



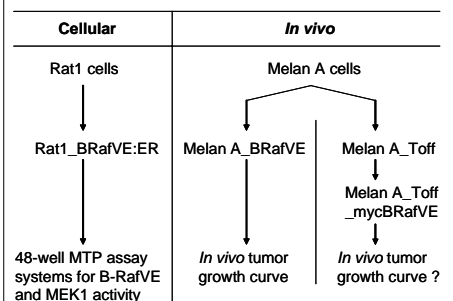
Introduction

B-Raf V600E (B-RafVE) is the most common activating mutation of B-Raf and plays an important role in oncogenic transformation of melanoma cells. In order to characterize inhibitors for B-Raf V600E kinase and its downstream kinase target MEK1 we went to develop cell systems for cellular assays as well as for *in vivo* models.

We here report on establishing a cellular assay that allows to assess B-RafVE activity by measuring phosphorylation of MEK1. At the same time, in this assay MEK1 activity itself can be monitored by measuring specific phosphorylation of ERKs. The assay is based on Rat1 cells in which B-Raf V600E activity is induced by addition of estrogen analogue 4-OH-Tamoxifen (OHT). This system was already reported for regulation of murine B-Raf (1). Functionally, sterical hindrance of kinase activity due to C-terminal fusion to a modified estrogen receptor (ER) domain is alleviated upon addition of OHT. We herein show that the OHT/ER-system can be used to regulate human B-RafVE activity as well. Tight regulation of BRafVE activity within the cell is important as high activities needed for detectable amounts of phosphorylated MEK1 can cause apoptosis.

We also report on establishing a B-RafVE-dependent *in vivo* model using the melanocyte cell line Melan A similar as published by Wellbrock et al. (2). We report on initial steps of generating a Doxycyclin (Dox) regulatable BRafVE-system in Melan A cells to apply *in vivo*.

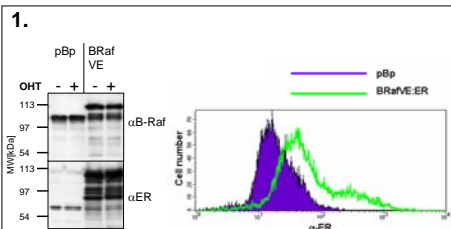
Generation of cellular assay systems for evaluating B-RafVE600 inhibitors



Material and Methods

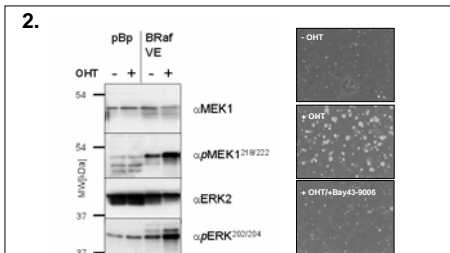
- **Plasmid:** pBp-BRafVE600 (kindly provided by D. Peepker), pBabePuro (pBp), pBp-BRafVE600:ER, pTRE-Tight, pTRE-2myc-BRafVE
- **Cell culture:** Melan A cells were kindly provided by I.Hart. All cell lines were cultured at 37° C/ 10% CO₂ in DMEM, 10% FCS, Pen/Strep. Melan A medium contained 200 nM TPA. For transduction, cells were treated with retroviral supernatant obtained upon transfection of Ampho293 cells with pVSV-G and either pBp,pBp-BRafVE:ER or pBp-BRafVE600. Transduced cells were selected by puromycin, cloned by limiting dilution, and best regulating clones used for further characterization. OHT treatment was performed as indicated. For generation of MelanA_Toff, Melan A cells were lipofected with pTetoff and subsequently selected with G418. For generation of MelanA_Toff_mycBRafVE, MelanA_Toff cells were cotransfected with pTRE_mycBRafVE and pBp and subsequently selected with Puromycin.
- **Western Blots and FACS:** Lysate protein was separated by 10% SDS-PAGE, blotted and analysed using the specific antibodies obtained from Santa Cruz and Cell Signaling Technology.
- ***In vivo* growth curve:** 1E7 MelanA_BRafVE and pBp in 100 µL PBS were subcutaneously injected into the flank of NMRI nude mice. Growth was regularly monitored by caliper measurements.
- **pS^{218/222}-MEK1-ELISA:** Cell lysates were loaded on microtiter plates precoated with MEK1 capturing antibody. Phosphorylations were detected by an antibody specific for pS^{218/222}-MEK1.
- **pT^{202/Y204}-ERK-ELISA:** Cell lysates were loaded on microtiter plates precoated with ERK capturing antibody. Phosphorylations were detected by an antibody specific for pT^{202/Y204}-ERK.
- **Softagar Assay:** Cells were cultured in Softagar medium and fresh OHT was added every other day. After 13 days cells were photographed at 40fold magnification.

Results



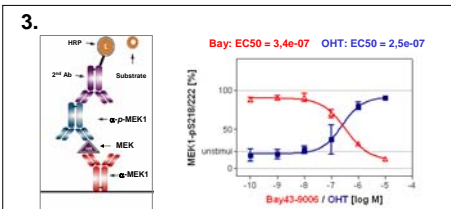
Clonal cell line Rat1_B-RafVE:ER expresses B-RafVE:ER protein independent of OHT-treatment.

Rat1 embryonal fibroblast cell line Rat1 was retrovirally transduced with vector pBp-BRafVE:ER or empty vector pBp. Clones were tested for expression of ER domain and B-Raf protein by Western Blot (left) and FACS (right). Our data show that Rat1_B-RafVE:ER express exogenous B-Raf mutant (MW~121 kDa) in addition to endogenous wildtype B-Raf (MW~84 kDa) independently of treatment with 4-Hydroxy-Tamoxifen (OHT: 0.1 µM; 1h).



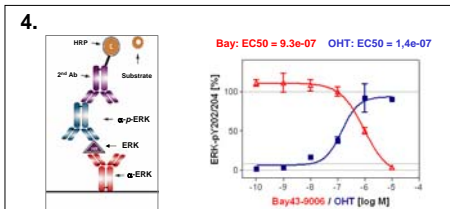
OHT-treatment of Rat1_B-RafVE:ER cells induces activation of MEK1 and softagar growth.

Rat1_B-RafVE:ER were treated +/- OHT and analysed for downstream activation. Western Blot analyses (left) revealed activating phosphorylation of MEK and ERK's upon OHT treatment (0.1 µM; 1h). Pathway activation results in transformation as assessed by soft agar growth (right). Transformation is susceptible to inhibition by BRaf-Inhibitor Bay43-9006 (Sorafenib).



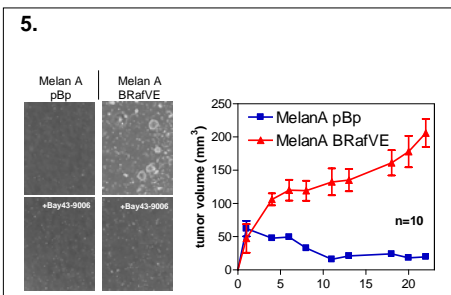
Measuring pS^{218/222}MEK1 in a 48 well format allows efficient analysis of potential B-RafVE inhibitors

As shown for BRaf-inhibitor Bay43-9006, Rat1_B-RafVE:ER cells are treated with inhibitor, stimulated with 1 µM OHT and finally lysates are analysed by pS^{218/222}-MEK1-ELISA (red line). Analyses of the activating potency of OHT is shown by the blue line. Shown are means +/- min/max; 100%=w/o inhibitor; 0%=cells treated w/ 1E-5M Staurosporine, unstim.=w/o OHT.



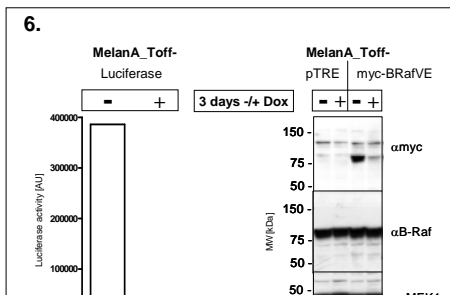
Measuring pT^{202/Y204} ERK in the same cells allows for simultaneous analysis of potential MEK1 inhibitors

As shown for BRaf-inhibitor Bay43-9006, Rat1_B-RafVE:ER cells are treated with inhibitor, stimulated with 1 µM OHT and finally lysates are analysed by pT^{202/Y204}-ERK-ELISA (red line). Analyses of the activating potency of OHT is shown by the blue line. Shown are means +/- min/max; 100%=w/o inhibitor; 0%=cells treated w/ 1E-5M Staurosporine, unstim.=w/o OHT.



Melan A cells transduced with BRafVE form tumors in nude mice.

Melan A cells transduced with BRafVE (w/o ER-domain) but not control cells (pBp) form softagar clones in a Bay43-9006-sensitive way (left) and grow as subcutaneous tumor in NMRI nude mice (right). Shown are means +/- SE of calipered tumor volumes.



Generation of MelanA_Toff cells expressing myc-tagged BRafVE in a Dox-dependent manner

Melan A cells stably transfected with pTetoff led to Dox-repressible MelanA_Toff cells as assessed by activity of transiently transfected Dox-regulatable luciferase (left). Stable transfection of MelanA_Toff with pTRE-myc-BRafVE results in MelanA_Toff_myc-BRafVE cells expressing low amounts of myc-tagged B-RafVE in a Dox dependent manner. However, there was no visible effect on total B-Raf and pMEK-levels acc. to Western Blot (right).

Conclusion:

- We have established an OHT-inducible cellular assay system for the analysis of B-RafV600E kinase activity using MEK1 phosphorylation at S^{218/222} as readout.
- The same cellular assay system can be applied for the simultaneous analysis of MEK1 kinase activity using ERK phosphorylation at T^{202/Y204} as readout.
- Both cellular kinase assays are established in 48 well format and suited for medium throughput analyses.
- B-RafVE600 transforms the benign murine melanocyte cell line Melan A, resulting in tumor growth in nude mice.
- Our aim is to establish a Dox-regulatable tumor model using MelanA_Toff-myc-B-RafVE cells

Acknowledgement: We thank M. McMahon for advice and A.Lingnau and S.Hoffmann for performing the *in vivo* study.