HelmholtzZentrum münchen
German Research Center for Environmental Health

# Bone morphogenetic protein signaling as novel therapeutic target in pheochromocytoma

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## Background

**MENX** is a spontaneous multiple endocrine neoplasia-like syndrome in the rat, which is caused by a biallelic germline mutation in the *Cdkn1b* gene, encoding a very unstable mutant p27 protein (Figure 1C) [1]. MENX predisposes, among other neoplasias, to the development of bilateral **pheochromocytomas (PCC)** with complete penetrance (Figure 1) [2]. Gene expression profiling of MENX rat PCCs identified the growth factor *Bmp7* (bone morphogenetic protein 7) as highly expressed in tumors versus normal adrenal medulla (Figure 1D) [3]. Previous work demonstrated that upregulation of ***Bmp7*** enhances proliferation, migration and invasion of PCC cells. In primary rat PCC cells *Bmp7* expression sustained cell viability. In PCC Bmp7 signals through the PI3K/AKT/mTOR pathway and integrin β1 [3]. The small molecule antagonist **DMH1** is a second-generation analog of dorsomorphin and inhibits most selectively BMP type I receptors (BMPR-I) (Figure 2) [4].

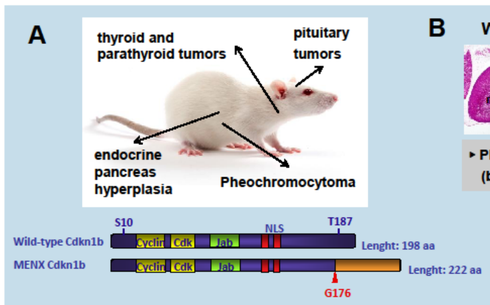


**Fig.1: Introduction of the MENX rat model. (A)** Illustration of the MENX rat model with all affected organs and the schematic structure of WT and MENX-mutant *Cdkn1b* (frameshift mutation). **(B)** H&E stainings of PCCs of MENX affected rats show compression of the adrenal cortex [cor] caused by the expansive growth of the adrenomedullary tumor [med]. **(C)** p27-IHC and **(D)** Bmp7-IHC of adrenomedullary cells of WT and mutant MENX rats. PCC development occurs due to a loss of function mutation of p27 and the overexpression of *Bmp7*.

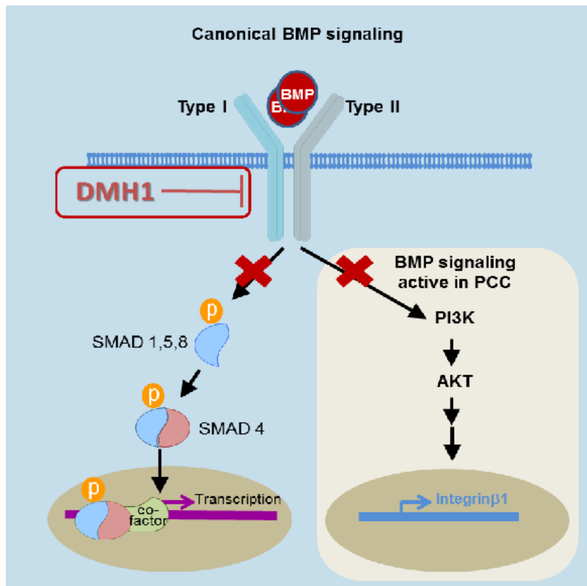


**Fig.2: Illustration of the canonical BMP pathway**. Upon BMPR stimulation the PI3K/AKT/mTOR pathway is activated and integrin β1 is expressed. DMH1 inhibits BMPR-I to avoid phosphorylation of Smad proteins and AKT, which inhibits further BMP signaling.

## Aim

The aim of the project was to assess the role of ***BMP7*** in PCCs of MENX rat and human PCC patients and to explore the effect of the small molecule compound, ***DMH1*** (BMPR-I analog), in *BMP7*-mediated PCC tumorigenesis *in vitro* and *ex vivo*.

## Experimental Plan

We used cell lines such as MPC (mouse PCC) and its aggressive derivative MTT, both with high levels of Bmp7 and primary rat PCC cells with high levels of Bmp7. To evaluate the effect of blocking Bmp7 signaling on PCC cells we treated these cells with DMH1, which selectively inhibits BMPR-I. *In vitro* assays assessing **proliferation** (MTT) and **migration** (Boyden chamber) were then performed. Additionally, we establishes an *ex vivo* system based on the **rotary cell culture system** from Synthecon-Cellon. With this system tissues will be cut in small pieces right after dissection of MENX rats and cultured *ex vivo* under DHM1 treatment.

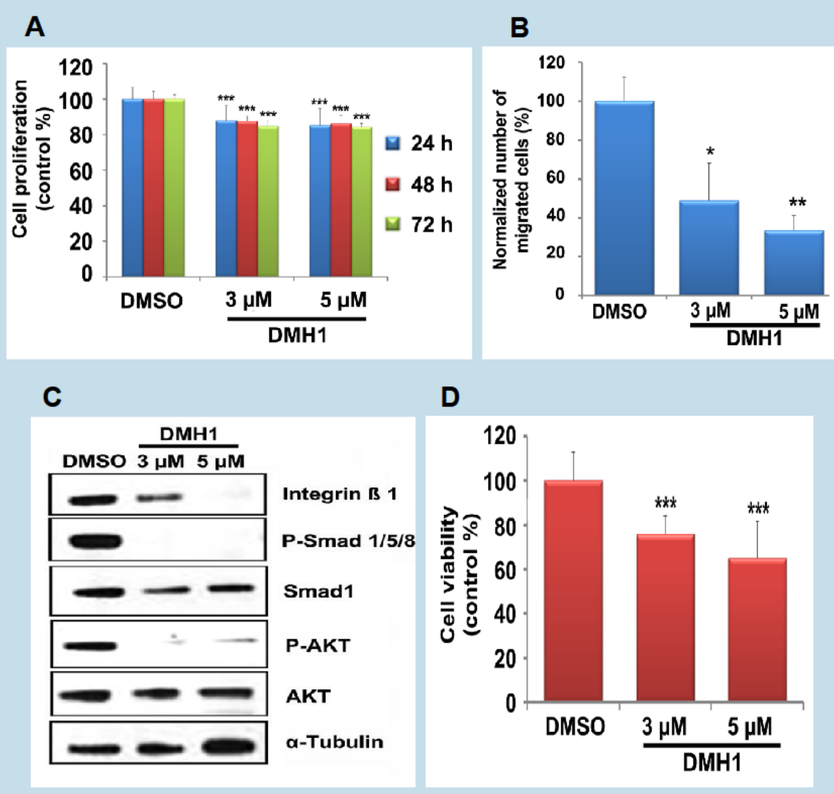


**Fig.3: Effect of DMH1 on BMP downstream sig-naling and PCC cell growth. (A)** MTT cells were treated with DMH1 (3 μM or 5 μM) or with DMSO. Proliferation was assessed at the indicated times. ***P<0.001. **(B)** MTT cells were treated with DMH1 (3 μM or 5 μM) or with DMSO for 24h. Proteins were collected and probed for the expression of integrin β1, P-AKT, AKT, P-Smad1/5/8 and Smad1. α-Tubulin was used as a loading control. **(C)** MTT cells treated as in A for 24h were used for migration assays. *P<0.05, **P<0.01. **(D)** Rat primary tumor cells were treated with DMH1 (3 μM and 5 μM) or DMSO for 72h and cell viability was assessed. Shown is the average of five independent cultures from five mutant rats. ***P<0.001

## Results

The small-molecule BMP antagonist DMH1 highly selectively inhibits BMP type I receptors, but no other off-target receptors [4]. To verify whether blocking BMP receptor signaling might be a potential strategy for targeted therapy of PCC, we treated MTT cells (high endogenous Bmp7 levels) with DMH1 and then figures out a significantly suppressed MTT cell proliferation (*Figure 3A*), and even more strongly inhibited cell migration (*Figure 3B*). Concomitantly, we observed a dose-dependent downregulation of the expression of P-Smad1/5/8 and integrin β1, both readouts of active BMP signaling in PCC cells, as well as of P-AKT (*Figure 3C*). Next, we determined a decreasing effect of DMH1 on rat primary PCC cells (high endogenous Bmp7 level) (*Figure 3D*). Furthermore we investigated the effect of DMH1 in PCC tissues of MENX affected rats *ex vivo* by using a rotary cell culture system. We could show a reduction of Bmp signaling downstream targets (P-AKT and P-S6) at the protein level by western blotting (reduction of around 20%) and by IF staining (P-Smad1/5/8) (*Figure 4*).

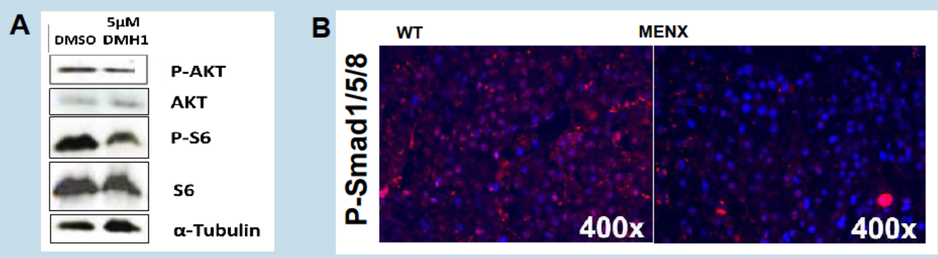


**Fig.4: *Ex vivo* effect of DMH1 on BMP downstream signaling. (A)** Adrenal glands of MENX rats were cultured *ex vivo* with medium containing DMH1 (5 μM) or DMSO vehicle for 4 days: Western blotting of P-AKT, AKT, P-S6 and S6 and **(B)** IF staining for P-Smads.

## Conclusions

**The Bmp pathway represents a novel therapeutic target in PCC! DMH1, a BMP receptor antagonist elicits anti-proliferative and anti-migratory responses in PCC cells with active BMP signaling *in vitro* and *ex vivo*. Future studies will address DMH1 effects on PCC *in vivo*.**

## References

[1] Pellegata, Quintanilla-Martinez *et al.*, *PNAS USA*, 2006
[2] Fritz, Walch *et al.*, *Cancer Research*, 2002
[3] Leinhäuser, Richter *et al.*, *Oncotarget*, 2015
[4] Hao, Ho *et al.*, *Chem Biol*, 2010

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