# M402, a Heparan Sulfate Mimetic, Inhibits Tumor Revascularization and Invasiveness after High-Dose Taxane Treatment in a Mouse Breast Cancer Model

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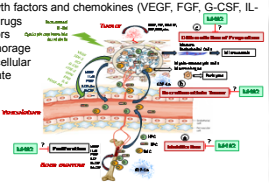
## BACKGROUND

Treatment with certain anti-cancer agents, particularly taxanes and sunitinib[1,2], can lead to mobilization of pro-angiogenic factors and subsequently Endothelial Progenitor Cells (EPCs), which home to the viable tumor rim where they can enhance tumor vascularization.[3,4] This phenomenon has been linked to rapid tumor regrowth following treatment and may thus diminish its long-term efficacy. [5,6] EPCs as well as other bone marrow-derived stromal cells are mobilized in response to circulating pro-angiogenic growth factors and chemokines (VEGF, FGF, G-CSF, IL-6, SDF-1α, etc.)[7] that are induced by certain drugs or the progressing tumor. Many of these factors contain heparin binding domains for their anchorage to proteoglycans on cell surfaces or the extracellular matrix.[8] Here, we tested a novel heparan sulfate mimetic, M402, for its ability to inhibit

a) EPC mobilization
b) EPC function on tumor angiogenesis, and
c) Tumor invasiveness and metastasis

as a result of interference with heparin-binding cytokines, chemokines and growth factors.

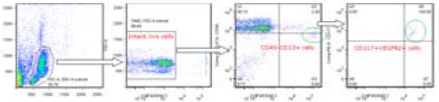



## MATERIALS / METHODS

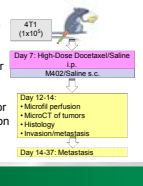
**Materials.** Docetaxel was purchased from Sanofi-Aventis. Antibodies for flow cytometry and histology were from Biolegend, BD Biosciences (CD13) or Biocare (CD31).

**M402.** M402 was prepared at Momenta by controlled depolymerization of unfractionated heparin with nitrous acid and then subjected to sequential periodate oxidation and borohydride reduction. The final product was isolated by salt-methanol precipitation to yield a glycol-split heparan sulfate mimetic of 5500-6500 Da with an anti-Xa activity of 2-10 IU/mg.

**EPC mobilization.** Female Balb/c mice (6-8 weeks) were injected i.p. with docetaxel (once, 40 mg/kg) or saline control. M402 (40 mg/kg) or saline control was injected s.c. 15-30 min. prior to the chemotherapeutic. Mice were serially bled via the submandibular plexus or terminally bled by cardiac puncture. Bone marrow was isolated from 1 femur after $CO_2$ asphyxia of the mice. EPCs were stained in lysed, washed whole blood or bone marrow after Fc block with a cocktail of anti-CD13-FITC, CD117-PE, 7-AAD, VEGFR2-A647, and CD45-PE/Cy7 and gated as outlined below.




**4T1 breast cancer model.** Balb/c mice (6-8 weeks) were injected with $1x10^5$ 4T1-luc cells into the 4th mammary fat pad. On Day 7, mice were treated i.p. with docetaxel (once, 40 mg/kg) or saline control. M402 (40 mg/kg) or saline control was dosed s.c. 15-30 min. prior to docetaxel. In some experiments, M402 was dosed also daily thereafter for 5 days. The effect on primary tumors was analyzed on Day 12-14. For analysis of tumor vasculature, mice were perfused with Microfil, tumors excised and analyzed by microCT (Numira Biosciences). Tumor growth, invasion, and metastasis was monitored by bioluminescence on A Xenogen Lumina system. To study the effect on metastasis, primary tumors were resected on Day 12-14 and lungs evaluated on Day 37.




## RESULTS

### EPC Mobilization

**M402 Inhibits High-Dose Docetaxel-Induced EPC Mobilization**

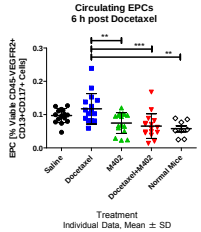



**Figure 1. Effect of M402 on docetaxel induced EPC mobilization.**
4T1-tumor bearing Balb/c mice were dosed with docetaxel (i.p., 40 mg/kg), or saline once ± M402 (s.c., 40 mg/kg) or saline control. 6 h after dosing, the mice were bled via the submandibular plexus (100 µL). Blood was lysed (erythrocytes) in 5 mL lysis buffer, washed twice and stained for live EPCs. 100,000 live cells were acquired on a FACSCanto. EPCs were defined as intact and live CD45-VEGFR2+ CD13+CD117+ cells.
** p<0.01 ***p<0.001 by 1-way ANOVA with Tukey's multiple comparison test
Note that tumor-bearing mice (see saline group) have increased circulating EPC levels as compared to normal, non-tumor bearing mice.

**M402 May Trap the Induced EPCs in the Bone Marrow**

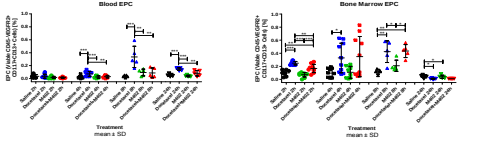



**Figure 2. Time course of docetaxel-induced EPC mobilization – blood vs. bone marrow**
Balb/c mice (tumor-free) were dosed with docetaxel (i.p., 40 mg/kg) or saline control once ± M402 (s.c., 40 mg/kg) or saline control. At 2 h, 4 h, 8 h, and 24 h after dosing, the mice were euthanized and blood taken by cardiac puncture. 100 µL of blood was lysed (erythrocytes) in 5 mL of lysis buffer, washed twice and stained for EPCs. Bone marrow cells were harvested from 1 femur, washed twice and stained for EPCs (7-AAD, CD45-PE/Cy7, VEGFR2-A647, CD13-FITC, and CD117-PE) and 100,000 live cells acquired on a FACSCanto. * p<0.05; ** p<0.01; *** p<0.001 by 1-way ANOVA and Tukey's post test

**EPC Mobilization Correlates with G-CSF, SDF-1 and IL-6 Plasma Levels**

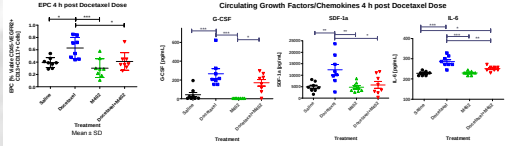
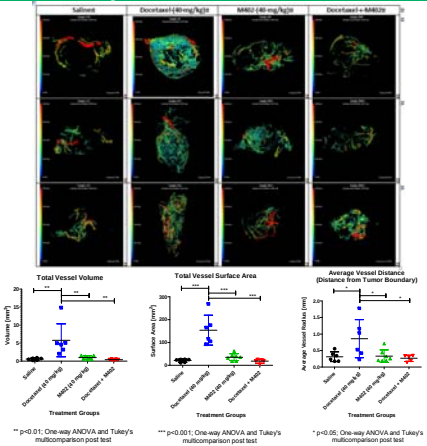



**Figure 3. Docetaxel-induced EPC mobilization in 4T1 tumor-bearing mice**
Balb/c mice were implanted with $1x10^5$ 4T1 tumor cells into the 4th mammary fat pad. On Day 7, mice were dosed with docetaxel (i.p., 40 mg/kg) or saline control once ± M402 (s.c., 40 mg/kg) or saline control. 4 h later, the mice were bled via the submandibular plexus. 100 µL of blood was lysed (erythrocytes) in 5 mL of lysis buffer, washed twice, stained for EPC, and 100,000 live cells were acquired. Chemokines/growth factors were measured by ELISA (R&D Systems) in plasma.
* p<0.05; ** p<0.01; *** p<0.001 by 1-way ANOVA and Tukey's post test

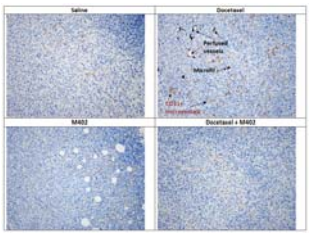
### Tumor Vascularization

**M402 Inhibits High-Dose Docetaxel-Induced Tumor Vascularization**




** p<0.01; One-way ANOVA and Tukey's multicomparison post test
*** p<0.001; One-way ANOVA and Tukey's multicomparison post test
* p<0.05; One-way ANOVA and Tukey's multicomparison post test

**Fig. 4. Tumor vascularization visualized by Microfil perfusion and microCT.**
Balb/c mice were implanted with $1x10^5$ 4T1 tumor cells into the 4th mammary fat pad. On Day 7, mice were dosed with docetaxel (i.p., 40 mg/kg) or saline control once ± M402 (s.c., 40 mg/kg, daily for 5 days) or saline control. On Day 14, mice were perfused with Saline (60 mL), followed by Microfil (30 mL). Tumors were excised and imaged by microCT.



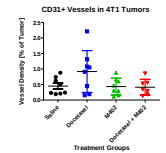
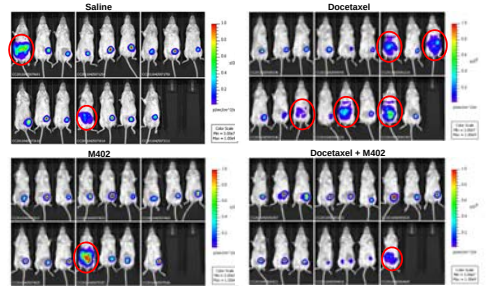




**Fig. 5. CD31 immunohistochemistry of Microfil perfused 4T1 tumors.**
4T1 tumors were perfused *in vivo* with Microfil, fixed in PFA/EtOH, imaged by microCT, then paraffin embedded, sectioned and stained for CD31 (Mec13.3, Biocare). 40x images.

### Tumor Invasion and Metastasis

**M402 Reduces Primary Tumor Invasion and Metastasis**



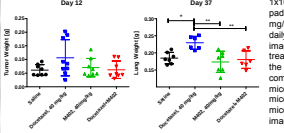




**Fig. 6. M402 attenuates invasion and metastasis.**
$1x10^5$ 4T1 cells were injected into the 4th mammary fat pad. On Day 7, mice were dosed with docetaxel (i.p., 40 mg/kg) or saline control once ± M402 (s.c., 40 mg/kg, daily for 5 days) or saline control. On Day 12, mice were imaged for bioluminescent tumor burden. Docetaxel-treated tumors showed a higher rate of invasion through the peritoneal wall as compared to vehicle, M402 or combination treated mice (5 of 17 mice vs. 1-2 of 16 mice, see circles). Primary tumors were resected and mice followed for metastasis development. On Day 37, mice were sacrificed and lungs excised, weighed, and imaged as measures of lung tumor burden.

## CONCLUSIONS

- **M402 inhibited EPC mobilization in response to docetaxel or 4T1-tumor secreted factors by trapping the EPCs in the bone marrow.**
- **M402 affected recruitment and outgrowth of EPCs/stromal cells in the tumor, leading to reduced tumor vascularization, invasion and metastasis in response to docetaxel.**
- **The experimental data provide a rationale for the clinical investigation of M402 in combination with taxanes or other agents that induce similar effects (such as radiation, 5-FU, cyclophosphamide, sunitinib, etc.[5]).**

## ACKNOWLEDGEMENTS / REFERENCES

**We would like to thank Sucharita Roy and Radouane Zouaoui for producing M402.**

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