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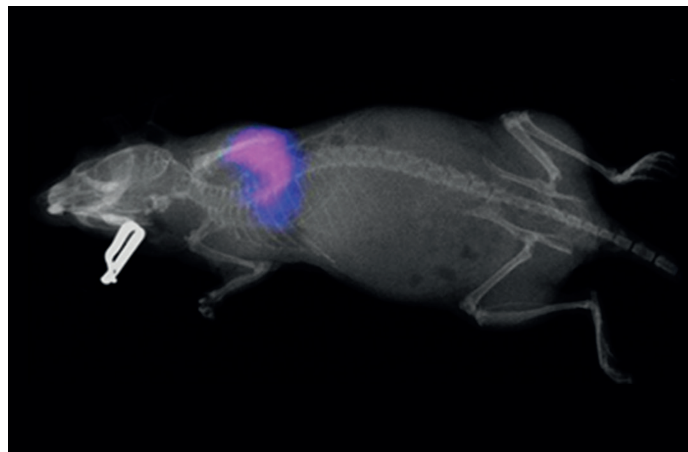

# Simultaneous Non-Invasive *in vivo* Imaging of Apoptosis and Angiogenesis in a Mouse MDA-MB-231 Xenograft Model


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GLPG1790, a small molecule nanomolar inhibitor of ephrin- and a few other tyrosine kinases, shows potent anti-tumor activity in vitro and *in vivo* in a mouse MDA-MB-231 xenograft model.[1] Here we report its effects on apoptosis and angiogenesis after 3 days of oral treatment using non-invasive *in vivo* fluorescence imaging. Correlation with immunohistology readouts was studied.


## Material and Methods

### *In vivo* model

5 mln MDA-MB-231 cells were subcutaneously injected in 50 % Matrigel + 50 % PBS to athymic female nude mice (Charles River, France). After randomization (mean tumor volume 100mm$^3$ for molecular imaging and 300mm$^3$ for tumor growth and histology analysis), mice were treated with GLPG1790 (30 mg/kg, p.o., q.d.), pro-apoptotic reference compound Flavopiridol (5 mg/kg, p.o., q.d.) or anti-angiogenesis reference compound Bevacizumab (5 mg/kg, i.p., 3x/wk). Tumor volumes were measured by caliper up to day 16.

## *In vivo* Imaging

Baseline angiogenesis and apoptotic activities were simultaneous evaluated before initiation of the treatment (D0) using the Bruker In-Vivo Xtreme imaging system, after i.p. administration of Angiosense680 EX and Annexin750 probes (Perkin Elmer), respectively, according to the instructions of the supplier. After 3 days of treatment (D3), angiogenesis and apoptotic activities were similarly assessed; GLPG1790, Flavopiridol or Bevacizumab treatments were given 2hr prior to imaging.

All images were captured by a cooled CCD camera with the following parameters: f-stop 1.1, binning 2, 2 sec acquisition time for Angiosense680 EX and 5 sec acquisition time for Annexin750.

For anatomical co-registration, a reflectance image (0.1 second acquisition time, f-stop 2.8, binning 1) and an X-ray image (1.2 second acquisition time, f-stop 4, 45 KVP energy, 2 mm filter) were performed.

For precise co-registration, all images were taken with a 190 mm field of view (FOV).

For each mouse and each probe, results were expressed as the difference between Net intensity at D3 and D0.

## Histology Analysis

At day 3 of treatment, tumors were fixed for 24h in 3.7% formaldehyde and embedded in paraffin prior to sectioning. 2µm-thick sections were processed by immunohistochemistry (IHC) with anti-cleaved caspase 3 antibody (TL) for apoptosis and anti CD34 antibody (Abcam) for angiogenesis.

Immunostainings were quantified by image analysis (software Sis'nCom, France). Apoptosis index was calculated as %: 100 x (immunopositive cell number / total cell number). Angiogenesis was calculated as vessel density (mm/mm$^2$): vessel length / tumor area.

## Image Analysis and Presentation

Semi-quantitative analysis was performed using Molecular Imaging Software version 7.1.2.20435 (Bruker BioSpin, Billerica, MA USA). A region of interest (ROI) was drawn around one tumor of each mice using the ROI freeform in the manual ROI tool in MI software and then applied on the image from acquired for both probe. After analysis of images obtained with control mice, threshold was set to 300 for Annexin750 and 500 for Angiosense680 EX. Data was exported and figures were compiled in Microsoft Excel®. Images were exported as JPEG files.

## Statistical Analysis

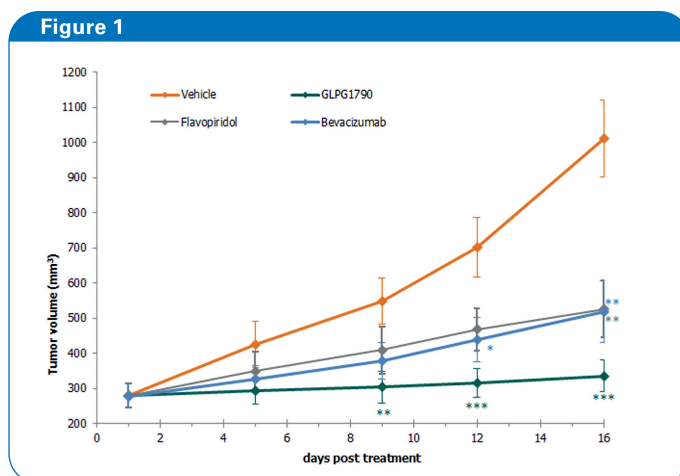
For each read-out, mean and standard error to the mean (s.e.m.) were calculated.

A statistically significant difference between vehicle and treated groups was evaluated with Prism® software using a one-way ANOVA followed by a Dunnett's multiple comparisons post-hoc test. *: $p<0.05$; **: $p<0.01$.

## Results and Discussion

In a preliminary experiment, randomization was performed when mean tumor volume reached 300mm3. Tumor volume evolution was followed up for 16 days (see Figure 1). GLPG1790 completely blocked tumor growth at 30 mg/kg, p.o., q.d. Flavopiridol (5 mg/kg, p.o., q.d.) and Bevacizumab (5 mg/kg, i.p., 3x/wk) delayed tumor growth significantly, but to a lower extent than GLPG1790.

**Figure 1**

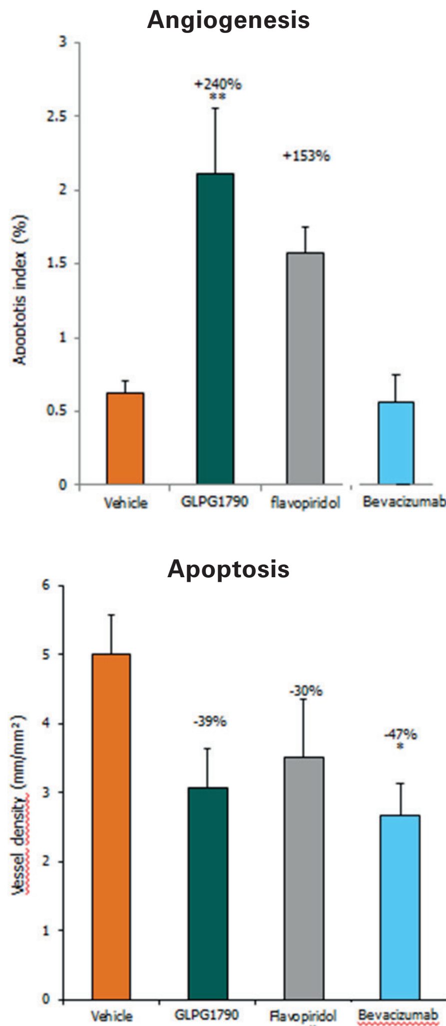



Follow up of tumor growth. 5.10$^6$ MDA-MB-231 cells were subcutaneously injected in athymic female nude mice (n=8 per group). After randomization (mean tumor volume 300 mm$^3$), mice were treated with GLPG1790 (30 mg/kg, p.o., q.d.), Flavopiridol (5 mg/kg, p.o., q.d.) or Bevacizumab (5 mg/kg, i.p., 3x/wk). Tumor volumes were measured by caliper.

Using classical histology readouts for both angiogenesis and apoptosis,we confirmed that GLPG1790 vs. Vehicle control treatments reduced angiogenesis by 39%, and increased apoptosis significantly (240%, $p<0.01$%, see Figure 2). Flavopiridol decreased angiogenesis (30%) and increased apoptosis (150%). Bevacizumab reduced angiogenesis significantly (47%, $p<0.05$%), while it had no effect on apoptosis.

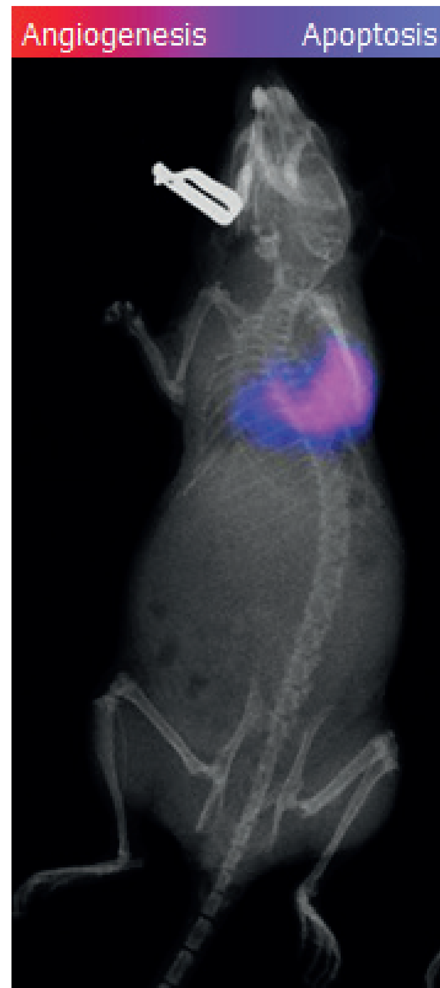
To avoid quantification biases due to effect of compounds on tumor growth or tumor necrosis commonly observed with MDA-MB-231 cells, *in vivo* imaging studies were performed after 3 days of treatment; randomized tumors had reached a mean volume of 100mm$^3$.

**Figure 2**




Measurement of angiogenesis (top panel) and apoptosis (bottom panel) by immunohistochemistry. $5.10^6$ MDA-MB-231 cells were subcutaneously injected in athymic female nude mice (n=8 per group). After randomization (mean tumor volume 300mm3), mice were treated with GLPG1790 (30 mg/kg, p.o., q.d.), Flavopiridol (5 mg/kg, p.o., q.d.) or Bevacizumab (5 mg/kg, i.p., 3x/wk) for 3 days. Immunohistochemistry was performed using anti-cleaved caspase 3 antibody for apoptosis and anti CD34 antibody for angiogenesis. Apoptosis index was calculated as %: 100 x (immunopositive cell number / total cell number). Angiogenesis was calculated as vessel density ($mm/mm^2$): vessel length / tumor area.

**Figure 3**

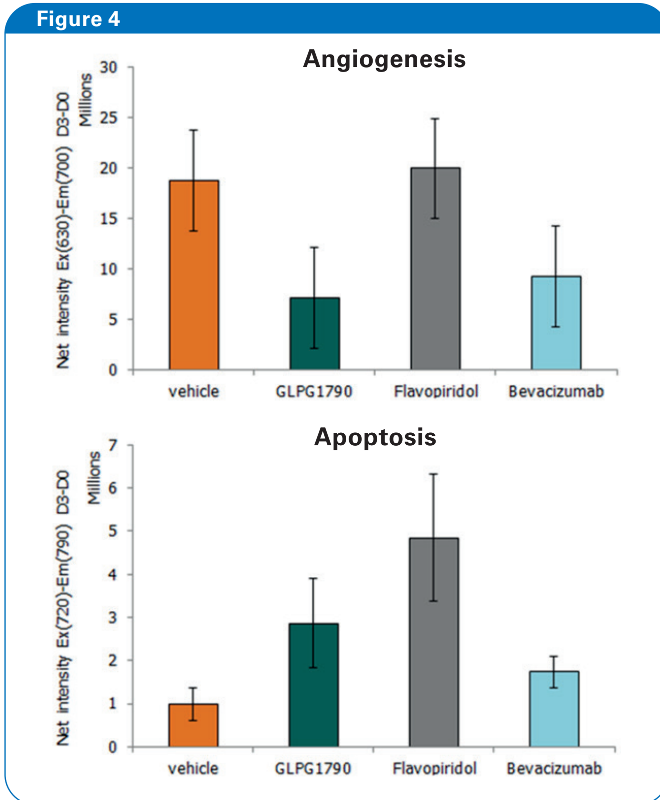



Simultaneous visualization of angiogenesis and apoptosis using the Multiplex functionality of the Bruker In-Vivo Xtreme imaging system. $5.10^6$ MDA-MB-231 cells were subcutaneously injected to athymic female nude mice (n=5 per group). After randomization (mean tumor volume $100mm^3$), mice were treated with GLPG1790 (30 mg/kg, p.o., q.d.). At D3, compound was given 2 hr prior to imaging.

The AngioSense680 probe has been designed for fluorescence imaging of blood vessels and angiogenesis. The Annexin750 probe has been developed to enable fluorescent visualization of phosphatidyl-serine exposed in the outer leaflet of the cell membrane lipid bilayer during the early stages of apoptosis. After image acquisition, both signals can be visualized using the Multiplex functionality of the Bruker In-vivo Xtreme imaging system (see Figure 3).

Angiogenesis and apoptotic activities were simultaneously evaluated at baseline and after 3 days of treatment by i.p. administration of Angiosense680 and Annexin750 probes, respectively. At D3, imaging compounds were given 2 hr prior to imaging. Results were expressed as the difference between net intensity at D3 and D0. By this fluorescent probe analytical approach, GLPG1790 decreased angiogenesis to the same extent as the anti-angiogenesis reference compound Bevacizumab, while Flavopiridol did not impact angiogenesis (see Figure 4, top panel). GLPG1790 induced apoptosis, as did the pro-apoptotic reference compound, Flavopiridol, and to a lower extent Bevacizumab. These results are in line with histological analysis (see Figure 2).

Figure 4




Simultaneous measurement of angiogenesis and apoptosis using the Bruker In-Vivo Xtreme imaging system. $5.10^6$ MDA-MB-231 cells were subcutaneously injected to athymic female nude mice (n=5 per group). After randomization (mean tumor volume $100mm^3$), mice were treated with GLPG1790 (30 mg/kg, p.o., q.d.), Flavopiridol (5 mg/kg, p.o., q.d.) or Bevacizumab (5 mg/kg, i.p., 3x/wk). Baseline angiogenesis and apoptotic activities were simultaneously evaluated before initiation of the treatment (D0), after i.p. administration of Angiosense680 EX and Annexin750 probes, respectively. After 3 days of treatment (D3), angiogenesis and apoptotic activities were similarly assessed with imaging probe compounds given 2hr prior to imaging. For each probe, results were expressed as the difference between Net intensity at D3 and D0.

## Conclusion

GLPG1790 is an anti-tumor reagent that can completely block established tumor growth in the mouse MDA-MB-231 xenograft model, at the well tolerated dose of 30 mg/kg. Flavopiridol and Bevacizumab delay significantly the tumor growth to a lower extent than GLPG1790.

In this application note, we have used a non-invasive, fluorescent *in vivo* imaging technique, at just day 3 into treatment, to identify two anti-tumorigenic mechanisms of action of GLPG1790: a reduction in angiogenesis, and an increase in apoptosis.

This non-invasive and early fluorescent readout approach has been cross-validated with immunohistological analyses, and it could be used, therefore, as a secondary endpoint in a wide range of preclinical, cancer studies designed to demonstrate tumor growth inhibition.

## References


[1] Pujuguet et al., GLPG1790: the first Ephrin (EPH) receptor tyrosine kinase inhibitor for the treatment of triple negative breast cancer, AACR 2014



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