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Instrument : Specific Targeted Research Project
PRIORITY 1: Life Sciences, Genomics and Biotechnology for Health

Publishable final activity report

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1. Project execution

1.1 Project overview

The failure of anti-cancer therapies generally results from locally intractable invasive growth or from the presence of metastases refractory to treatment with curative intent. A novel therapeutic strategy is to develop new anti-cancer drugs specifically targeting the invasive or metastatic phenotype of tumour cells. We propose to validate at the preclinical level a strategy that targets the critical mechanism allowing apoptosis evasion and survival of invasive or metastatic tumour cells. Anoikis is a process by which a cell detached from its resident tissue undergoes apoptosis as a result of loss of normal cell/matrix interactions. Loss of anoikis allows survival of cancer cells in abnormal microenvironments, such as tissue compartments invaded by the primary tumour, and the intravascular compartment during the metastatic process. Participant 2 has recently discovered that the BDNF receptor TrkB is a potent suppressor of anoikis and is responsible for apoptosis evasion that occurs in aggressive human tumours overexpressing TrkB (Douma, Nature 2004). The aim of the present proposal was to validate TrkB as a target for new anticancer drugs, aiming to restore anoikis and thereby destroy the invasive and metastatic cancer cells. This validation included the identification TrkB-expressing tumour cell lines amongst a collection of resected human cancers. Then, abrogation of TrkB mediated signalling was achieved by either selective TrkB mRNA-targeting small interfering RNAs (siRNAs or shRNAs) delivered by viral vectors, or by novel and potent small molecule inhibitors that were designed and synthesized. The efficacy of these tools were assessed by using anoikis-sensitive cell models and xenograft mouse models, particularly orthotopic metastasis models derived from human tumours. The mechanisms of TrkB-dependent anoikis suppression were also investigated in order to identify additional markers for invasive and metastatic capability and novel drug targets.

At the end of the contract, TrkB not validated as a target for anti-cancer drugs, but encouraging results obtained yet and critical experiments still on-going. Three potent and selective TrkB inhibitors were selected for in vivo evaluation of antimetastatic properties. New mouse models for metastasis were in use and several alternative models were identified. New potential biomarkers for metastasis were identified as critical factors of TrkB pathway.

1.2 Project objectives

We proposed to evaluate the relevance of a strategy of selective blocking of TrkB, thereby restoring anoikis sensitivity and thus inducing apoptosis in invasive and metastatic malignant tumours, by the complementary approaches achieving the following project objectives:

- *Objective 1: Generation and selection of suitable (i.e., anoikis-resistant) and clinically relevant tumour cell lines expressing high levels of TrkB, or expressing mutant TrkB.* These cell lines were selected from a collection of human tumours derived from clinical biopsies, and will represent the basis for cell and animal models used for validation of TrkB (both by RNA interference and TrkB-inhibitory small molecules) as a target for anti-cancer drugs.

- *Objective 2: Evaluation in vivo of the metastatic capacity of human, TrkB-expressing tumour cells.* We made use of retroviral vectors for transducing TrkB to cells that subsequently will be implanted in mice as xenografts. The objective is to assess the capability of TrkB to render human, non-metastatic tumour cells metastatic.

- *Objective 3: Evaluation of the effects of selective abrogation of TrkB signalling on the metastatic potential of TrkB-expressing xenografts.* The methodology made use of retro- or lentiviral vectors to transduce, TrkB-expressing cells with TrkB-specific RNAi expressed from an inducible promoter. The *in vivo* effects of TrkB shRNAs were assessed after implantation in mice of the tumoral TrkB-expressing tumour cells and induction of shRNA

expression. The objective was to assess whether selective invalidation of TrkB is sufficient to reduce the metastatic potential of tumorigenic cells. These approaches used cellular, built or "natural" models expressing TrkB, as well as animal models carrying human-derived tumours with strong metastatic capability. The anti-metastatic effects were assessed by classical approaches (survival curves, microscopic examination) as well as by recently developed non-invasive imaging techniques using bioluminescent tumour cells in live intact animals.

- *Objective 4: High-throughput phenotypic characterization of tumours (tumour profiling).* The methodology made use of tumour tissue microarrays and rapid quantification of biological markers. A specific fluorescent marker for TrkB was developed with the Luminex technology. Expression of TrkB was confirmed with conventional immunohistochemistry techniques. This approach also made use of high-throughput transcriptome analysis with DNA expression microarrays. The first objective was to evaluate the correlation between the presence of TrkB and the metastatic potential of the tumour. The second objective was to identify proteins downstream the TrkB signalling pathway that are important for suppression of anoikis. This information was also used for decrypting the mechanisms of anoikis suppression (see below) and to find new biological markers for metastasis (including biomarkers for testing the efficacy of therapeutic agents in metastasis inhibition).

- *Objective 5: Functional assessment of the TrkB downstream proteins identified above in TrkB-dependent cells* by using a knock-down approach (siRNA and shRNA). The objective was to unravel the mechanisms of anoikis suppression by TrkB and to identify novel players critically involved in this process that is instigated by TrkB. This information was also used to define potential new “druggable” targets for anti-invasive or anti-metastatic agents. These targets were considered as alternative targets to TrkB, in case the latter fails for any reason.

- *Objective 6: Identification and use of powerful, selective and “developable” TrkB antagonists/inhibitors*. The methodology made use of medium- and/or high-throughput screening of drug-like small molecules. This was performed in a variety of *in vitro* and *in vivo* assays having progressively increasing pathophysiological and clinical relevance, so that the most interesting compounds were progressively selected. The objective was to generate accurate pharmacological tools for inhibiting TrkB and to evaluate the therapeutic interest of these drugs.

- *Objective 7: Early stages of preclinical development* (preliminary ADMET, preliminary safety, physico-chemical and metabolic stability tests) in order to assess the “developability” of the novel TrkB inhibitors. The long-term objective (beyond the scope of the present project) is to develop these drugs as medications.

1.3 Contractors involved

Partic. Role	Partic. N°	Participant name	Participant short name	Country	Date enter project	Date exit project
CO	1	Institut de Recherche Pierre Fabre	IRPF	FR	Month 1	Month 36
CR	2	Het Nederlands Kanker Instituut	NKI	NL	Month 1	Month 36
CR	3	Oncotest GmbH	ONCO	DE	Month 1	Month 36
CR	4	Vrije Universiteit Medisch Centrum	VUMC	NL	Month 1	Month 36

1.4 Co-ordinator contact details

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1.5 Work performed and results achieved during the whole contract

Toward achievement of Objective #1

A series of tumoral cell lines available by contractors have been screened for TrkB expression. Only a few cell lines have size approaching the full-length size. A large number of xenografts in nude mice were found to express TrkB transcripts, although no correlation exists between TrkB gene and protein expression. In addition, mutant forms of TrkB were detected, which maybe implicated in metastasis (e.g. TrkB-T1). Expression of full-length TrkB was more readily detected by immunohistochemistry in human tumours (biopsies). The results so far achieve suggest that TrkB expression may depend on the context of tumour growth (ex: *in vivo* vs. *in vitro*).

This objective was partially achieved: no clear expression of TrkB was found in cultured tumour cell lines, although a few cell lines were found to spontaneously express low levels of full-length, kinase-active TrkB.

Toward achievement of Objective #2

Viral vectors for TrkB expression or invalidation have been obtained. So far, out of 30 shRNA vectors constructed, 3 vectors have been found to produce almost complete extinction of TrkB expression and are available for further use on in TrkB-overexpressing cell lines. Transduction of TrkB into cell lines prove to be difficult (the expression is not stable with time), a problem that has been solved by adding special sequences (mir).

A series of experimental and spontaneous metastasis models in the mouse have been established (11 different models). In a human neuroblastoma tumor (SH-SY-5Y) xenografted in mouse, TrkB and BDNF overexpression did not influence metastasis formation.

This objective was achieved, although the results did not support a role of TrkB in metastasis formation

Toward achievement of Objective #3

Efficient lentiviral vectors for TrkB knockdown have been produced. The effects of TrkB knockdown have been assessed on some tumour cell lines. *In vitro,* TrkB knockdown did not change proliferation and anoikis resistance. *In vivo,* preliminary results suggest that TrkB knockdown reduces metastasis formation.

This objective was achieved, although the results did not fully support a role of TrkB in metastasis formation

Toward achievement of Objective #4

Human tumours samples have been collected for tissue microarray (TMA). Technology of TMA preparation was optimized so that high-quality specimens are routinely produced. A number of human tumour samples have been prepared for TMA from various tissues and immunohistochemistry performed.

At the VUMC, a wide variety of archival specimens of normal human tissues and human tumour specimens were processed for tissue microarray (TMA), with optimalisation of the TMA workup and processing procedures. All tumours were classified by histopathology, using WHO criteria, and this was confirmed at revision in the context of TMA preparation. At least two samples of each specimen, containing the highest percentage of tumour cells and the areas best preserved in the tissue block, were selected for TMA. Thus, a very wide spectrum of normal tissues as well as at least ten samples each of the following tumour types: cutaneous melanoma (metastasis); invasive ductal carcinoma of the breast; invasive lobular carcinoma of the breast; gastric adenocarcinoma (sinet ring cell type; intestinal type); pancreatic adenocarcinoma NOS; prostatic adenocarcinoma; colorectal adenocarcinoma; adrenocortical carcinoma; pituitary adenomas of various types. In addition, nephroblastomas, neuroblastomas and ganglioneuroblastromas were investigated. We extended of tumour types, to include hepatic carcinoma, pulmonary squamous cell carcinoma, pulmonary adenocarcinoma, pulmonary large cell carcinoma, squamous cell carcinoma, cutaneous basal cell carcinoma, as well as a variety of soft tissue sarcoma types.

Bead Suspension Assays have been established for various markers and adaptation of the method for measuring TrkB expression is on-going.

This objective was achieved, but brought little support for a role of TrkB in tumorigenicity: in general a minority of samples from the same tumour type express TrkB.

Toward achievement of Objective #5

TrkB mutants lacking various receptor domains have been constructed to assess which domains are required for metastasis. We set out to study the relative contributions of TrkB's kinase activity and its adhesion domains to anoikis suppression and oncogenicity. On the basis of a structure-function analysis, we report that TrkB kinase activity is required and, unexpectedly, also sufficient for anoikis suppression, tumor formation, and experimental metastasis. Thus, TrkB can act tumorigenically independent of its adhesion motifs. These results suggest that targeting the enzymatic activity of TrkB might be beneficial in cancer therapy.

By combining genetic and functional analysis NKI has identified the Fos-family Fra-1 as critical mediator of Trb-induced EMT (epithelial to mesenchymal transition), invasion and metastasis. In support of these observations, we demonstrated that Fra-1 depletion from human breast cancer cells abolished their ability to form metastases. Microarray analysis of Fra-1-depleted breast cancer cells identified a gene-expression signature that predicted tumor recurrence with high accuracy, underscoring a key role for Fra-1 in breast cancer metastasis.

Next, NKI has used the Fra-1 signature as a platform to search for Fra-1-regulated genes critically involved in metastasis. We generated a shortlist of 31 genes whose expression is suppressed by Fra-1 depletion, in two human breast cancer cell lines, and which are highly expressed in poor prognosis patients. We systematically silenced the expression of these genes in breast cancer cells and analyzed

the impact of this on metastasis *in vivo*. We have identified several genes whose silencing dramatically suppressed metastasis.

This objective was fully achieved: we demonstrated that TrkB kinase activity is required and, unexpectedly, also sufficient for anoikis suppression, tumor formation, and experimental metastasis. Potential biomarkers of metastasis were identified among key targets of TrkB signalling pathway.

Toward achievement of Objective #6

New cell-based assays for TrkB have been established to measure TrkB inhibition. A first screening on ~800 potential anti-TrkB inhibitors has been performed, hits identified, resynthesized and validated. Optimization of hits has been performed through chemical synthesis in order to build a structure-activity relationship (SAR) and to optimise anti-TrkB activity. New synthetic routes have thus been developed to overcome problem of the limitations of chemical variations scope and solubility. A total of 196 new molecules were synthesized and most potent inhibitors are subject to optimization. A second screening on a chemical library of 6,400 molecules has been performed and 92 new hits preselected. Chemical modulation (54 analogs) did not provide any exploitable new hits.

Optimization of lead compounds provide 3 TrkB inhibitors ready for pharmacological evaluation in vivo. Metastatic models in which TrkB is overexpressed have been set up and characterized. Antimetastatic effects of TrkB inhibitors are currently investigated using a dedicated imaging platform.

This objective was fully achieved: A total of 196 new molecules were designed and synthezised. Among them, 6 compouds show high potency (IC_{50} < 1 µM in cell-based assays) and specificity among various kinases.

Toward achievement of Objective #7

Metabolic and plasmatic stability of most potent TrkB inhibitors have been assessed and most suitable compounds selected for further evaluation in *in vivo* models.

This objective was fully achieved: three compounds were selected for pharmacological tests in vivo after assessment of chemical, metabolic and plasma stability, pharmacokinetics and preliminary toxicology (Maximal Tolerated Dose)

1.6 Relevance of these achievement against the current state-of-the-art

Although TrkB expression has reported in various cell lines and tumours (notably neuroblastomas), no systematic survey is available. The achievements of the present programme make a comprehensive inventory. During the course of the contract, two studies pointed to a potential role of TrkB in tumorigenicity of ovarian cancers (Yu et al., Cancer Sci., 2008, 99:543-552; Au et al., Cancer Lett., 2009, 281:151-161). So far these results have not been confirmed. Our results also demonstrate that TrkB kinase activity is required and, unexpectedly, also sufficient for anoikis suppression, tumor formation, and experimental metastasis. However, the truncated form TrkBT1, devoid of the kinase domain, has been shown to induce liver metastasis (Li et al., Cancer Res, 2009, 69:7851-7859).
Numerous mixed TrkA/TrkB inhibitors have been described during recent years (Siu et al., Expert Opin Ther. Targets, 2009, 13: 1169-1178), but none has been shown to exert anti-cancer activity, except AZ-23 in a neuroblastoma model in the mouse (Thress et al., Mol

Cancer Ther, 2009, 8:1818-1827). For the time being, it cannot be ascertained whether TrkA or TrkB is the critical target for antièncancer activity.

1.7 Intentions for use and impact

Work on mutants TrkB used for defining the TrkB domains required for anoikis suppression have recently been published (Geiger and Peeper, Cancer Research *67*, 6221-6229, 2007). Furthermore we found two downstream targets of TrkB, Twist and Snail to be cricital for TrkB-induced metastasis. This work has recently been published (Smit *et al.* MCB *29*, 3722-3737, 2009). Finally, a comprehensive review has been written on metastasis mechanisms (Geiger and Peeper, BBA Reviews Cancer, 1796(2):293-308, 2009).The other pieces of knowledge are not ready to be published.

An international patent application on TrkB inhibitors synthesised during this program will be deposited by IRPF. If efficacy of TrkB inhibitors is later confirmed in several *in vivo* mouse models, further optimization of the compounds will be undertook and selected compounds may enter preclinical and clinical developments.

2. Dissemination and use

2.1 Publishable results of the Final plan for using and disseminating the knowledge

- Work on mutants TrkB used for defining the TrkB domains required for anoikis suppression have been published. A manuscript describing the role of Snail and Twist in TrkB-induced epithelial-to-mesenchymal transition and metastasis has been published, as well as a comprehensive review on metastasis mechanisms. The other pieces of knowledge are in preparation.

Publications:

Geiger T.R. and Peeper D.S. Critical role for TrkB kinase function in anoikis suppression, tumorigenesis, and metastasis. *Cancer Research* **67**, 6221-6229, 2007

Marjon A. Smit, Thomas R. Geiger, Ji-Ying Song, Inna Gitelman and Daniel S. Peeper
A Twist–Snail axis critical for TrkB-induced EMT-like transformation, anoikis resistance and metastasis. *Molecular and Cellular Biology* **29**, 3722-3737, 2009

Geiger TR, Peeper DS., Metastasis mechanisms. *Biochim Biophys Acta*. 2009; 1796(2):293-308.

Christophe J. Desmet, Alexandre Prieur, Fabien Reyal, Thomas R. Geiger, Marjon A. Smit, Hugo Horlings, Abderrahrim Ajouaou, John Zevenhoven, Martin van Vliet, Lodewyk F.A. Wessels & Daniel S. Peeper. Fra-1 and its associated transcriptome are central determinants of human breast cancer metastasis (*submitted*)

Zeb1 is a critical target of TrkB-induced EMT-like transformation and metastasis
Marjon A. Smit and Daniel S. Peeper. (*in preparation*).

- Work on the new cellular TrkB model (BRET) has been published:

De Vries L, Finana F, Cachoux F, Vacher B, Sokoloff P, Cussac D. Cellular BRET assay suggests a conformational rearrangement of preformed TrkB/Shc complexes following BDNF-dependent activation. *Cell Signal*. 2010; 22(1):158-65.