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TITLE: Receptor Tyrosine Kinases as Targets for Treatment of Peripheral Nerve Sheath Tumors in NF 1 Patients

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14. ABSTRACT The purpose of this study is the preclinical testing of multiple available tyrosine kinase inhibitors for NF1-associated benign and malignant tumors in vitro and in vivo. We found frequent copy number changes for EGFR and ERBB2, and also for the tumor suppressor genes PTEN, CDKN2A and TP53 in MPNST. CDKN2A loss was associated with metastasis. EGFR and ERBB2 were frequently expressed in MPNST on the protein level. MPNST and neurofibroma xenograft mouse models have been established. Erlotinib, and combined AMN107/Gefinitib inhibited cell proliferation in vitro. In the xenograft model, Gefinintib and Glivec suppressed growth of the xenograft tumors. Glivec treatment led also to shrinkage of plexiform neurofibromas xenograft in mice.						
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INTRODUCTION

NF1 is characterized by the appearance of multiple tumors of the peripheral nerve, and occasionally the malignant transformation of these tumors. The only available but unsatisfying therapy is surgical tumor resection. The purpose of this study is the preclinical testing of multiple available tyrosine kinase inhibitors for NF1-associated MPNST using *in vitro* and *in vivo* systems. MPNST cell lines and xenograft in nude mice will be established. Molecular analysis of the targeted receptor tyrosine kinases will show the incidence of alterations in the receptors and provide a profile of activation of the associated pathways in MPNST. According to the patterns of receptor tyrosine kinase receptor activity appropriate inhibitors will be tested for effects on tumor growth. The findings will substantiate causal therapy attempts based on the tumor specific tyrosine kinase receptor activity profile.

BODY

Task 1 -to establish cell lines from MPNST from NF1 patients

Cells were cultured from 18 MPNSTs from unrelated NF1 patients. One cell line (462) has been established in passages over 50. Another MPNST cell culture (1507) has been verified as containing tumor cells as the same somatic genetic alteration of the original tumor were found in the cells. This culture is still in early passage and needs to be further established. Additional two cultures in early passages look promising and are being further passaged.

For xenograft tumor models and for *in vitro* drug testing, we are using our earlier MPNST-derived cell line S462

- recruitment and consenting of 20 NF1 patients with MPNST and 20 patients with pNF

19 MPNST and 30 PNF patients with NF1 were recruited. Clinical examination and genetic characterization of patients has been completed. Tumors have been collected and frozen for further mutation analysis (*NF1*, *TP53* and *INK4A*). Primary cell cultures were established for some of these tumors. All cells cultured from MPNST were frozen at various passages for further characterization (table 1)

Table 1 MPNSTs in culture

	ID	Passages	LOH in NF1 / P53 / <i>INK4A</i>	Status
1	462	50	Yes / yes / no	Established
2	1507-2	23	No / yes / no	Established
3	520	28	Yes / yes / yes	Note 1
4	805	21	- / yes / yes	Note 1
5	1268,1	17	Yes / no / no	Note 1
6	1482	10	No / no / no	Frozen stored
7	1483	16	- / yes / no	In culture
8	1511	6	No / no / no	Frozen stored
9	1565.1	0	Yes / yes / no	In culture
10	1565.3	10	Yes / no / no	In culture

11	1569.1	6	Yes / no / no	In culture
12	1602	4	No / no / no	Frozen stored
13	1627	3	No / yes / no	Frozen stored
14	1639	2	No / not clear / yes	In culture
15	1640	4	No / no / no	Frozen stored
16	1559	3	No / no / no	Frozen stored
17	1593	0	No / no / no	Frozen stored
18	1594	0	No / no / no	Frozen stored
19	1599	0	No / no / no	Frozen stored

Note 1: Growth decline over passages

In summary, patient recruitment is completed. Primary cells and tumor tissue has been archived and is ready for molecular characterization. Genetic characterization for tumor tissues (LOH of *NF1*, *TP53*, *INK4*) is complete. One additional MPNST cell line was established. Two others are promising and being enriched for tumor cells.

Task 2 - to determine genetic alterations and expression of PDGFR- α , c-Kit, EGFR and Neu (ERBB2) in MPNST, pNF, and cultured MPNST cells

We studied expression and genetic alterations of EGFR and erbB2 in MPNST from 37 patients. No mutations were found within exons encoding the respective kinase domain. Gene dosage analysis was performed by multiplex ligation-dependent probe amplification for *EGFR* and *ERBB2*, and also for the tumor suppressor genes *PTEN*, *CDKN2A* and *TP53*. MLPA revealed increased *EGFR* copy number in 28% of MPNST, which was confirmed by FISH. Loss of genetic material was detected for *ERBB2* (32%), *PTEN* (58%), *CDKN2A* (58%), and *TP53* (39%). *CDKN2A* loss was associated with metastasis. Comparison of corresponding benign neurofibromas and MPNST suggested that genetic alterations occurred during progression from benign tumors to the malignant ones. On the protein level frequent expression of EGFR and erbB2 was detected in MPNST. Stronger EGFR expression was associated with increased *EGFR* gene copy numbers. In contrast, benign neurofibromas expressed EGFR, but rarely erbB2. The EGFR ligands *TGFA* and *EGF* were expressed stronger in MPNST than in neurofibromas.

Task 3 -to test single and multiple (combinations) tyrosine inhibitors *in vitro*

The effect of erlotinib and trastuzumab targeting EGFR and erbB2 on MPNST was examined in established MPNST cell cultures. Erlotinib was found to be effective for suppressing proliferation of MPNST cells *in vitro*, while trastuzumab did not. EGF induced EGFR phosphorylation was attenuated by erlotinib. Combinations of erlotinib and imatinib yielded additive effects. This study provides evidence for a molecular basis justifying application of EGFR and erbB2 inhibitors in MPNST patients.

Proliferation assays were performed on S462 cell line to test Glivec, Gefinitib and AMN107, alone or in combination (Figure 1). At 48h in culture Glivec and Gefinitib alone did not mediate an anti-proliferative effect. When, however, tested in combination, these drugs reduced cell proliferation with an Ic_{50} of ~10 μ M. AMN107 alone reduced cell proliferation (Ic_{50} ~9 μ M), and in combination with Glivec and Gefinitib the reduction in cell proliferation was much higher (Glivec + AMN107 Ic_{50} ~4 μ M and Gefinitib + AMN107 Ic_{50} ~50

~2µM) (Figure 1). We have shown previously that longer incubation periods with Glivec (4 and 7 days) led to efficient growth inhibition (Holtkamp et al. 2006).

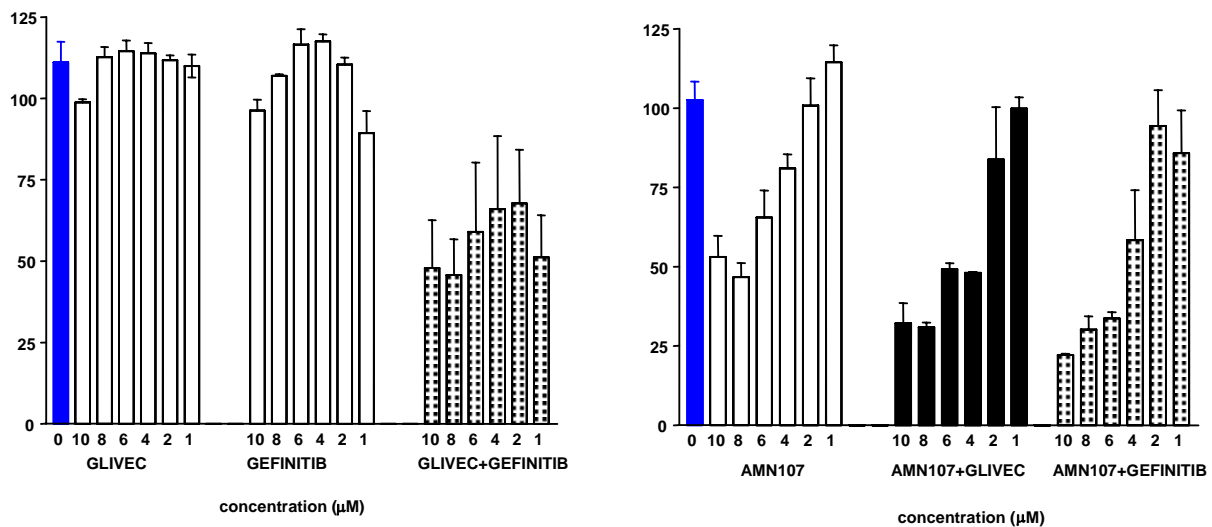


Figure 1 : Effects of Glivec (inhibits PDGFR and Kit) and Gefitinib (inhibits EGFR) and AMN107 (targets Kit and PDGFR) on proliferation of the MPNST cell line S462 (XTT assay, 48h). At this short incubation time only AMN107 showed an effect when tested on its own. When they were tested in combination, AMN107+Gefitinib showed the most marked effect.

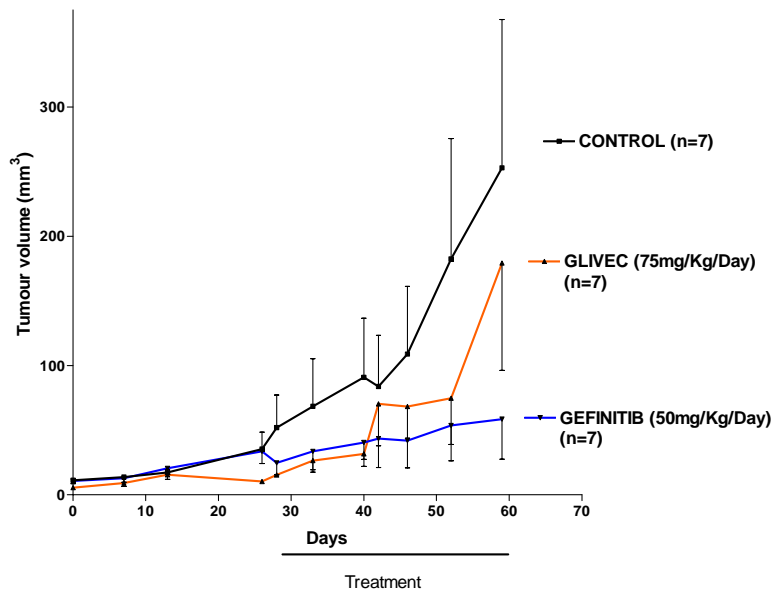
Task 4 - to inhibit receptor tyrosine kinases *in vivo*

Last year we established xenograft tumors in mice by injecting cultured MPNST cells S462 into the flank. Tumors developed in 80% of the mice within 2-3 weeks. Recently, we also tried to re-implant pieces of tumors grown in mice again back to other mice, and obtained new tumors in shorter period than in previous model using cultured cells. These mice with MPNST were treated with Glivec (75mg/Kg/Day) and Gefitinib (50mg/Kg/day) (Figure 2A) and AMN107 (1mg/Kg/day).

Gefinintib and Glivec suppressed growth of the tumors. However, the difference to the control groups did not reach statistical significance (Figure 2A). One reason could be the highly variable tumor growth. These experiments will be repeated with a larger number of mice. AMN107 (data not shown) did not produce any effects on tumor growth.

Pieces of plexiform neurofibromas (pNF), freshly obtained after surgical resection from NF1 patients, were implanted to the sciatic nerve of nude mice. We observed that these pieces of tumors survived at least up to 60 day (longer periods of time have not been tested), up to 10% enlargement of the implants was observed after 30 days. We have found mouse blood vessel in the tumor implants, indicating vascularisation by the host. At present, we are examining whether the enlargement is due to growth of the implanted tumor pieces, or due to invasion of inflammatory cells.

Animals with implanted pieces of pNF fragments were treated with Glivec (75mg/kg/day) for various periods starting one week after the implantation. Four weeks of treatment with Glivec (and left without treatment for further 30days) suppressed tumor growth compared to those mice without treatment. In 2 out of these 3 mice, the implanted tumors disappeared.



B

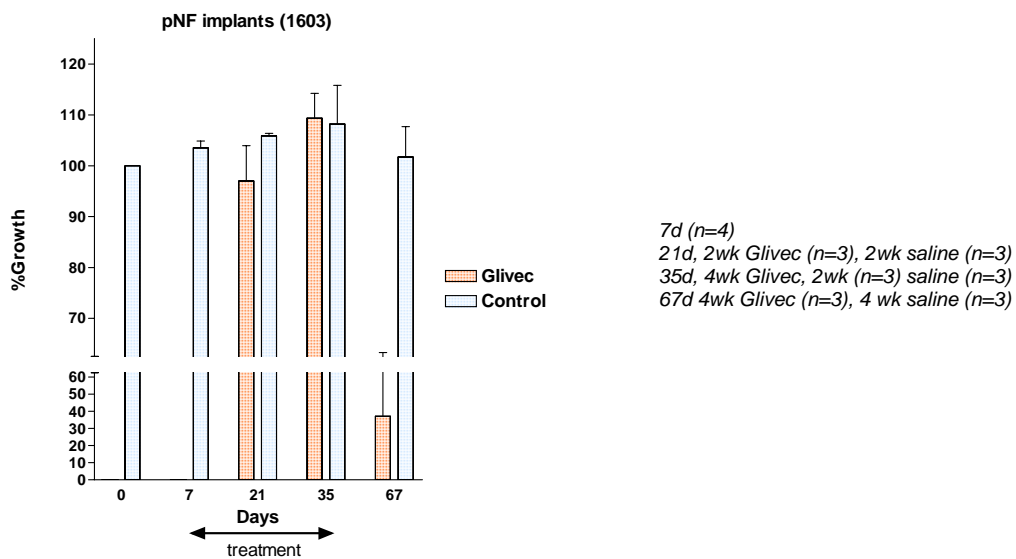


Figure 2. (A). MPNST tumors were treated with Glivec and Gefinitib for 28 days. When mice were treated with Gefinitib a reduction in tumor volume was observed. **(B)** pNF were implanted next to the sciatic nerve of mice. After 1 week mice were treated with Glivec for 2 and 4 weeks. 3 mice were treated for 4 weeks and left till day 67. In 2 of them, the implanted tumor pieces disappeared.

In summary, Gefinitib was more efficient in xenograft treatment than Glivec. Further, we have demonstrated that our pNF fragments implanted next to the sciatic nerve of nude mice induce neo-angiogenesis. Hence, this model appears suitable to test anti-angiogenic compounds. In accordance to this assumption, Glivec, which mediates anti-angiogenic effects, suppressed pNF growth and survival *in vivo*.

KEY RESEARCH ACCOMPLISHMENTS

- completed recruit of 19 MPNST and 30 PNF patients.
- set up cultures from all the 19 MPNST. Genotyped these tumors regarding LOH of *NF1*, *p53* and *INK4A*
- established two stable, genetically verified MPNST cell cultures.
- found altered gene dosage and frequent expression of EGFR and erbB2 in MPNST.
- found that erlotinib, AMN107, and combined Glivec/Gefinitib, Glivec/AMN107 and AMN107/Gefinitib were effective in suppressing MPNST cell growth *in vitro*.
- found that Gefinitib and Glivec affected MPNST xenograft growth. Glivec was effective against pNF in a xenograft mouse model.

REPORTABLE OUTCOMES

Holtkamp, N., Malzer, E., Zietsch, J., Okuducu, A., Mucha, J., Mawrin, C., Mautner, V.-F., H-U, S., and von Deimling, A. EGFR and erbB2 in malignant peripheral nerve sheath tumors and implications for targeted therapy. In revision for Neuro-oncology.

CONCLUSION

We have completed the recruit of 19 MPNST and 30 PNF patients and set up cultures MPNST and some of the pNFs. By tracing LOH of *NF1*, *p53* and *INK4A* from tumor to cultured cells, we established two MPNST cell lines, which are not only useful for the present project, but are also valuable for other studies in other research groups. We found altered gene dosage and expression of EGFR and erbB2 in MPNST. Erlotinib, AMN107, and combined Glivec/Gefinitib, Glivec/AMN107 and AMN107/Gefinitib were effective in suppressing MPNST cell growth *in vitro*. Gefinitib and Glivec suppressed MPNST xenograft growth in mice and Glivec led to shrinkage of implanted pNF pieces in two mice. Our results suggest, that the tested tyrosine kinase inhibitors may be effective as single drug regimen or in combination for treatment of patients with MPNST and pNF.

REFERENCES

Holtkamp N, Okuducu AF, Mucha J, Afanasieva A, Hartmann C, Atallah I, Estevez-Schwarz L, Mawrin C, Friedrich RE, Mautner VF, von Deimling A. Mutation and expression of PDGFRA and KIT in malignant peripheral nerve sheath tumors, and its implications for imatinib sensitivity. *Carcinogenesis*. 2006 Mar;27(3):664-71.

Appendices

none