±10.85*	5.64±0.26	9.67±0.28*
Control	3	female	24.67±0.33	155.3±10.33	174.00±14.60	6.31±0.47	7.05±0.57
AG-1478	5	female	22.50±1.05	141.20±12.08	172.29±13.55	6.30±0.53	7.78±0.86
Control	4	male	31.50±1.71	145.20±8.30	166.40±3.20	4.65±0.38	5.31±0.19
AG-1478	6	male	33.30±0.62	151.20±18.50	177.80±17.0	4.53±0.50	5.34±0.55

* $p < 0.05$;

** $p < 0.01$ compared to respective controls.

BW, body weight; HW, heart weight; LuW, lung weight; H:BW; normalized heart weight; Lu:BW, normalized lung weight.

Toxicol Appl Pharmacol. Author manuscript; available in PMC 2009 May 18.

Table 3

Echocardiographic parameters

Treatment	N	Sex	LVED,d	LVPWTh,d	%FS	HR
Baseline	16	female	3.40±0.07	0.70±0.08	39.80±2.38	455±20
Control	6	female	3.35±0.08	0.81±0.07	42.42±5.53	467±15
EKB-569	5	female	3.58±0.05$^{*,£}$	0.59±0.03$^{£δ}$	36.24±2.10$^{£}$	444±30
AG-1478	5	female	3.60±0.27$^{£}$	0.75±0.13	29.34±2.56,$^{¥}$	452±12
ANOVA	p<	female	0.05	0.01	ns	ns
Control	4	male	3.80±0.13	0.80±0.06	35.17±3.94	414±20
AG-1478	5	male	3.55±0.06	1.12±0.18	35.07±2.00	430±17
ANOVA	p<	male	ns	ns	ns	ns

$^{*}p < 0.05$, inhibitor versus baseline;

$^{**}p < 0.01$, inhibitor versus baseline;

$^{£}p < 0.05$, inhibitor versus control;

$^{¥}p < 0.01$, inhibitor versus control;

$^{δ}p < 0.05$, EKB-569 versus AG-1478.

LVED,d, left ventricular end diastolic diameter; LVPWTh,d, left ventricular posterior diastolic wall thickness; %FS, percent fractional shortening; HR, heart rate; ns, not significant.