Title

Lower gefitinib dose led to earlier resistance acquisition before emergence of T790M mutation in EGFR mutated lung cancer model

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Abstract

Non-small cell lung cancers (NSCLCs) with epidermal growth factor receptor (EGFR) mutations are sensitive to EGFR tyrosine kinase inhibitors (TKIs); however, unlike cytotoxic agents, it is generally accepted that minimal doses of drugs inhibiting target molecules are sufficient when molecular-targeted agents, including EGFR-TKIs, are administered. Thus, any utility of higher doses remains unclear. We compared low-dose (15 mg/kg) gefitinib therapy with high-dose (50 mg/kg) therapy using an EGFR-mutated lung cancer xenograft model. Both gefitinib doses induced tumor shrinkage, but tumors regrew in the low-dose group within 1 month, whereas tumors in the high-dose group did not. Neither the T790M mutation nor MET amplification was apparent in regrown tumors. We also compared outcomes after administration of two doses of gefitinib (5 and 25 mg/kg) in a transgenic EGFR-mutated lung cancer mouse model. In line with the results obtained using the xenograft model, both gefitinib doses completely inhibited tumor growth, but tumors treated with the lower dose of gefitinib developed earlier drug resistance.

In conclusion, administration of a low gefitinib dose caused tumors to become drug-resistant prior to acquisition of the T790M mutation or MET amplification in EGFR-mutated models of lung cancer. This suggests that it is important to optimize the EGFR-TKI dose for treatment of EGFR mutation-associated lung cancer. Gefitinib may need to be administered at a dose greater than the minimum required for inhibition of target molecules.

1. *Introduction*

Targeted molecular therapy has advanced greatly, and dramatic responses to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) have been observed in Non-small-cell lung cancer (NSCLC) patients, particularly in those bearing activating EGFR mutations [1-3]. Several phase III trials have shown that EGFR-TKIs afford beneficial effects in NSCLC patients with EGFR mutations, and EGFR-TKIs have become indispensable therapeutic agents for patients with EGFR-mutated NSCLCs [4–6]. The standard gefitinib dose is 250 mg/day, as determined by two Phase II studies (the IDEAL-1 and IDEAL-2 studies) that compared the 250 mg/day dose with 500 mg/day. In the cited works, gefitinib at 250 mg/day was as effective as a dose of 500 mg/day, but the lower dose was less toxic. Based on these data, 250 mg/day is now considered to be the standard dose [7, 8]. However, these studies were conducted using patients regardless of their EGFR mutation status, as the EGFR mutation and its relationship to EGFR-TKI response was not evident at that time. Thus, the current standard 250 mg/day dose is not optimized for NSCLC with EGFR mutation [9].

Cytotoxic agents generally exert dose-dependent effects, and standard dosages are determined according to the principle of use of as high a dose as possible. On the other hand, the existence of a positive correlation between effect and dosage for small molecular-targeted agents, including EGFR-TKIs, remains unclear, as administration of the minimal dose that inhibits target molecules is necessary. Thus, in this study we investigated the effects of gefitinib dose using xenograft and transgenic mouse models.

2. *Materials and Methods*

2.1 Cell culture and reagents

Cells were cultured at 37°C under 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS).

2.2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Growth inhibition was measured using a modified assay employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as the substrate. Briefly, cells were seeded into 96-well plates at a density of 1,500 per well and exposed to drug for 96 h. Each assay was performed in quadruplicate.

2.3 Reagents and antibodies

Gefitinib was purchased from Tocris Bioscience. Rabbit antisera against EGFR; phospho-EGFR (pY1068); Akt; phospho-Akt (pSer473); insulin-like growth factor-1 receptor (IGF-IR); phospho-IGF-IR (pIGF-IR; pY1131); Ki-67; and β-actin, were purchased from Cell Signaling Technology. A polyclonal antibody against mouse hepatocyte growth factor (HGF) was purchased from Sigma-Aldrich Corporation.

2.4 Immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer. Proteins were separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, probed with specific antibodies, and detected using appropriate ECL-Plus reagents (GE Healthcare Biosciences).

2.5 cDNA synthesis from mRNA

Total mRNA was isolated using RNeasy Mini kits (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen).

2.6 Quantitative PCR

Quantitative polymerase chain reaction (PCR) was performed using a GeneAmp 5700 platform (Japan Applied Biosystems). The ratio of the MET copy number ratio to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated using genomic DNA samples. Primer sequences were as described previously [10]. The ratio of the levels of mRNAs encoding HGF and GAPDH were calculated using cDNA data.

2.7 Immunostaining

Slices from formalin-fixed paraffin-embedded tissue blocks of xenograft samples were cut to a thickness of 5 μm. The sections were next deparaffinized. Antigen retrieval was performed and endogenous peroxidase activity was blocked. Tissues were next treated with 0.03% trypsin for 10 min. After, sections were blocked with 2% bovine serum albumin for 60 min. Sections were next incubated with an anti-HGF polyclonal antibody (Institute of Immunology Co.) overnight at 4°C. Staining was amplified by addition of a biotinylated anti-rabbit antibody and an avidin-biotinylated horseradish peroxidase conjugate, for 10 min (LSAB^{TM}2 Kit, DakoCytomation). Development used 3, 3′-diaminobenzidine (DAB). Sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted.

2.8 Enzyme immunoassay (EIA)

Human and mouse HGF concentrations were determined using EIA kits (Institute of Immunology Co.) following the protocols of the manufacturer.

2.9 Xenograft model

Cells (2×10^6) were subcutaneously injected into the backs of 7-week-old female athymic mice purchased from Japan Charles River Co. One week after injection, mice were randomly assigned to one of three groups (n = 12–15 mice/group) that received either vehicle, or 15 or 50 mg/kg/day gefitinib. Vehicle and gefitinib were administered once per day, five times per week, by gavage. Tumor volume ($\text{width}^2 \times \text{length}/2$) was determined regularly. In addition, to evaluate the efficacy of administration of anti-HGF antibody, mice were randomly assigned to four xenograft groups that received either a combination of vehicle and control antibody (8 mg/kg), a combination of vehicle and anti-HGF antibody (8 mg/kg), a combination of gefitinib and control antibody, or a combination of gefitinib and anti-HGF antibody. Rabbit IgG or anti-HGF antibody was intraperitoneally (i.p.) injected three times per week.

2.10 Transgenic mice

Transgenic mice expressing an in-frame deletion (*del*) of the E748-A752 mutant version of mouse EGFR (corresponding to the *del*E746-A750 mutation of human EGFR) [11] were housed under pathogen-free conditions. Seven-week-old transgenic mice were randomly assigned to one of three groups (n = 8–9 mice/group) that received vehicle, or 5 or 25 mg/kg/day gefitinib. Vehicle and gefitinib were given once daily, five times per week, by gavage.

After 7 weeks of treatment, vehicle-treated controls were sacrificed. After 29 weeks of treatment, gefitinib-treated groups were likewise sacrificed. Tumor specimens were preserved for analysis.

In addition, to assess survival, 7-week-old transgenic mice were divided into three groups (n = 12–13 mice/group), and treated with either vehicle, or 5 or 25 mg/kg/day gefitinib. Gefitinib was administered by gavage five times per week from 7 weeks of age until death.

3. *Results*

3.1 Administration of a low dose of gefitinib caused acquisition of earlier resistance in the xenograft model

First, we administered two doses of gefitinib (low dose: 15 mg/kg, high dose: 50 mg/kg) in a xenograft model using the human NSCLC cell line, PC-9 [12]. This line was derived from an untreated Japanese patient with pulmonary adenocarcinoma who carried an in-frame deletion in EGFR exon 19, and the cells are highly sensitive to EGFR-TKIs. Both the low- and high-dose groups exhibited tumor regression (Fig. 1A). However, only 2 weeks after treatment initiation, tumors in the low-dose gefitinib group began to regrow and exceeded pretreatment size at 4 weeks, whereas tumors in the high-dose group did not regrow (Fig. 1A).

To investigate whether low-dose gefitinib could inhibit EGFR signaling in this model, we examined p-EGFR expression and downstream signaling status by immunoblotting, commencing 3 days after treatment. EGFR phosphorylation in tumor tissues was completely suppressed and p-Akt was also suppressed in both groups (Fig. 1B, upper panel). To determine the extent of tumor growth, we also evaluated Ki-67 levels by immunostaining of samples of tumor tissue treated with vehicle, or low- or high-dose gefitinib, for 3 days. Expression of Ki-67 in the low- and high-dose groups was suppressed to the same extent (Fig. 1B, lower panel). These results indicated that low-dose gefitinib (15 mg/kg) adequately inhibited EGFR signaling in the xenograft model.

3.2 Neither the EGFR T790M mutation nor MET amplification was evident in regrown tumors

To investigate the mechanisms by which gefitinib resistance was acquired by regrown tumors treated with low-dose drug, we resected the tumors 10 weeks after gefitinib treatment. Phosphorylation of EGFR was suppressed in tumor tissues treated with low-dose gefitinib (Fig. 1C). Tumors in the high-dose group were too small to conduct

immunoblotting analysis. Fifty percent of acquired resistance to EGFR-TKIs is reportedly attributable to the EGFR T790M mutation at exon 20 [13, 14] and 10% to MET amplification [15, 16]. To assess the presence of the secondary T790M mutation, we performed both direct sequencing and peptide nucleic acid-locked nucleic acid PCR clamp-based detection testing (Mitsubishi Chemical Medicine Corp.) [17]. The T790M mutation was not detected in any of the three groups (data not shown). MET amplification was also explored using quantitative PCR and did not differ significantly among the groups (Fig. 1D). IGF-IR activation is another reported cause of acquired EGFR-TKI resistance [18]. But pIGF-IR activation was not observed in tumors treated with low-dose gefitinib compared to controls (Fig. S1). Together, the data showed that none of the T790M mutation, MET amplification, or IGF-IR activation explained the development of resistance in the low-dose gefitinib group.

3.3 A cell line derived from a regrown tumor was highly sensitive to gefitinib

To explore the resistance mechanism in more detail, we established three cell lines from regrown tumors treated with low-dose gefitinib, and termed them PC-9/RX-1, PC-9/RX-2, and PC-9/RX-3. PC-9/RX cells were highly sensitive to gefitinib, as were parental PC-9 cells. The IC_{50} values of gefitinib (± SD) in PC-9, PC-9/RX-1, -2, and -3 cells, determined using the MTT assay, were 0.017 ± 0.017, 0.030 ± 0.012, 0.033 ± 0.008, and 0.043 ± 0.005 μmol/l, respectively. This implied that the mechanism triggering resistance to low-dose drug resistance was reversible and not attributable to permanent molecular changes inside cells.

To determine whether such a reversible resistance mechanism actually developed in humans, we next conducted experiments with human tissue. First, we established a cell line (ABC-6-1) harboring an exon 19 in-frame deletion mutation in EGFR. The line was derived from a pleural effusion of a treatment-naïve patient. The patient was treated

with vandetanib, an EGFR-TKI that exerts additional inhibitory effects on the vascular endothelial growth factor receptor (VEGFR) and "rearranged during transfection" (RET). Marked tumor shrinkage was apparent. Next, we established ABC-6-2 and ABC-6-3 cell lines derived from pleural effusions (taken at different times) of the same patient after acquisition of vandetanib resistance. The sensitivities of the ABC-6-1, ABC-6-2, and ABC-6-3 lines to vandetanib were very similar. The IC_{50} values (± SDs) for vandetanib of the ABC-6-1, ABC-6-2, and ABC-6-3 lines were 0.13 ± 0.020, 0.27 ± 0.016, and 0.27 ± 0.031 μmol/l, respectively. (Fig. S2). All of the ABC-6-1, ABC-6-2, and ABC-6-3 lines had a deletion in exon 19, but neither a secondary T790M mutation nor MET amplification (data not shown). These data indicated that development of reversible resistance occurred not only in our mouse xenograft model, but also in humans.

3.4 Elevated HGF expression in xenografts established after gefitinib treatment

We hypothesized that the relevant resistance mechanism was caused by environmental factors, perhaps growth factors secreted by stromal cells. HGF is an extracellular growth factor that has been reported to cause gefitinib resistance [19]. HGF triggers various biological actions including mitogenic, motogenic, and morphogenic activities [20]. We explored the contribution of HGF to development of gefitinib resistance in our current model. First, we determined whether HGF was overexpressed by immunostaining using anti-mouse HGF antibody. Compared to the vehicle control, HGF was overexpressed in both the low- and high-dose drug groups (Fig. 2A). Immunoblotting and EIA also revealed HGF overexpression in tumor tissue treated with low-dose gefitinib (Fig. 2B, C). Tumors treated with a high dose of the drug were too small to permit evaluation. In addition, the level of mRNA encoding mouse HGF was significantly higher in tumors treated with low-dose gefitinib than in tumors treated with the vehicle control (Fig. 2D). We also used EIA to

determine whether human HGF was expressed in PC-9 cell culture supernatants and PC-9 xenograft tumors treated with gefitinib. No human HGF was detectable. As the PC-9 line was established from human cancer cells, the results suggested that the elevated HGF level in geftinib-treated tumors was attributable to HGF secretion not by tumor cells but rather by mouse stromal cells.

Next, we explored whether HGF overexpression indeed caused development of resistance to low-dose gefitinib. PC9/RX-1 cells, derived from a regrown tumor, were exposed to HGF and the proliferation capacity of the cells was evaluated using the MTT assay. PC-9/RX-1 cells became modestly resistant to gefitinib following HGF treatment. The IC_{50} values (± SDs) for gefitinib in control PC-9/RX-1 cells and such cells treated with HGF were 0.030 ± 0.012 and 0.209 ± 0.14 μmol/l, respectively (Fig. 3A). ABC-6-3 cells also became moderately resistant to vandetanib in the presence of 50 ng/ml HGF (IC_{50} ± SD: 0.90 ± 0.067 μmol/l).

Finally, we determined whether HGF inhibition restored gefitinib sensitivity. PC-9 xenografts were treated with a combination of vehicle and control antibody, vehicle and anti-HGF antibody, low-dose gefitinib (15 mg/kg) and control antibody, or low-dose gefitinib and anti-HGF antibody. The combination of gefitinib and anti-HGF antibody significantly inhibited tumor growth compared to the combination of gefitinib and control antibody ($p<0.05$, Student's *t*-test; Fig. 3B).

These data thus suggested that treatment with low-dose gefitinib caused earlier development of tumor resistance via an HGF-mediated mechanism.

3.5 EGFR-mutated lung cancer cells treated with low-dose gefitinib acquired earlier drug resistance in a transgenic mouse model

We next compared two gefitinib doses using an EGFR-mutated lung cancer transgenic mouse model. This model was created to constitutively express mouse EGFR *del* E748-A752 in type II pneumocytes. The mice develop multifocal lung adenocarcinomas between 5 and 6 weeks of age [11]. Seven-week-old mice were treated with two doses of gefitinib (low-dose group: 5 mg/kg, high dose group: 25 mg/kg; 5 mg/kg was the lowest dosage causing tumor shrinkage). At commencement of treatment, multifocal adenocarcinomas were microscopically identified (Fig. 4A, upper panel). Two weeks after treatment initiation, adenocarcinomas had almost completely disappeared in both groups (Fig. 4A, middle panel). However, in the low-dose group, tumors began to regrow 14 weeks after treatment initiation, and multiple tumor nodules were apparent after 29 weeks, whereas almost no tumors were detected in the high-dose group (Fig. 4A, lower panel). At this time, the number (mean ± SE) of superficial left lung tumors with long axes exceeding 1 mm was significantly greater in the low-dose group than in the high-dose group (7.6 ± 1.0 and 0.67 ± 0.18, respectively, $p < 0.001$; Fig. 4B: left panel). Left lung weights (mean ± SE) in the low- and high-dose groups were 0.35 ± 0.04 g and 0.19 ± 0.0036 g, respectively ($p < 0.005$, *t*-test; Fig. 4B: right panel). We also investigated the effect of different gefitinib doses on survival. Mice in the low-dose group died significantly earlier than did animals in the high-dose group, although both dosages significantly extended survival time compared to that of vehicle-treated mice (Fig. 4C). The median survival time of each group was as follows: vehicle treatment, 17.3 weeks; low-dose group, 51.7 weeks; and high-dose group, more than 70 weeks ($p < 0.005$, log-rank test). We also examined the mechanism of resistance that was in play in this model. None of the T792M secondary mutation (corresponding to the T790M mutation in human EGFR), MET amplification, or elevated HGF expression, was detected (Fig. S3). The precise mechanism of the observed drug resistance thus remains unclear.

Discussion

In the present study, we showed that a lower dosage of gefitinib was efficacious for a shorter period of time than was a higher dose. Both doses initially exerted antitumor effects in both a xenograft and a transgenic mouse model, suggesting that prescription of an inadequate gefitinib dose causes early development of drug resistance in clinical practice.

In our xenograft model, tumors shrunk in both the low- and high-dose groups soon after initiation of gefitinib treatment, suggesting that both doses effectively inhibited signaling by mutated EGFR. However, a few weeks after treatment commenced, tumors regrew in the low-dose group but not in the high-dose group. Neither EGFR T790M mutation nor MET amplification was detected in regrown tumors. It is possible that tumors in the low-dose group became drug resistant before acquiring these genetic alternations because they are generally considered to take at least several months [21]. Growth factors secreted by the tumor microenvironment are important in terms of both the progression and drug sensitivity of various malignancies [22, 23]. HGF overexpression is reportedly involved in development of EGFR-TKI resistance [19, 24] and HGF was overexpressed in our xenograft model. Thus, we hypothesized that HGF overexpression might cause the observed resistance. Indeed, HGF exposure caused development of gefitinib resistance in PC-9/RX cells derived from regrown tumors, whereas control PC-9/RX cells remained highly sensitive to the drug. In addition, treatment with HGF-neutralizing antibody restored gefitinib sensitivity. These results indicated that the acquired resistance in xenografts derived from cells treated with low-dose gefitinib was likely caused by HGF expression.

HGF is produced by stromal fibroblasts [25] but it remains unclear why HGF is overexpressed in EGFR-TKI-resistant tumors. In our model, HGF was expressed in gefitinib-treated tissue irrespective of drug dosage, but not in vehicle-treated tissue. Inhibition of EGFR action might directly or indirectly influence stromal fibroblast

activity, inducing HGF overexpression. Unlike what was observed in the low-dose group, the high-dose group did not acquire resistance, although HGF expression was detected by immunostaining (Fig. 2A). Although no clear explanation can yet be advanced, the *in vitro* PC-9/RX data revealed that relatively moderate drug resistance was induced by HGF (the IC_{50} of PC-9/RX cells treated with HGF was less than 1 μmol/l). It is thus possible that tissues treated with high-dose gefitinib might be affected by the resistance mechanism described only minimally.

Treatment with low-dose gefitinib also caused earlier emergence of acquired resistance in a transgenic mouse model. However, HGF was not elevated in this model. Recently, various growth factors, thus not only HGF, have been shown to cause development of resistance to EGFR-TKIs [23]. The resistance observed in the transgenic mouse model may be attributable to production of some other growth factor(s).

Unlike what is true of cytotoxic agents, it is generally considered that the minimal dose required to inhibit targeted molecules is sufficient when targeting agents (*e.g.*, gefitinib) are prescribed [9]. However, some studies have found it important to use high doses of such agents. The significance of imatinib dose choice (imatinib specifically targets BCR-ABL and KIT) was demonstrated in a phase III clinical trial [26]. High-dose imatinib (800 mg daily) afforded better PFS than did standard-dose imatinib (400 mg daily). Work with patients who had recurrent or metastatic squamous cell carcinoma of the head and neck showed that those given 250-mg gefitinib daily seemed to experience poorer PFS and overall survival compared with those given 500 mg daily [27, 28]. In addition, patients who achieved disease control had higher gefitinib trough levels than did those experiencing disease progression [29]. Turning to lung cancer, a patient with EGFR-mutated NSCLC responded to high-dose (500 mg daily) gefitinib after acquisition of resistance to the standard daily dose of 250 mg gefitinib [30]. Recently, we investigated the association between gefitinib efficacy and the physical size of patients with EGFR-mutated NSCLC [31]. In the cited study, the median PFS of the

patients with large physical size was significantly worse than that of those with small physical size. This may be attributable to the relatively lower dosage of gefitinib given to physically larger patients. These data suggest that even small-molecule-targeting agents should be given at a level above the minimum dose required to inhibit target molecules.

In conclusion, low-dose gefitinib treatment caused earlier development of drug resistance than when a high dose was administered. Such resistance became apparent prior to acquisition of the T790M mutation or MET amplification in EGFR-mutant lung cancer models. Our results suggest that it is important to optimize the EGFR-TKI dose when treating patients with EGFR-mutated lung cancer.

Disclosure Statement

NT and KK have received honoraria from AstraZeneca. All other authors declare that they have no conflict of interest related to this study.

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Figure Legends

Figure 1.

A; PC-9 xenograft. Bars: standard errors (SEs). B; Immunoblotting (upper) and immunostaining (lower). Lysates were extracted from PC-9 xenografts after 3 days of treatment. C; Immunoblotting. Lysates were extracted from PC-9 xenograft tumors after 10 weeks of treatment. The level of EGFR expression and the extent of EGFR phosphorylation were compared between the vehicle control and low-dose gefitinib groups. D; Quantitative PCR of the MET gene. The MET gene copy number was assessed using genomic DNA from PC-9 xenograft tumors in three independent experiments. Bars: standard errors (SEs).

Figure 2.

A; Immunohistochemical staining of HGF. Samples were taken from xenograft tumors treated with vehicle, or 15 or 50 mg/kg/d gefitinib. Magnification: ×400. B; HGF immunoblotting. Mouse HGF expression levels were using immunoblotting. C; Mouse HGF expression levels. HGF expression was measured by ELISA. The significance was evaluated using Student's *t*-test. D; Mouse HGF mRNA levels. mRNA levels were assessed by quantitative PCR using cDNA from xenograft tumors.

Bars: standard errors (SEs).

Figure 3.

A; Effects of mouse HGF on gefitinib sensitivity. Cells were seeded (1,500 per well) into 96-well plates and, after 96 h

of culture, were subjected to MTT assay. Points: mean values of four cultures; bars: standard deviations (SDs). B; Effect of a combination of gefitinib and anti-HGF neutralizing antibody. Tumor volumes after 3 weeks of drug treatment are shown. Tumor volumes on day 21 relative to those on day 0 are indicated. Bars: standard errors (SEs).

Figure 4.

A; Comparison of the effects of low- (5 mg/kg/d) and high- (25 mg/kg/d) dose gefitinib in transgenic mice. Lung tissue from transgenic mice obtained at 7, 9, and 36 weeks of age was subjected to histological examination using hematoxylin-eosin (HE) staining. Magnification: ×40. B, left; The numbers (means ± standard errors; SEs) of superficial left lung tumors in which the long axis exceeded 1 mm in the low- and high-dose gefitinib groups was assessed at 36 weeks of age. The significance of differences in tumor volume was assessed using Student's *t*-test. B, right; Weights (g; means ± SEs) of the left lungs were measured in each group. The significance of differences in weights was assessed using Student's *t*-test. C; Survival curves of the vehicle group (dotted line),the low-dose gefitinib group (solid line), and the high-dose gefitinib group (dashed line).

List of supporting information

Figure S1. IGF-IR immunoblotting

Figure S2. Vandetanib sensitivity

Figure S3. MET gene copy number and HGF expression

Figure 1.

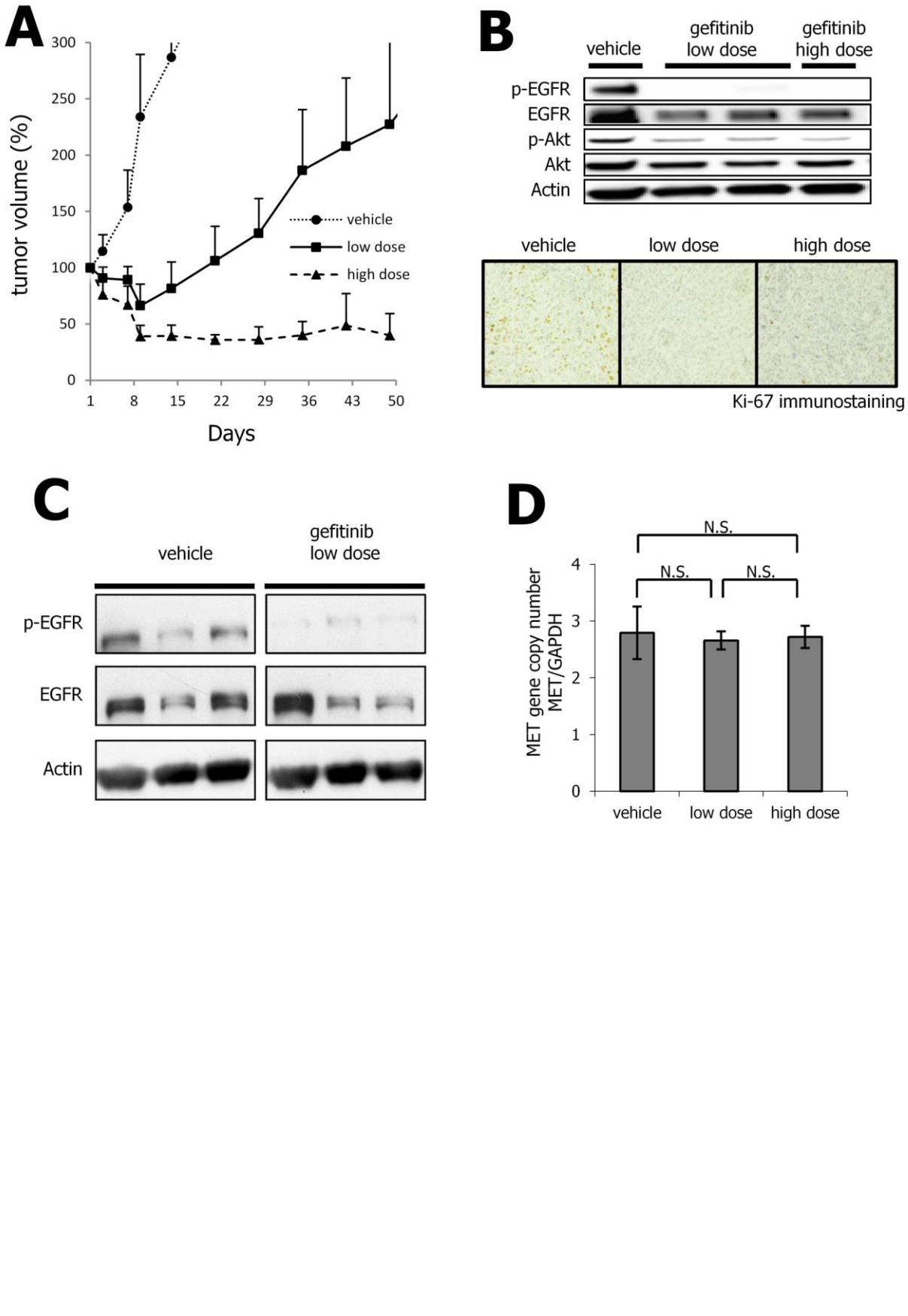

A
tumor volume (%)
300
250
200
150
100
50
0
1
8
15
22
29
36
43
50
Days
vehicle
low dose
high dose
B
vehicle
gefitinib low dose
gefitinib high dose
p-EGFR
EGFR
p-Akt
Akt
Actin
vehicle
low dose
high dose
Ki-67 immunostaining
C
vehicle
gefitinib low dose
p-EGFR
EGFR
Actin
D
N.S.
N.S.
N.S.
MET gene copy number MET/GAPDH
4
3
2
1
0
vehicle
low dose
high dose

Figure 2.

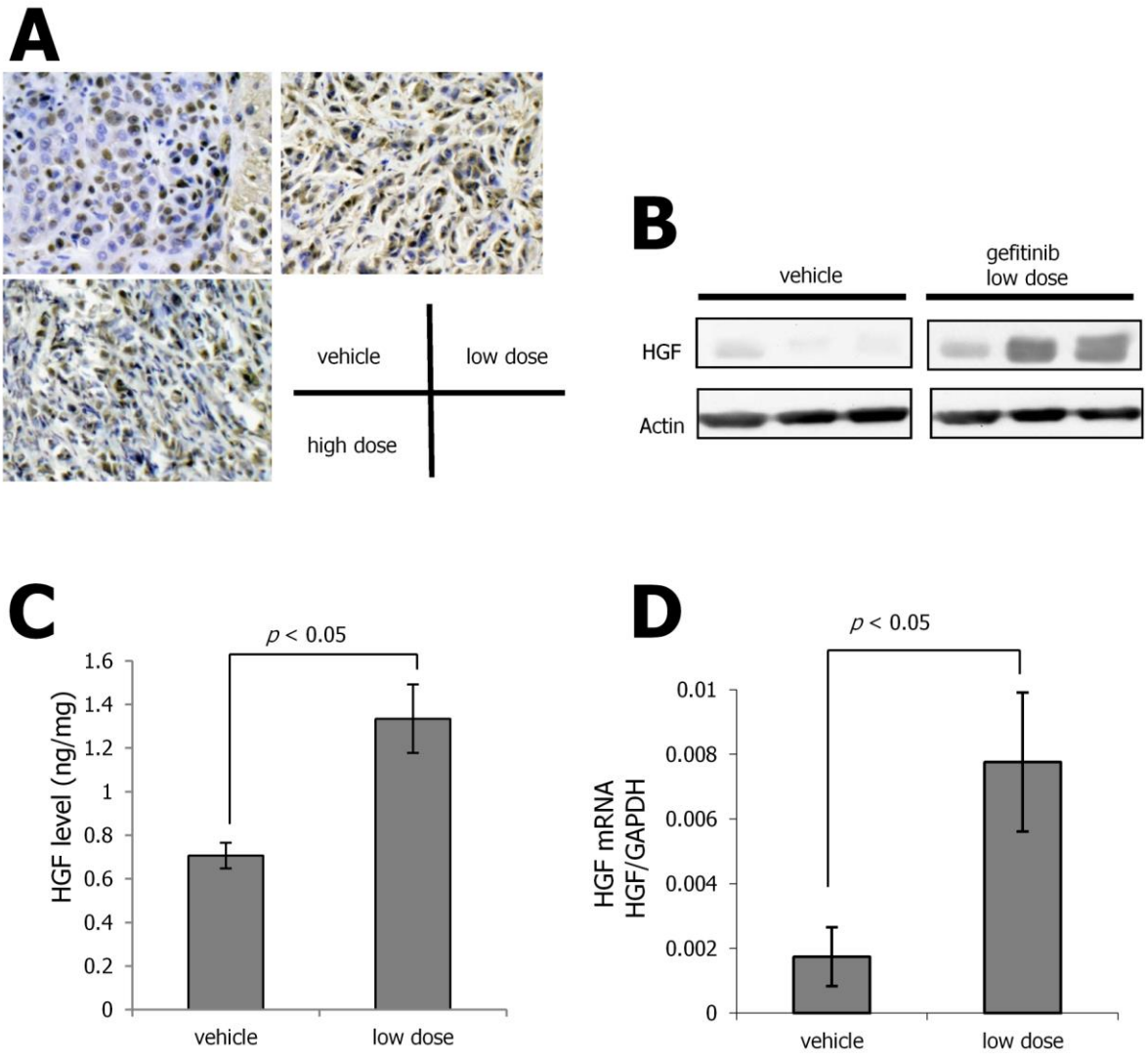

A
vehicle
low dose
high dose
B
vehicle
gefitinib
low dose
HGF
Actin
C
p < 0.05
HGF level (ng/mg)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
vehicle
low dose
D
p < 0.05
HGF mRNA
HGF/GAPDH
0.01
0.008
0.006
0.004
0.002
0
vehicle
low dose

Figure 3.

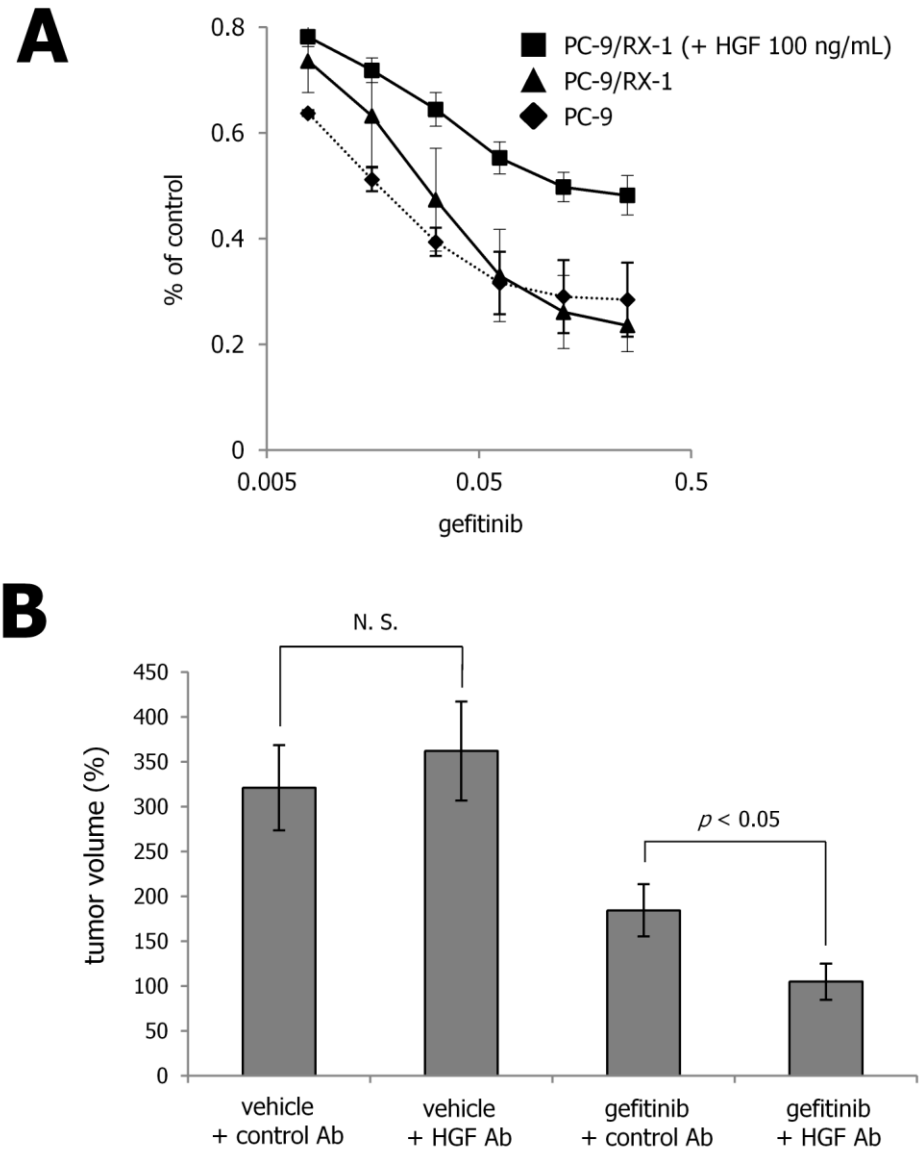

A
0.8
0.6
0.4
0.2
0
% of control
0.005
0.05
0.5
gefitinib
PC-9/RX-1 (+ HGF 100 ng/mL)
PC-9/RX-1
PC-9
B
N. S.
$p < 0.05$
450
400
350
300
250
200
150
100
50
0
tumor volume (%)
vehicle
+ control Ab
vehicle
+ HGF Ab
gefitinib
+ control Ab
gefitinib
+ HGF Ab

Figure 4.

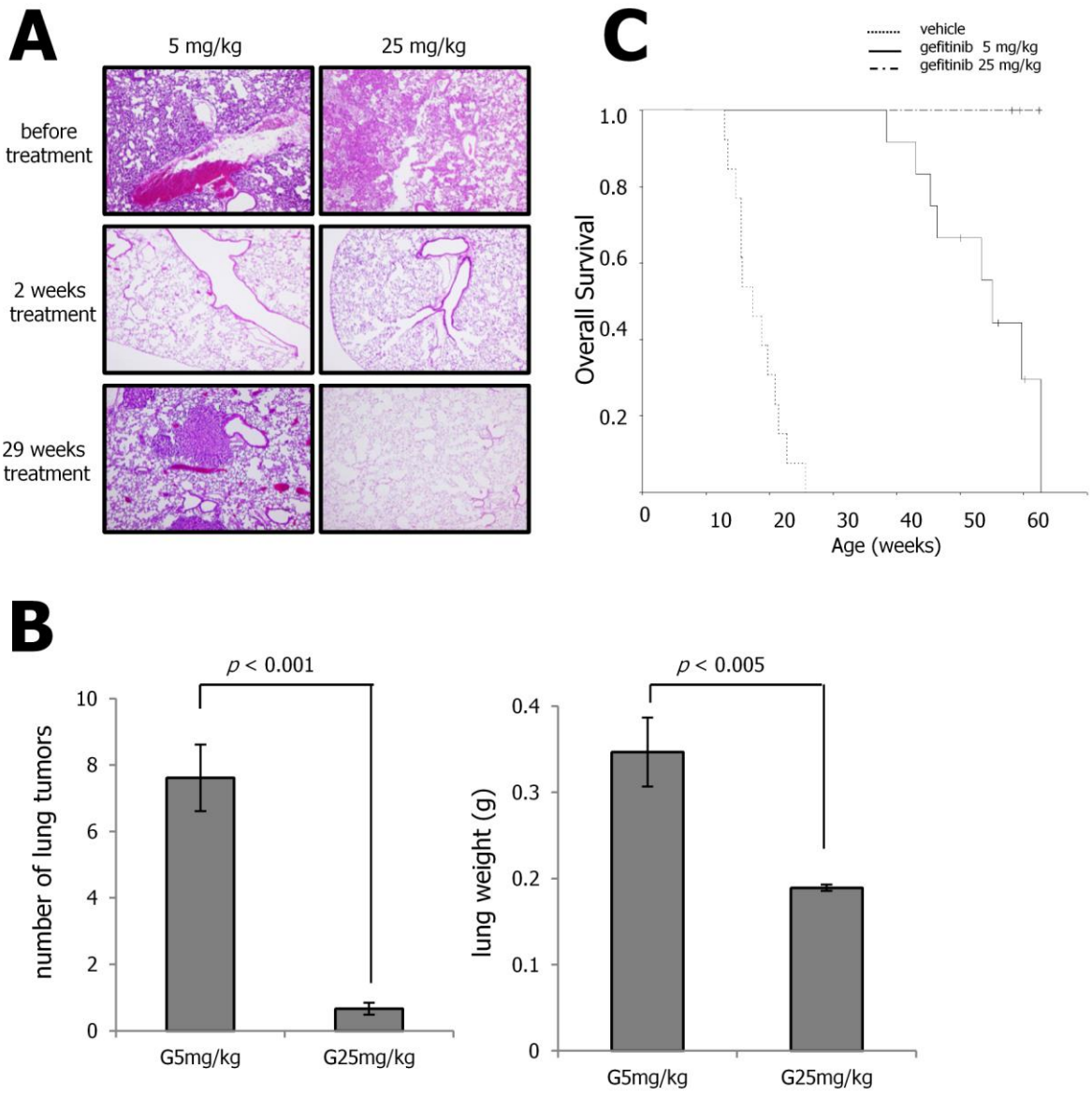

A
5 mg/kg
25 mg/kg
before treatment
2 weeks treatment
29 weeks treatment
C
vehicle
gefitinib 5 mg/kg
gefitinib 25 mg/kg
Overall Survival
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
Age (weeks)
B
p < 0.001
number of lung tumors
10
8
6
4
2
0
G5mg/kg
G25mg/kg
p < 0.005
lung weight (g)
0.4
0.3
0.2
0.1
0
G5mg/kg
G25mg/kg

Figure S1.

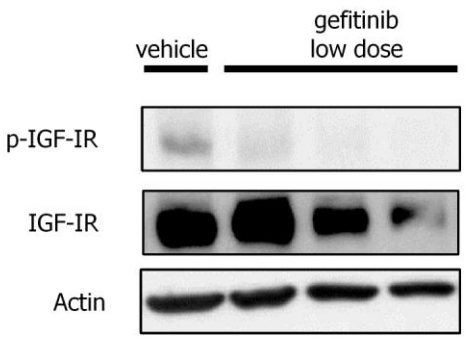


Figure S2.

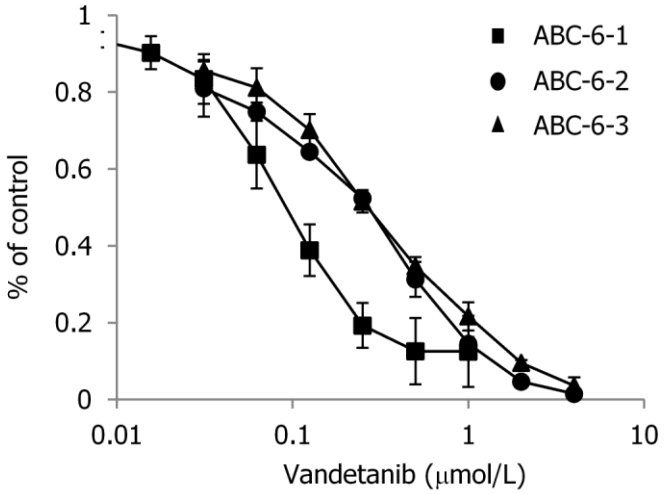
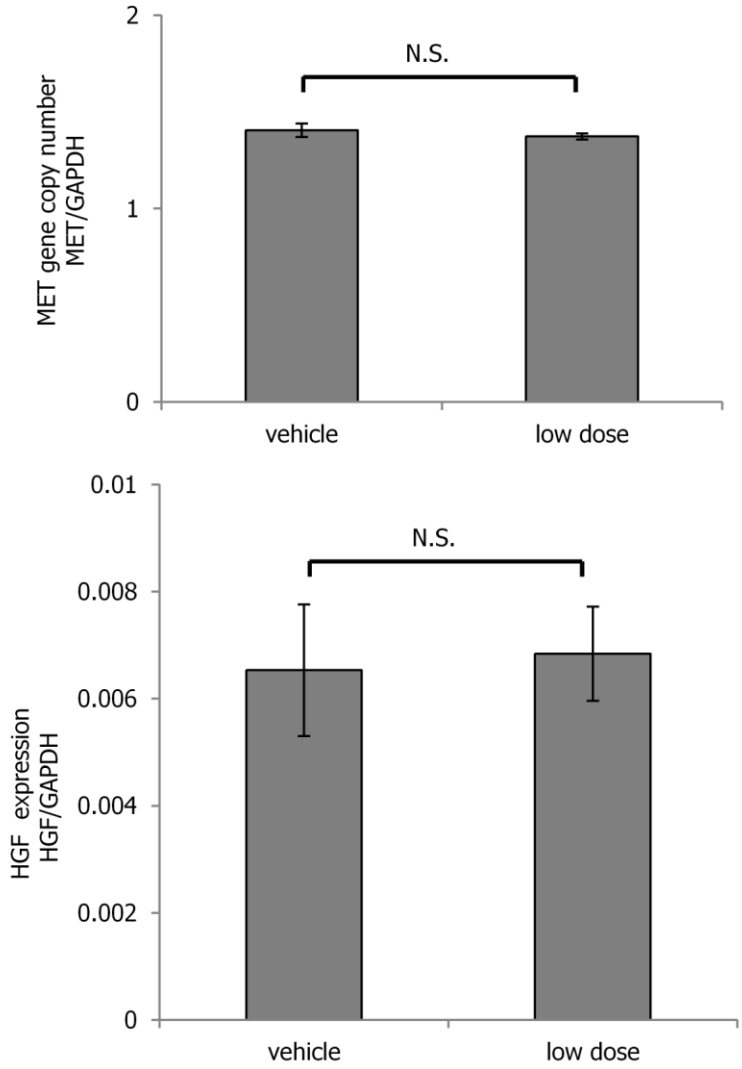

ABC-6-1
ABC-6-2
ABC-6-3
% of control
1
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
Vandetanib (μmol/L)

Figure S3.


MET gene copy number
MET/GAPDH
2
1
0
N.S.
vehicle
low dose
HGF expression
HGF/GAPDH
0.01
0.008
0.006
0.004
0.002
0
N.S.
vehicle
low dose