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TITLE PAGE

Transcriptome analysis and *in vivo* activiy of Fluvastatin versus Zoledronic acid in a murine breast cancer metastasis model

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RUNNING TITLE PAGE

Running title: Fluvastatin versus Zoledronate antimetastatic potentials

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List of abbreviation:
Zol: zoledronate
Fluva: fluvastatin
BP: bisphosphonate

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ABSTRACT

Statins and bisphosphonates (BPs) are two distinct classes of isoprenoid pathway inhibitors targeting downstream enzyme of HMG-CoA reductase (upstream enzyme) and Farnesyl-pyrophosphate synthase, respectively. Here, we studied Fluvastatin (Fluva) and Zoledronate (Zol), representative molecules of each class, respectively. *In vivo* metastatic potentials of both molecules were assessed. For the first time, we observed a significant reduction of established metastasis progression under Fluva treatment. Treatment with Zol at 100 µg/kg and Fluva at 15 mg/kg both inhibited 80% of metastasis bioluminescence signal and increased mice survival. The Zol and Fluva transcriptomic profiles of MDA-MB-231 treated cells, revealed analogous patterns of affected genes but each of them reached with different kinetics. The observable changes in gene expression started after 24 for Fluva $IC50_{72h}$ and only after 48h for Zol $IC50_{72h}$. To obtain early changes in gene expression of Zol treated cells, three times higher dose of Zol $IC50_{72h}$ had to be applied. Combining Fluva and Zol *in vivo* showed no synergy but a several days benefit in mice survival. This study demonstrated that Zol or Fluva are of potential clinical use for the treatment of established metastasis.

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INTRODUCTION

Breast cancers are frequently characterized by overexpression of Rho family GTPases and constitutive activation of Ras or other small GTP-binding proteins that are mutated in a variety of tumors, thereby regulating cancer cell migration and invasion (Fritz et al., 1999). To be functional, these proteins require prenylation, a lipid post-translational modification that assures their translocation and attachment to the plasma membrane crucial for their ultimate involvement in signal transduction. In human cells, isoprenoids (farnesyl-pyrophosphate, FPP, and geranylgeranyl-pyrophosphate, GGPP) are derived from the mevalonate pathway that starts from the reaction of conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonic acid catalyzed by the HMG-CoA reductase (the rate-limiting reaction in cholesterol biosynthesis). Recent evidence has accumulated on the direct inhibiting effects of bisphosphonates (BPs) and statins, initially known as potent inhibitors of osteoclast-mediated bone resorption and cholesterol lowering drugs respectively, on cancer progression and metastasis (Fig. 1).

Use of bisphosphonates (Russell, 2011) in solid tumours to prevent or treat bone metastases is supported by numerous clinical studies and is recommended by international consensus conferences (Aapro et al., 2008). Effects of bisphosphonates on soft tissue metastases are less clear (Fournier et al., 2010) Moreover, recent studies show that (Zometa) , Zoledronic acid (Zol) could induce adverse cytotoxic effects as osteonecrosis of the jaw. This effect could be bypassed using Zol in combination with other chemotherapeutic molecules. (Zol), the more potent drug among aminobisphosphonates (NBPs), is described to suppress prenylation of several intracellular small GTPases essential for many cellular functions important in carcinogenesis (Caraglia et al., 2006). However, the inhibition of breast cancer cell proliferation by NBPs, including zoledronic acid (Zol), appears to involve other mechanisms besides the inhibition of the mevalonate pathway as none of the mevalonate pathway intermediates completely reversed the effect of NBPs on MDA-MB-231 proliferation (Reinholz et al., 2002). On the other hand, novel non-nitrogen containing BP (BP7033) has been shown to prevent post-translational Ras processing similarly to NBPs (Hamma-Kourbali et al., 2003). In addition, Zol, has been found to induce formation of a novel ANT-inhibiting cytotoxic ATP analogue, ApppI. Thus, Zol could act on cell apoptosis both via inhibition of the mevalonate pathway and by the blockage of mitochondrial ANT (Monkkonen et al., 2006). Zol has been demonstrated to inhibit proliferation and induce apoptosis (Jagdev et al., 2001; Senaratne et al., 2002), inhibit cell invasion (Denoyelle et al., 2003), and potently

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diminishes bone, but also visceral metastasis *in vivo* (Duivenvoorden et al., 2007; Green et al., 2000; Hiraga et al., 2004; Peyruchaud et al., 2001). In addition, accumulating evidences indicate that bisphosphonates antitumor effects could be potentiated by other chemotherapeutic drugs (Yano et al., 2003). Several cases of possible synergy between bisphosphonates and statins or other mevalonate pathway inhibitors *in vitro* (Budman and Calabro, 2006; Dudakovic et al., 2008; Issat et al., 2007) and *in vivo* (Issat et al., 2007) have been reported showing potentiated effects.

Lescol, Fluvastatin (Fluva) represents lipophilic statins able to directly cross cell membranes in contrast to hydrophilic statins, like pravastatin, unable to penetrate membrane lipid bilayers (Istvan, 2003). Interestingly, recent results from epidemiological studies conclude in the protective effects of lipophilic statins on breast cancer risk (Ahern et al., 2011, Cauley et al., 2006). In breast cancer cells, Fluva is shown to exert significant growth inhibitory activity (Campbell et al., 2006), and reduce transendothelial migration of MDA-MB-231 cells in correlation with the inhibition of the membrane localization of RhoA and RhoC, but not with Ras (Kusama et al., 2006).

Fluva and Zol are used to treat and prevent atherosclerosis and osteoporosis respectively, mainly in elderly at an age where the prevalence of cancers increases sharply. Both drugs are therefore often co-prescribed in cancerous patients and they might influence the progression of the disease (Jadhav *et al.*, 2006). Since *in vivo* potential effects of Fluva have not been described yet on breast cancer established metastases and since statins and bisphosphonates are frequently co-administrated, we proposed to investigate effects of these drugs using *in vivo* imaging system with a highly invasive bioluminescent MDA-MB-231 breast cancer subpopulation characterized by oncogenic KRAS mutation (Kozma et al., 1987) and constitutive activation of Ras as well as overexpression of RhoA (Fritz et al., 1999). Furthermore, as BPs induced apoptosis by forming ATP analogues (AppI) that could be complementary to Fluvastatin inhibition effect involving small G protein, we also evaluated the combination of these two drugs on established MDA-MB-231 metastases (fig 1).

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MATERIALS AND METHODS

Cell culture and Drugs

The human breast adenocarcinoma MDA-MB-231 cells, obtained from the American Type Culture Collection (Manassas, VA, USA), were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate and antibiotics (1% penicillin sodium and 1% streptomycin) at 37°C in a humidified atmosphere containing 5% carbon dioxide. D3H2LN cell line isolated from MDA-MB-231 lymph node metastasis was purchased from Caliper Life Sciences (Alameda, CA, USA). D3H2LN cell line was a clone selected from a MDA-MB-231 stable clone expressing luciferase. MDA-MB-231 cells expressing luciferase were injected into the mammary fad pad of nude mice and after 12 weeks of growth *in vivo*, they were harvested and re-propagated *in vitro*. This subclone was injected once more into the mammary fad pad of mice to yield a second cell line D3H2LN, harvested from a lymph node metastasis (Jenkins et al., 2005). D3H2LN cells were cultured in Minimum Essential Medium with Earl's Balanced Salts Solution MEM/EBSS medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, and 1% sodium pyruvate and antibiotics (all from Hyclone, Logan, UT, USA) at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Fluvastatin was purchased from (Axxora, Coger S.A.R.L., Paris), Zoledronic Acid (Zol), was synthesized as described earlier (Lecouvey et al., 2001; Monteil et al., 2005).

In vitro viability inhibition assay

MDA-MB-231 and D3H2LN cell viability was assessed using the MTT-microculture tetrazolium assay (Mosmann, 1983). The cells (5000 cells per well in 96 well plate) were then incubated with different concentrations of Zol or Fluva, for 24h, 48h, and 72h at 37°C in a 5% CO_2-incubator. Optical density was measured at 570 nm using a Labsystems Multiskan MS microplate reader.

Intracardiac experimental metastasis model

Fluva and Zol effects were evaluated on already established metastasis. Zol administration of 100 µg/kg, corresponding to 2.5 µg/mouse, was chosen to be inferior to the effective dose described earlier in 4T1/luc mouse breast cancer model (5 µg/mouse, i.e. 150-200 µg/kg) (Hiraga et al., 2004). The highest Fluva dose (15 mg/kg/d), equivalent to a human dose of 80mg/d, was chosen according to previous publication (Campbell et al., 2006). Drug Curative effects were evaluated on the progression of established metastases as following: Female nude

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mice 8-10 weeks old (Janvier) were anesthetized intraperitoneally with 120 mg/kg ketamine and 6 mg/kg xylazine and were injected with D3H2LN ($1x10^5$cells) in 100µl sterile PBS into the left ventricle of the heart by non surgical means. A successful intracardiac injection (IC) was immediately confirmed by systemic bioluminescence signal distributed throughout the animal. Only mice with satisfactory injection continued the experiment. Once metastatic colonisation had been confirmed 7-10 days after cells IC injection (the baseline of metastasis bioluminescent signal was set to 300 photon counts/sec maintained for 2-3 days, i.e. seen at two subsequent acquisitions), treatments were administered by intraperitoneal injection with 100 µl of PBS solutions of Zol (100 µg/kg three times a week) or Fluva (1 or 15 mg/kg daily) or PBS alone (daily for control group). The first drug administration was denoted as Day 0, and the treatments lasted for 3 weeks (Day 21).

Bioluminescent Imaging

Anesthetized mice by exposure to 1-3% Isoflurane, were placed in the IVIS™ Imaging System (Xenogen) and imaged from both dorsal and ventral views approximately 5 min after intraperitoneal injection of 150µl D-luciferin (Caliper Life Science) at 30 mg/ml per mice. Assessment of established metastasis was evaluated by imaging during the treatment period (3 weeks). Acquisition time was 5 min at the beginning of the time course and was progressively reduced afterwards in accordance with signal strength to avoid saturation. Analysis was performed using Living Image software (Xenogen) by measurement of photon flux (photon/s/cm^2) with a region of interest (ROI) drawn around the whole animal bioluminescence signal. Signals of both dorsal and ventral views at early stages, and from 4 views (dorsal, ventral, left and right) at later stages were averaged for each animal in order to avoid the discrepancy related to the depth of metastases location.

Gene Expression Profiling

MDA-MB-321 cells were lysed after 12h and 24h treatment with 2 µM Fluvastatine, or 30 µM Zoledronate (concentrations corresponding to IC_{50} at 72h treatment), or treated with Zoledronate for longer time (48h at 30 µM) or with its higher concentration (24h at 100 µM of Zoledronate). Control were treated with equivalent volume of PBS 1X added to each drug solution. Total RNA from these treated or control cell cultures (four independent replicates for each condition) were isolated using RNeasy mini kit (Qiagen, Valencia, CA, Germany) by direct lyses on the 10 cm culture dish with 600 µl RLT Buffer following manufacturer's instructions. Purified RNA was quantified with the Nanodrop ND-1000 spectrophotometer

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(Thermo Fisher Scientific, Hudson, NH, USA) and its quality was checked by running the Eukaryotic Total RNA Nano Assay in the Bioanalyser 2100 (Agilent Technologies, Chandler AZ, USA) using Agilent RNA 6000 Nano Kit. Samples were prepared for hybridization to Affymetrix GeneChip® Human Gene 1.0 ST arrays according to manufacturer's instructions starting from 300 ng of total RNAs. Quality Control of cRNA synthesis, hybridization and data acquisition was performed according to the manufacturer's protocol completed with personal QC and data visualization.

Data Analysis

Raw data were processed with the apt-probeset-summarize program from the Affymetrix Power Tools (v1.10.2) for background correction and normalization. RMA (Robust Multichip Analysis) algorithm (Irizarry et al., 2003) with full quantile normalization was applied using the array design library files V1 release 4 and normalised values expressed on a log base 2 scale. Transcripts and genes were annotated with the Affymetrix release 29 annotation files based on the human genome version 18 assemblies. Data analysis was performed with the R Bioconductor LIMMA package. The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE33552 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33552). Probe sets corresponding to control genes or having a low intensity signal (median log2 of intensity <4) or having low variability (trimmed range excluding the min-max values <0.5) were not considered for further analyses, yielding a total of 22289 probe sets. Only genes with a false discovery rate<0.05 and fold change≥1.5 in paired comparison between treatments and their corresponding controls were considered as differentially regulated. Analysis of genes associated with cell functions was carried out using Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems Inc.) to identify biological processes and pathways which may be associated with modulated gene expression.

Quantitative real-time RT-PCR analysis

Total RNA (20 μg) was reverse-transcribed using MMLV RT (Invitrogen, Carlsbad, CA, USA) and Random primers from Roche Applied Science (Roche, Indianapolis, IN, USA); 40 ng of reverse transcribed RNA was used as template for each reaction. qRCR assays were designed using Universal Probe Library site https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp (Roche Applied Science). Real-time PCR was carried out

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on LightCycler ® 1.5 Real-Time PCR System (Roche, Indianapolis, IN, USA) either using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit with primers purchased from Tib Molbiol Syntheselabor GmbH (Germany), or LightCycler TaqMan Master Kit and Universal ProbeLibrary probes (Roche Applied Science) with primers from Tib Molbiol. The reactions were cycled 45 times (95ºC, 10 sec; 60ºC, 30 sec) after initial 10 min incubation at 95ºC for TaqMan procedure or 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 60°C for 10 sec and 72°C for 10 sec for SYBR Green procedure. Results were normalised to the expression of the *PPia* or *beta2M*, two genes that exhibit little variation in this data set out of five tested house-keeping genes. Similar results were obtained for both genes of reference. The mRNA transcript level for target genes was calculated as outlined in previously (Pfaffl et al., 2002)

Ratio = (Eff target) $^{\Delta Cp\ insulin(MEANcontrol - MEANsample)}$ / (Eff reference) $^{\Delta Cp\ 18S(MEANcontrol-MEANsample)}$

Statistical Analysis

Analysis of *in vitro* and *in vivo* metastasis progression (bioluminescent signal or number of metastatic sites) was performed using the ANOVA Tukey-Kramer Multiple Comparisons Test for and Student's t-test. $P<0.05$ was considered significant. Kaplan-Meyer survival curves were generated using Graph Pad Prism software.

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RESULTS

In vivo anti-metastatic potential of Zol and Fluva

Drug effect was evaluated on the progression of established metastases (Fig. 2 and 3). Mice with successful intracardiac injection (Fig. 2A) developed metastases within 2 weeks. Once metastasis detected (Bioluminescent signal > 3 00 photon counts), mice were randomly divided in four groups and received intra-peritoneal injections three times a week with Zol at 100 µg/kg (n=8), or daily with Fluva at 1 mg/kg (n=8) or 15 mg/kg (n=7) or treated daily with PBS for control group (n=8) during 3 w eeks. While no significant effect on metastasis bioluminescence was observed with F luva at 1mg/kg (supplemental Figure S1), treatment with Fluva at 15 mg/kg or Zol 1 00 µg/kg induced about 80 % in hibition of luminescence signalling at the end of treatment (p=0.01 and 0.02, respectively, Fig. 2B,C). During last (third) week of treatment, 3 out of 7 Fluva-treated (15 mg/kg) mice manifested a decrease of established metastatic sites, and 5 ou t of 7 mice showed visible reduced/stabilized bioluminescent signal (Fig. 3A and Supplementary Table 1); this decrease of metastasis luminescence was not observed with Zol.

In order to visualize the organs affected by metastases, we performed *ex vivo* analysis. *Ex vivo* imaging of the different tissues after the final imaging *in vivo*, showed bone lesions (legs, scull, spine) as well as ovary, brain, lung, kidney, or lymph node ones (Fig 3B).

Mice survival curves were generated from the first drug administration followed by 3-week-treatment and until they died (Fig. 3C). Fluva (15 mg/kg) or Zol (100µg/kg) treatment significantly reduced the death of mice as compared to control with median survival of 21 days for control, 33 for Zol and 28 for Fluva (P=0.0245, Fig.3C).

Transcriptomic profiling of Zol or Fluva

In order to evaluate the different genes regulated by Zol and Fluva, we performed a transcriptomic analysis. To find a comparable treatment condition for each drug, their effects on MDA-MB-231 breast cancer cells survival/proliferation were first tested using a standard MTT assay at 12h, 24h, 48h, and 72h (supplemental Figure S2). Initially, we chose the concentration as the half maximal inhibitory concentration detected after 72 h treatment **(**$IC50_{72h}$), corresponding to 2 µM for Fluvastatin and 30 µM for Zoledronate. Early treatment was chosen (12h and 24h). RNAs extracted from four replicates (four independent treatments

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and respective controls for each condition) at each time point were subjected to hybridization on HuGene 1.0ST Affymetrix arrays (Fig.4). Analysis of genes functions common to Zol and Fluva (540 probesets: 373 up- and 167 down-regulated annotated genes, Supplementary Table 4A,B) showed that the majority of them were involved in functions critical for metastatic steps such as cellular proliferation, migration, invasion and apoptosis (Fig 4 A , B). In addition, only 9 genes (DKK1, FGF1, FST, G0S2, HIF1A, LGALS3, PVR, TNFRSF12, UAP1) dependenting on Ras activation were modified as compared to previous work (Loboda et al., 2010). In addition, by compiling genes whose expression is dependent on MAPK/PI3K pathways as described in a previous data (Bild et al., 2006) only 9% of the concerning genes whose expression depends on prenylation of Ras protein or Rho activation were modified. Transcriptomic data were validated by qPCR on 13 differentially regulated by Zol or Fluva genes (5 Zol-specific and 8 common Zol/Fluva genes asterisked in supplemental Tables 3-4).

The analysis of significant differentially expressed genes between treated and control cells (FDR<0.05, fold change>1.5) revealed 29 probe sets (18+11 on the diagram) for Fluva at 12h, additional 526 probe sets for Fluva at 24h (16+365+145 on the diagram) (supplemental Figure S3A) and no significant genes for Zol at 12h nor at 24h (supplemental Figure S3B) Analysis of genes specifically regulated by each drug, revealed 16 probe sets for Fluva corresponding to 12 annotated genes (supplemental Figure S3A, C, Supplementary Table 2) and a more important number (1032 probe sets corresponding to 951 annotated genes) for Zol treatments (supplemental Figure. S3A, D, Supplementary Table 3). The majority of genes regulated by Fluva at 12h or 24h (100% and 97%, respectively), were common to Zol100 24h gene profile (supplemental Figure S3A). Clustering of common Fluva and Zol genes (supplemental Figure S3B) reveals that for Zol treatment the pattern starts to appear at 24h with the shortest distance found between Zol at 48h (ZOLCONT48) and Fluva at 12h (FLUVACONT12) indicating early changes. It might indicate retarded Zol kinetics as changes induced by Zol at 48h were most similar to those induced by Fluva at 12h.

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In vivo anti-metastatic potential of Zol and Fluva combination

Since Zol can inhibited apoptosis differently from Fluva by inducing cytotoxic analog AppI (Fig 1) and since the combination of these two drugs was also described synergistic *in vitro* (Budman and Calabro, 2006) we tested the combination treatment *in vivo* using the same concentrations as described above for these drugs alone. Mice were treated with Zol (100 µg/kg, three times a week) combined with Fluva at 1 mg/kg or 15 mg/kg administered daily (Supplemental Fig. S1, and Fig 5). The combination of Zol with the highest Fluva concentration (15mg/kg) did not change significantly the level of bioluminescence metastasis signal (Fig. 5A) nor metastatic site number (data not shown) as compared to Zol or Fluva alone. Survival analysis showed no significant differences between Zol, Fluva (at 15mg/kg) or their combination (Fig. 5B), with median survival of 33, 3 8, and 34 days respectively. Moreover, combinatorial treatment did not result in any improvement of the effect seen with Fluva when administered at a non-efficient 1 mg/Kg concentration (supplemental Figure S1).

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DISCUSSION

For the first time, we demonstrate the curative effect of Fluva on breast cancer metastases *in vivo* using MDA-MB-231 population (D3H2LN) metastasis model. Indeed, Fluva induces a strong inhibition of the progression of established metastases (80%) when administered at 15 mg/kg. As compared to Zol, Fluva alone delayed the growth of established metastases as soon as the first week of the treatment. It is to note that we observed a partial metastasis regression in three out of seven mice characterized by decay of bioluminescent signal. However this effect was not observed in all mice since we only detected 40% of inhibition in the mean number of metastatis sites (data not shown). Concerning organs affected by metastases both Zol and Fluva affected bone as well as visceral metastases localisation.

Transcriptomic analyses of MDA-MB-231 cells treated with Fluva or Zol demonstrate common regulation of genes implicated in metastatic capacities of tumor cells. The majority of differentially expressed genes under 12h and 24h Fluva (2 µM for $IC50_{72h}$ concentration) treatment were regulated in the same manner by Zol when incubated at its $IC50_{72h}$ (30µM) for a longer time (48h), or with higher concentration (100µM) for the same time treatment (24h). This kinetic difference between proliferation and transcriptomic activity could be explained by the higher lipophily of Fluva versus Zol (LogP 3.69 versus -0.93, respectively) that allowed a better penetration of Fluva into the cells. In addition, proliferation might involve protein post translational changes that cannot be directly evaluated in transcriptome analysis. Genes implicated in cellular movement, cellular growth and proliferation, development as well as apoptosis are regulated in the same manner and provide target metastatic genes for both molecules. For instance gene expression is consistent with the pro-apoptotic activity of both drugs : the expression of 14 pro-apoptotic genes (TP53INP1, BTG1, TNS4, PGEP1, TP5313, MAP3K5, BINP3L, IFIH1, IER3, PTPRH, TMEM173, SLK,CD14, TNFRSF1A) is increased while only 6 pro-apoptotic genes are down-regulated (TGM2, ERCC2, PAWR, AEN, TNFRSF12A, FAM176A).

To note that some genes modified by both drugs are also involved in immune and inflammatory responses. Genes modulating the immune response are up regulated when they stimulate the immune system (CMKLR1, APOBE3CF, TLR6, REG, IFIH1, ANXA11, TMEM173, CD14) and down regulated when they are immuno-suppressive (PTGER4, PDCD1LG2, CD174), attributing globally to both drugs an immuno-stimulant profiles. This effect of Fluva on cancer cells is notably different of what is observed in immune cells, in

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which statins inhibit the immune system (Greenwood et al., 2006). This point is important since it demonstrates that these molecules could interact in concert with the immune system to induce suppression of cancer cells. To verify this finding, it would be interesting to further study drug effects on metastases processes in immuno-competent mice model.

Only one *in vivo* study of statins combined with bisphosphonates showed a delay in pancreatic tumor growth and an increase in mouse survival when combining lovastatin and pamidronate (Issat et al., 2007). Also, Fluva alone or in association has been described to inhibit tumor growth and metastases formation of hepatocarcinoma cell lines (Issat et al., 2007; Paragh et al., 2003). In addition, Fluva is effective on other cancers such as pancreatic and head and neck carcinomas (Bocci et al., 2005; Fujiwara et al., 2008). For breast cancer cells, the use of Fluva in combination with other chemotherapeutic molecules was also described *in vitro* (Budman et al., 2007) lacking for *in vivo* data. In our *in vivo* study of Fluva combination, we observed no differences in metastatic growth in combination treatments. Association of Fluva and Zol at their efficient concentrations (15 mg/kg and 100 µg/kg, respectively) resulted in no significant differences compared to the effects of these drugs alone, as well as combination treatment with Fluva at non-efficient 1mg/kg concentration. Observation of common transcriptomic profiles between Zol and Fluva supported this finding of the absence of synergy *in vivo*. The differences observed between our work on metastasis and other ones on tumor xenograft can also be explained by these molecule activities since they both target Rho/Ras proteins. However, concerning genes whose expression depends on prenylation of Ras protein or Rho activation (Bild et al., 2006), only 9% of the total number of these genes was modified by Zol or Fluva. This finding suggested that other mechanisms are involved in the inhibition of metastasis formation by the two drugs. Futhermore the specific capacity of Zol to inhibit DNA synthesis could complete the Fluva one in primary tumor rather than in metastatic cell growth. In conclusion, this study demonstrates the potential benefit of the use of Fluva associated or not with bisphosphonates in the context of established breast cancer metastases. It also suggests that the frequent association of statins and bisphosphonates in old patients could influence the course of breast metastasis

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Vintonenko, Crepin, Perret, and Di Benedetto

Conducted experiments: Vintonenko, Kassis, and Abdelkarim

Contributed new reagents or analytic tools: Lecouvey

Performed data analysis: Di Benedetto, Vintonenko and Jais.

Wrote or contributed to the writing of the manuscript: Vintonenko, Jais, Perret, and Di Benedetto

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FOOTNOTES

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LEGENDS OF FIGURES

Figure 1. Mevalonate pathway inhibition scheme. Prenylation of small G-proteins can be inhibited either by statins acting on upstream enzyme HMG-CoA reductase, or by Bisphosphonates targeting downstream enzyme Farnesyl-pyrophosphate synthase which could lead to cytotoxic ATP analog ApppI accumulation.

Figure 2. Fluvastatin or Zoledronate alone reduce metastatic burden in breast cancer metastasis mice model. *A,* Bioluminescent MDA-MB-231 (D3H2LN) cells (10^5) were injected into the left ventricle of nude mice. Only mice with successful intracardiac cells injection, witnessed by overall luminescent signal, continued the experiment. *B,* Plot of bioluminescent signal for control group treated (daily with PBS 1x) and treated with Zol (at 100µg/kg three times a week) and Fluva (at 15mg/kg daily) mice; each point represents a mean (±SEM) signal of eight (Control or Zol) or seven (Fluva) mice** $p<0.01$ vs Control (ANOVA Tukey-Kramer Multiple Comparisons Test for j21 Control, Zol, Fluva) *C,* Treatment by Zol, Fluva, or PBS for control group was started upon confirmation of detectable established metastasis within two weeks after injections (denoted as Day 0 for treatment); representative bioluminescent images for indicated days of treatment are rendered at the same photon scale for control group, and mice treated with Zol (100µg/kg) and Fluva (15mg/kg).

Figure 3. Effects of Fluva on number of metastatic sites and mice survival.
A. The number of detected metastatic sites in treated Fluva at 15mg/kg and control mice. The duration of treatment was 21 days**.** *B,* Ex vivo representative images of metastatic sites in mice treated with Fluva (15mg/kg). *C,* Kaplan-Meyer survival curves for control, Zol (100µg/kg), and Fluva (15mg/kg) treated mice (median survival of 21, 33, and 38 days respectively) generated for 3-weeks treatment period and followed up until they died.

Figure 4. Functional analysis of genes commonly regulated by Fluva or Zol.
Ingenuity Pathways Analysis for 540 probe sets common between Fluva (either at 12h or 24h) and Zol (either at 30 µM at 48h or 100 µM at 24h) treatments. *A,* 373 up-regulated probe sets corresponding to 370 annotated genes. *B,* 167 down-regulated probe sets corresponding to 166 annotated genes.

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Figure 5. Effect of Fluva and Zol combination *in vivo*.

Bioluminescent MDA-MB-231 (D3H2LN) cells (10^5) were injected into the left ventricle of nude mice. Only mice with successful intracardiac cells injection, witnessed by overall luminescent signal, continued the experiment. As described in Fig. 2, mice were treated upon detection of established metastasis (referred as Day 0) with Zol (at 100µg/kg three times a week), Fluva (at 15mg/kg daily), their combination (100µg/kg Zol trice a week + 15mg/kg Fluva daily), or PBS (daily for control group) during 3 weeks. *A,* Plot of bioluminescent signal; each point represents a mean (±SEM) signal of eight (Control, Zol, Fluva or Zol with Fluva combination) or seven (Fluva) mice. ** p<0,01 vs Control (ANOVA Tukey-Kramer Multiple Comparisons Test for Day 21 Control, Zol, Fluva15, Zol+Fluva15). *B,* Kaplan-Meyer survival curves for control and treated mice (median survival of 21 days for Control, and 33, 38, and 34 days for Zol, Fluva, or Zol with Fluva combination respectively) generated for 3-weeks treatment period and followed up until they died.

Figure 1.

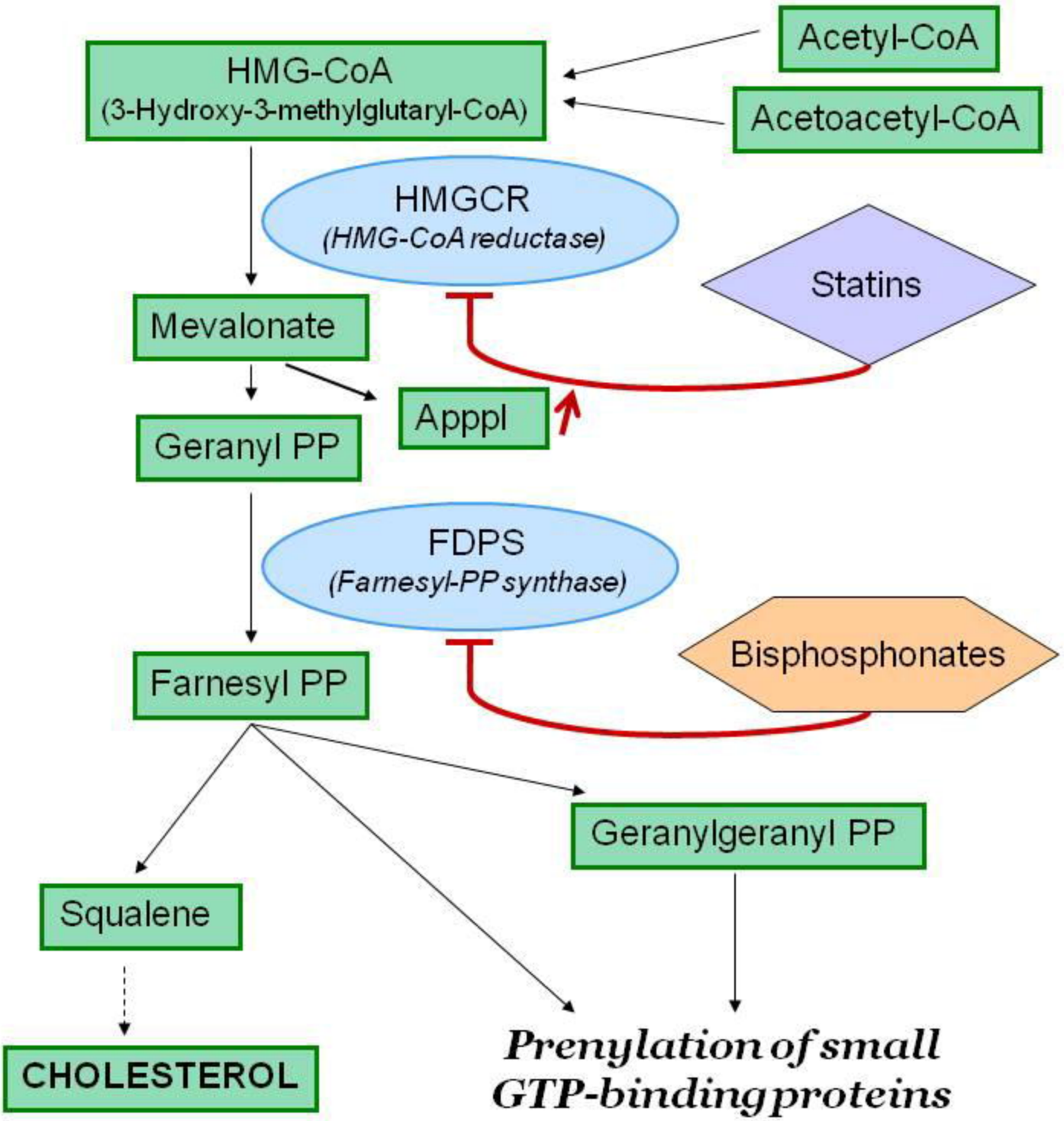

HMG-CoA
(3-Hydroxy-3-methylglutaryl-CoA)
Acetyl-CoA
Acetoacetyl-CoA
HMGCR
(HMG-CoA reductase)
Statins
Mevalonate
Apppl
Geranyl PP
FDPS
(Farnesyl-PP synthase)
Bisphosphonates
Farnesyl PP
Geranylgeranyl PP
Squalene
CHOLESTEROL
Prenylation of small
GTP-binding proteins

Figure 2.

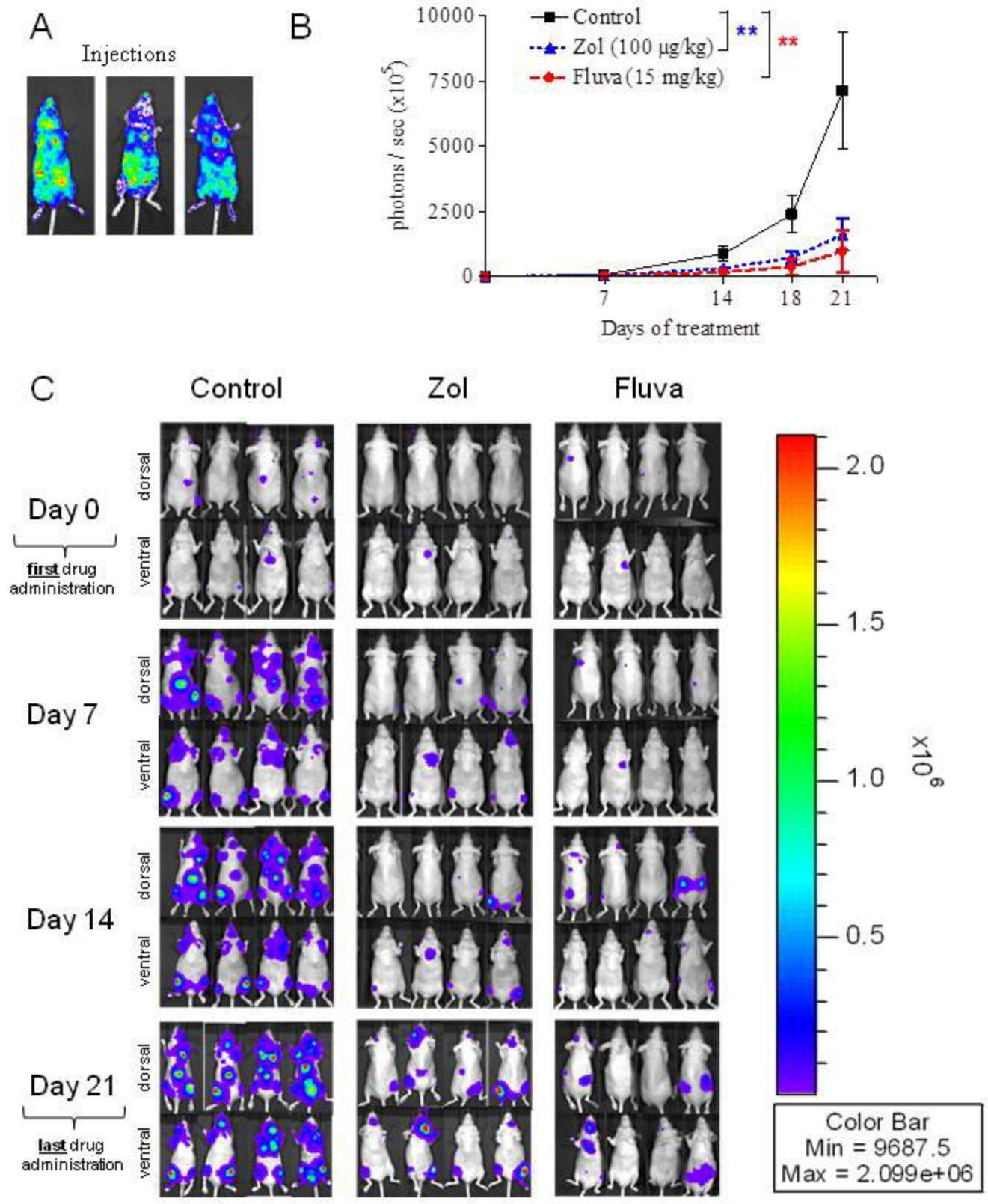
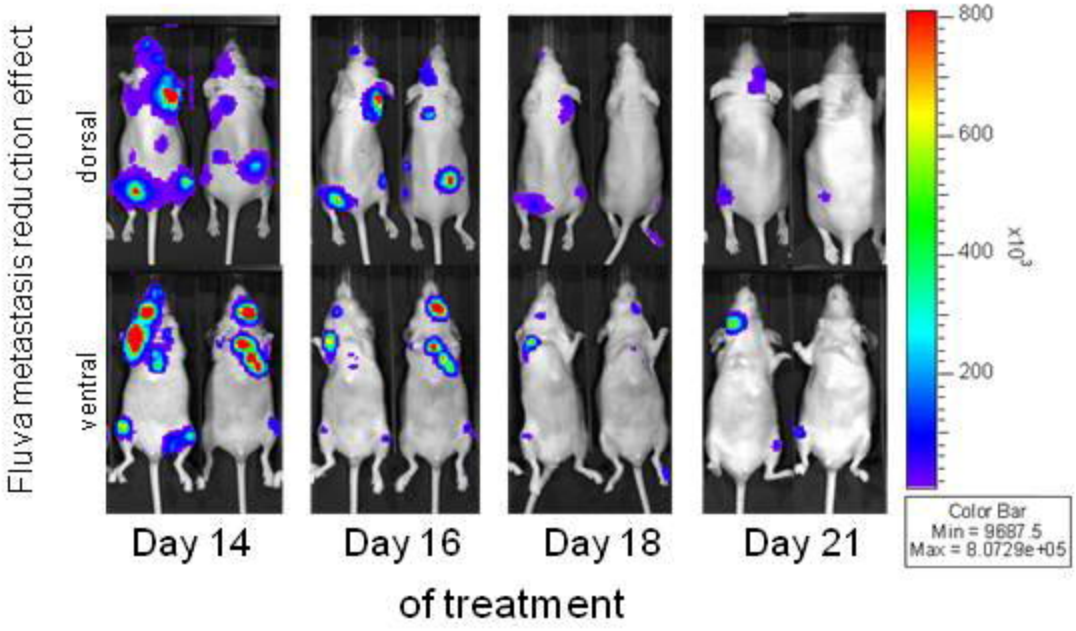
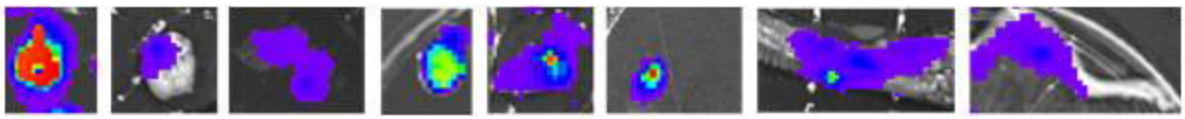

A
Injections
B
Control
Zol (100 µg/kg)
Fluva (15 mg/kg)
**
**
photons / sec (x10^5)
10000
7500
5000
2500
0
7
14
18
21
Days of treatment
C
Control
Zol
Fluva
Day 0
first drug administration
Day 7
Day 14
Day 21
last drug administration
dorsal
ventral
2.0
1.5
1.0
0.5
x10^6
Color Bar
Min = 9687.5
Max = 2.099e+06

Figure 3.

A


Fluva metastasis reduction effect
dorsal
ventral
Day 14
Day 16
Day 18
Day 21
of treatment
800
600
400
200
x10³
Color Bar
Min = 9687.5
Max = 8.0729e+05

B



C

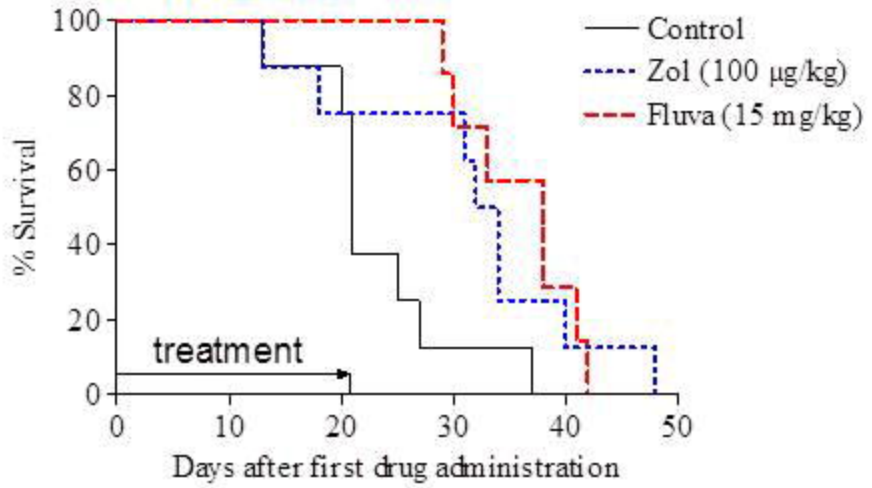
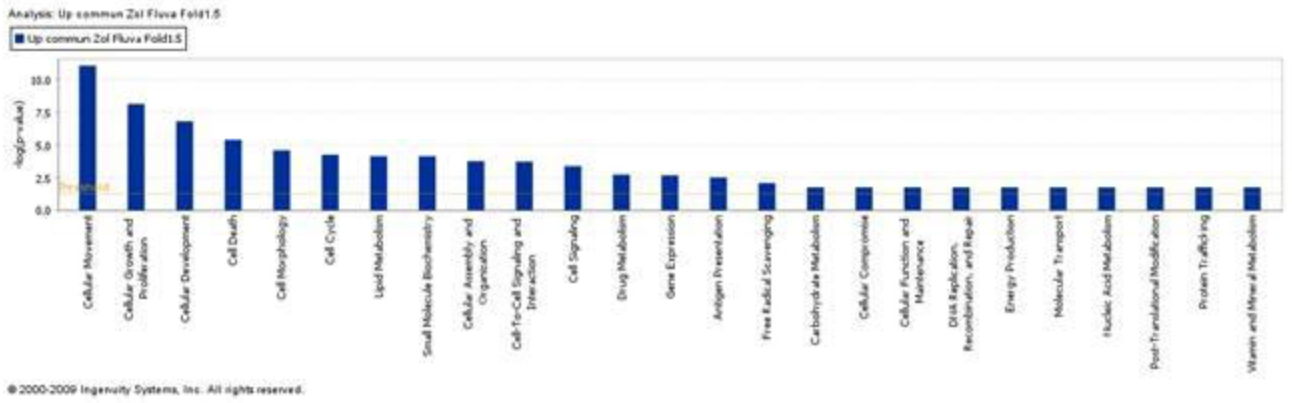

100
80
60
40
20
0
% Survival
treatment
0
10
20
30
40
50
Days after first drug administration
Control
Zol (100 µg/kg)
Fluva (15 mg/kg)

Figure 4.

A



B

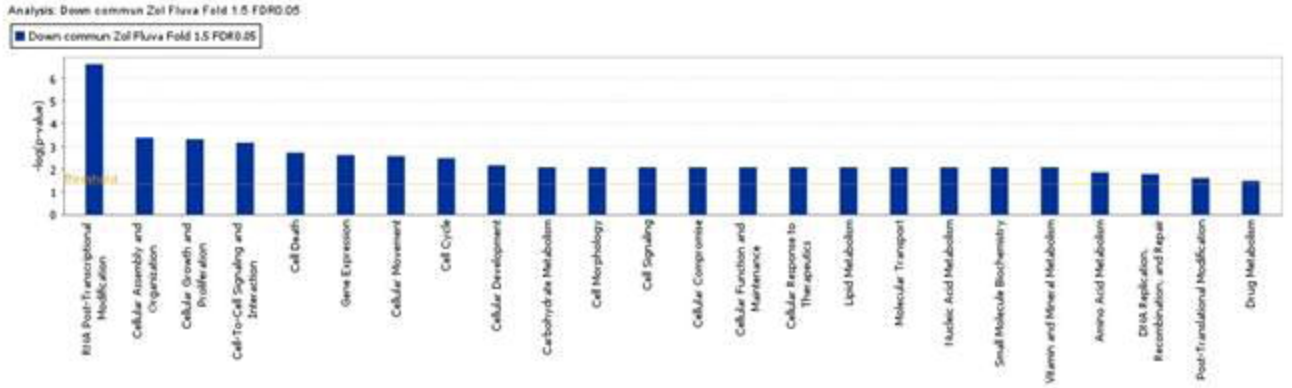
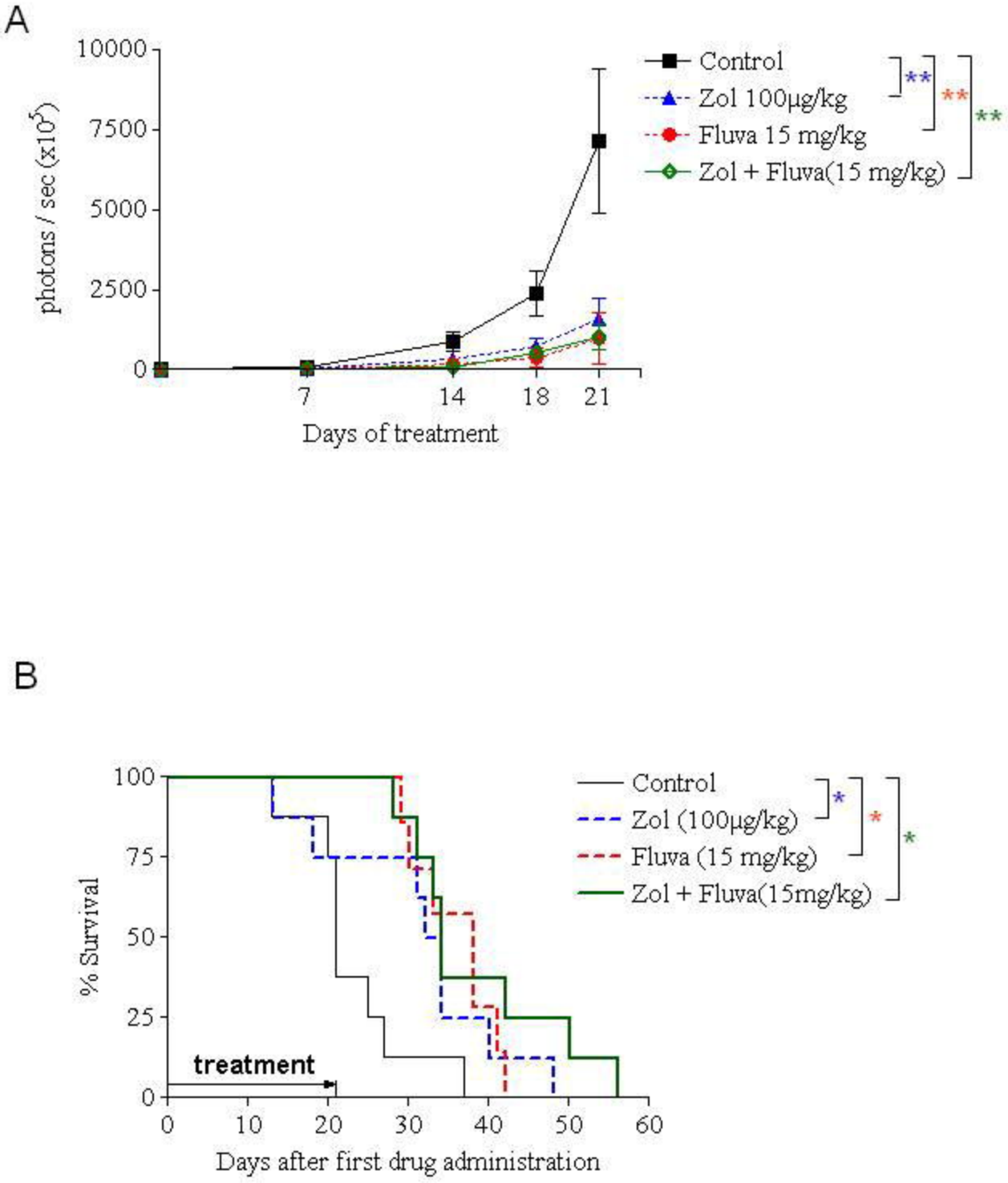


Figure 5.


A
10000
7500
5000
2500
0
photons / sec (x10^5)
7
14
18
21
Days of treatment
Control
Zol 100µg/kg
Fluva 15 mg/kg
Zol + Fluva(15 mg/kg)
**
**
**
B
100
75
50
25
0
% Survival
treatment
0
10
20
30
40
50
60
Days after first drug administration
Control
Zol (100µg/kg)
Fluva (15 mg/kg)
Zol + Fluva(15mg/kg)
*
*
*