Anaerobic bacteria in cancer treatment

"Clostridia: from Old Diseases to New Threats"
Villars-sur-Ollon, October 8th

Jan Theys, PhD

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"Clostridia: from Old Diseases to New Opportunities"
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Hypoxia – poor oxygenation

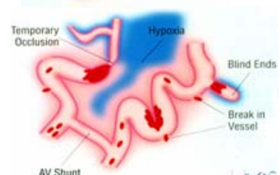
Air: 21% O2
Tissue normoxia: 5-7% O2
Tissue hypoxia: < 3% O2

Physiology

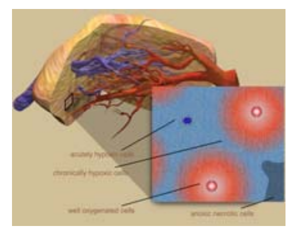
- **Development**
- **Exercise**
- **Altitude**

Pathology

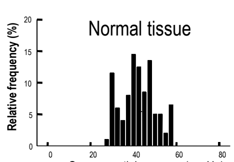
- **Wound**
- **Stroke**
- **Solid tumors**

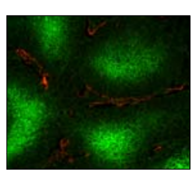


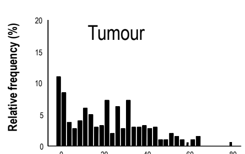
Exploit hypoxia to direct treatment

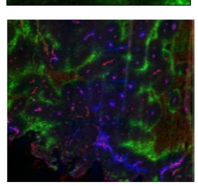


Heterogeneity in hypoxia amongst patients









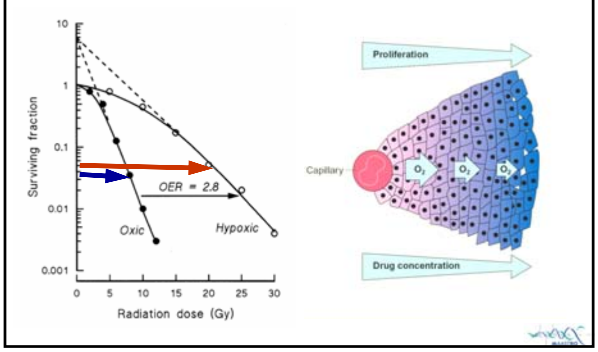
The clinical importance of tumor hypoxia

1. Resistance to radiotherapy
2. Resistance to chemotherapy
3. Contribution to 'malignancy'

Treatment Resistance

Radiotherapy

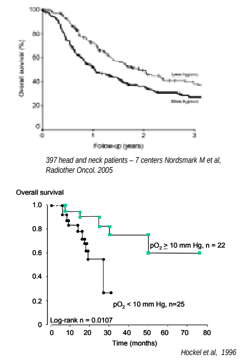
Chemotherapy



Hypoxia is a prognostic factor

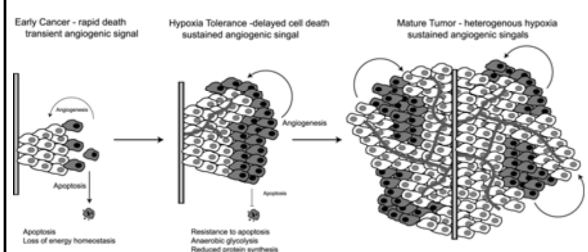
Hypoxic tumors are more malignant

- Cervix tumors have larger extensions, more frequent parametrial spread, more lymph-vascular space involvement
- recurrent tumors are more hypoxic than primary tumors
- predicts for the likelihood of distant metastases in soft tissue sarcomas
- hypoxia is a strong prognostic factor (*Independent of primary mode of treatment)*



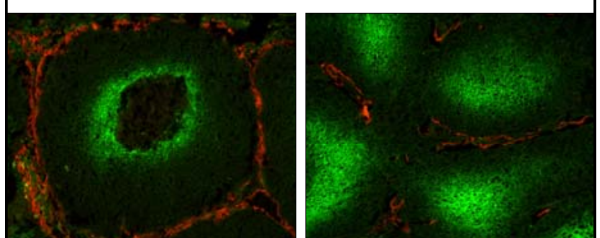
The concept of hypoxia tolerance

"Persistence of hypoxia in tumors is a reflection of the fact that hypoxia can act as a net positive factor in tumor growth"



Hypothesis: the amount of hypoxia is a direct reflection of the degree of hypoxia tolerance

Hypoxia tolerance varies amongst tumors



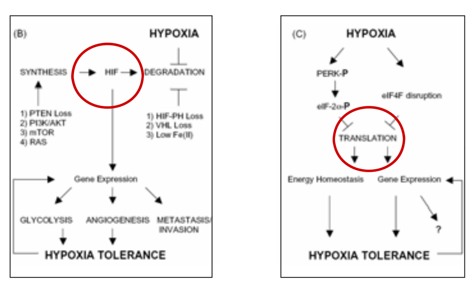
Cellular responses to hypoxia promote malignancy

Hypoxia causes biological changes that promote

- angiogenesis
- metastasis
- genetic instability

These changes are due to changes in gene expression

Hypoxia response pathways



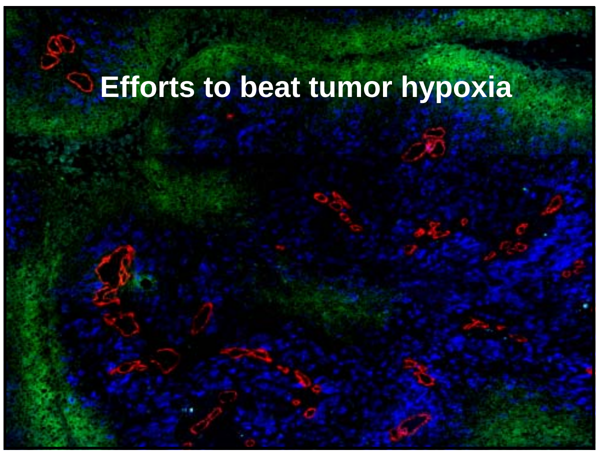
Hypoxia as the Grim Reaper

- Hypoxic cells more radioresistant
- Hypoxic cells more chemoresistant
- Hypoxic cells drive neovascularization
- Hypoxic cells drive disease progression

Hypoxia as cancer's Achilles' Heel

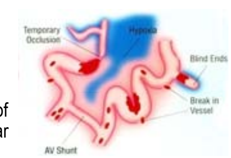
- Hypoxic cells can reoxygenate/get killed
- Hypoxic cells die
- Hypoxic cells express different genes
- Hypoxic cells are specific to tumors

Efforts to beat tumor hypoxia



Strategies for overcoming tumor hypoxia

1. Restore/replace oxygen
   - EPO
   - ARCON
   - Nimorazole
2. Target biological responses
   - requires a good understanding of these responses at the molecular level
3. Exploit hypoxia
   - Bioreductive drugs
   - **Anaerobic bacteria**



Exploit hypoxia to direct treatment

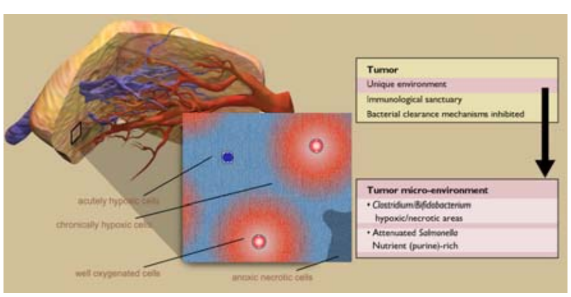


Table 1 – Anaerobic bacteria tested as anticancer agents

Groups	Genus/specie	Features	Advantages	Disadvantages
Group I. Lactic acid-producing bacteria	*Bifidobacterium*	Gram+, obligate anaerobes	Non-toxic – common flora of humans and have been	No obvious oncolytic effect
	B. longum		Used in dairy industry for many years	Non spore former
	B. adolescentis		Have been used as probiotics	More susceptible to non permissive conditions
	B. infantis		Can be used for intravenous or oral administration	More difficult to store and handle
Group II. Intracellular Bacteria	*Salmonella*	Gram−, facultative anaerobes	Attenuated vaccine strain has been proved safe clinically in human	May have difficulty to infect and lyse quiescent cells
	S. typhimurium			
	S. choleraesuis	Agents for intestine infection	Biochemical pathways and genomes are well characterised Auxotrophic isolates for solid tumours have intrinsic anti-tumour activity Can target both large and small tumours Intracellular bacteria can enter target cells or professional antigen presenting cells and induce strong innate immune response	Have a tumour to normal tissue ratio of 1000:1, therefore a significant number of bacteria colonise normal organs Wild type *S. typhimurium* was associated with Gallbladder cancer Virulence factors exist, safety is an issue
	Listeria *L. monocytogenes*	Gram+, facultative anaerobes		
Group III. Strictly anaerobic bacteria	*Clostridium* Proteolytic: *C. sporogenes* *C. oncolyticum* *C. novyi* Saccharolytic: *C. butyricum* *C. acetobutylicum*	Gram+ Normal habitat in the soil, aquatic sediments, and intestinal tract of both animals and humans	Spore former Spores are easy to produce, stable and economical to use, can be used for intravenous delivery Safety has been shown in early human trials Shown oncolytic ability in animal experiments and human trials	Some strains are pathogenic Strain variation exists

Wei et al., EJC, 43, 490-496, 2007

Bacteria in cancer therapy: early history

1813 Cancer patients suffering from gas gangrene
- tumor regressions

1890 "Coley's toxins" (Gram+, heat-killed *Streptococcus pyogenes* & Gram-, heat killed *Serratia marcescens*)
- reproducible therapeutic effects

1935 sterile filtrates of *Clostridium histolyticum*
- proteolysis of neoplastic tissue

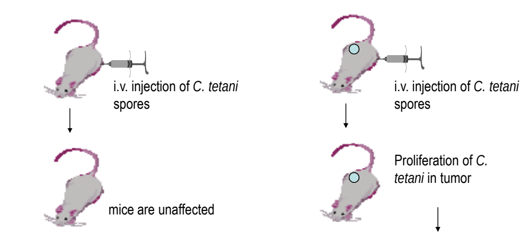
1947 direct injection into mouse sarcomas of *Clostridium histolyticum*
- extended survival times (co-treat penicillin and antitoxin)

1955 intravenous injection of *Clostridium tetani*
- direct injection into tumor not necessary

Clostridia in cancer therapy: early history

Tumor tetanus phenomenon

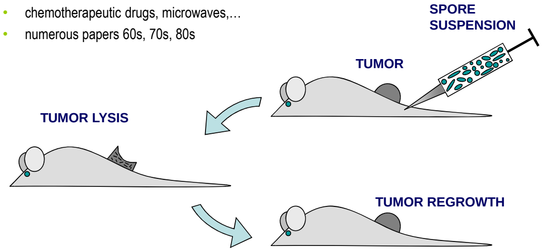
Malmgren & Flanigan, 1955



Clostridial tumor oncolysis

Combination therapy

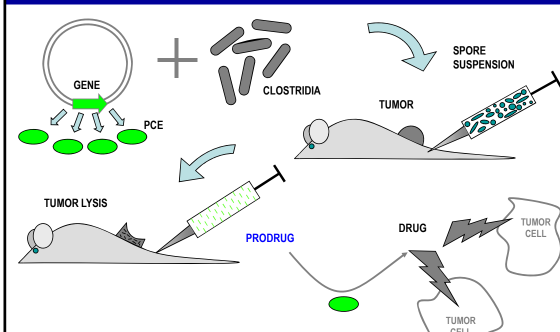
- chemotherapeutic drugs, microwaves,…
- numerous papers 60s, 70s, 80s



Addition of genes encoding anticancer agents

- Human cytokines (TNFα, IL2)
- Prodrug-converting enzymes (NfnB, CodA, CPG2)

Clostridial Directed Enzyme Prodrug Therapy (CDEPT)



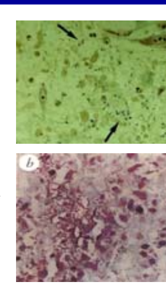
Minton (2003) Clostridia in Cancer Therapy. *Nature Microbiol Rev* **1:** 237-242
Theys et al (2007) Potential and limitations of bacterial-mediated cancer therapy.Front Biosci. 2007 May 1;12:3880-91.

Is the vegetative form of *Clostridium* (metabolically active) specific for tumors?

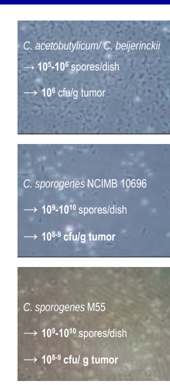
Tumor-selective colonization

Administration (recombinant) clostridia to tumor-bearing animals

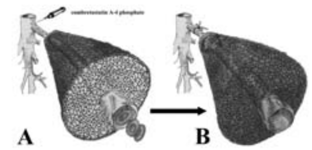
Tumor-specific localization of recombinant bacteria

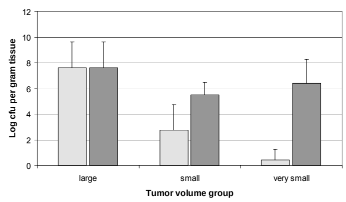


no vegetative cells detected in other tissues

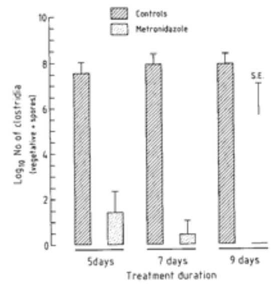


Tumor colonization ↑↑: effect of vascular targeting





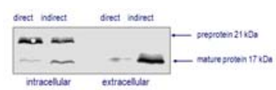
Tumor colonization ↓↓: effect of antibiotic treatment

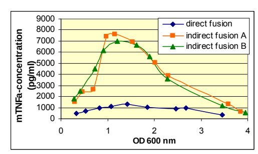


Is it possible to express therapeutic gene products in *Clostridium*?

Direct cytotoxic agents: cytokines

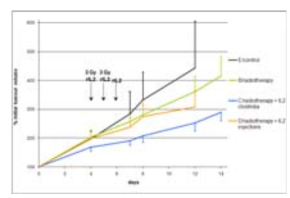
TNFα



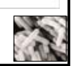


extracellular

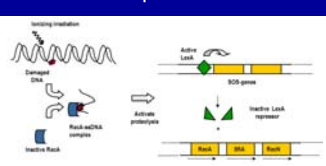
IL-2

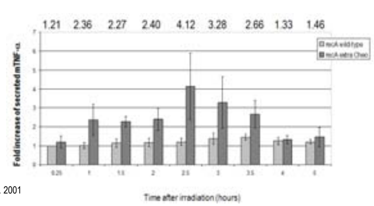


KATHOLIEKE UNIVERSITEIT LEUVEN



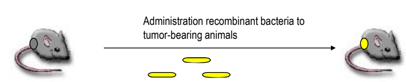
Radiation-induced promoter in *Clostridium*?



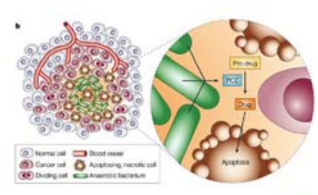


Nuyts et al, Gene Ther. 2001

CDEPT: Clostridium-directed enzyme prodrug therapy

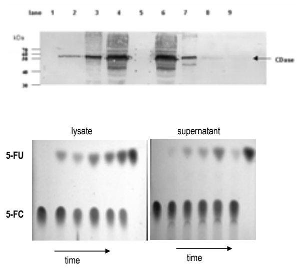


1. CDase
2. NTR

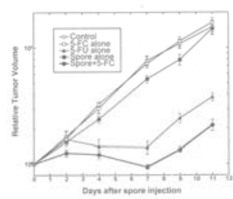


CDEPT: CDase

In vitro | *In vivo*

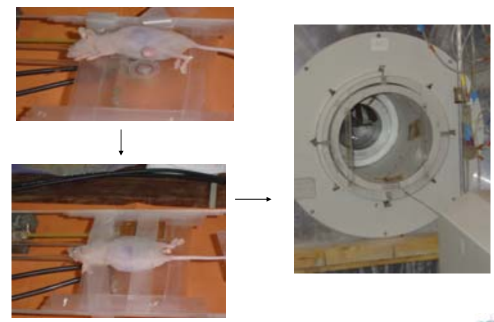


Theys et al., Cancer Gene Ther, 2001



Liu et al., Gene Ther, 2002

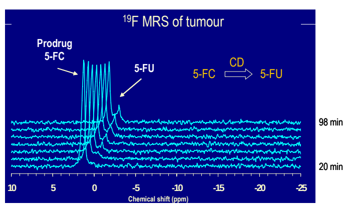
Non-invasive evaluation of bacterial-mediated prodrug conversion: MRS



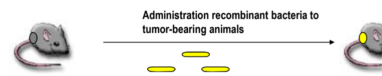
Non-invasive evaluation of bacterial-mediated prodrug conversion: MRS

Feasibility *in vivo*: detection conversion 5-FC/5-FU by recombinant bacteria

- injection of bacteria
- injection of prodrug 5-FC (t = 0)
- 13 min/spectrum

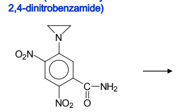


CDEPT: Clostridium-directed enzyme prodrug therapy



1. E. coli CDase: 5-FC → 5-FU
2. **NTR**

CB 1954 (5-aziridin-1-yl-2,4-dinitrobenzamide)



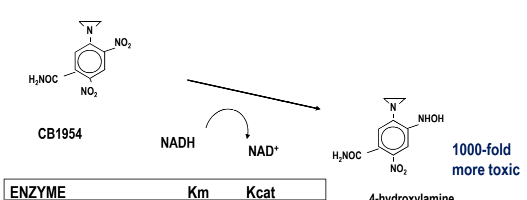
$C_9H_8N_4O_5$
252.18

C 42.9% H 3.2% N 22.2% O 31.7%

- 2- and 4-hydroxylamino derivatives
- Cellular coenzymeA converts the latter into powerful bifunctional alkylating agent
- Molecular level: induction of interstrand DNA cross links and apoptosis

Enzyme-prodrug combination

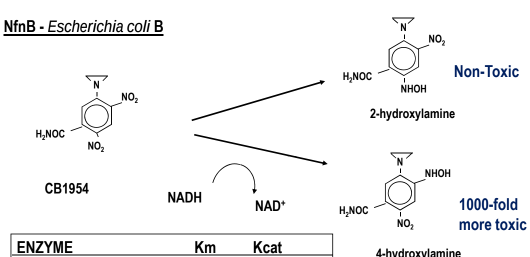
DT Diaphorase (RAT Walker Cell Line)



ENZYME	Km	Kcat
DTD - Rat	826	0.07

Enzyme-prodrug combination

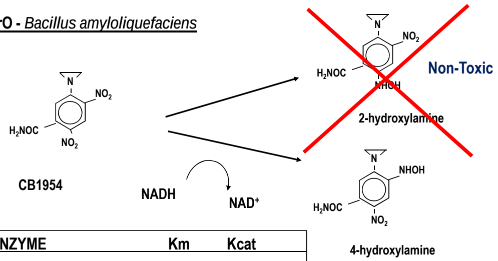
NfnB - *Escherichia coli* B



ENZYME	Km	Kcat
DTD - Rat	826	0.07
NfnB - E.coli	862	6.0

Enzyme-prodrug combination

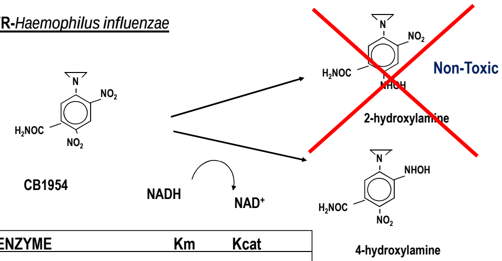
YwrO - *Bacillus amyloliquefaciens*



ENZYME	Km	Kcat
DTD - Rat	826	0.07
NfnB - E.coli	862	6.0
YwrO – Bacillus	617	2.0

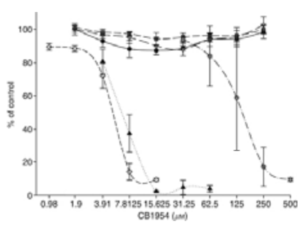
Enzyme-prodrug combination

NTR-*Haemophilus influenzae*



ENZYME	Km	Kcat
DTD - Rat	826	0.07
NfnB - E.coli	862	6.0
YwrO – Bacillus	617	2.0
NTR- Haemophilus	**680**	**56.2**

Optimizing therapeutic gene product



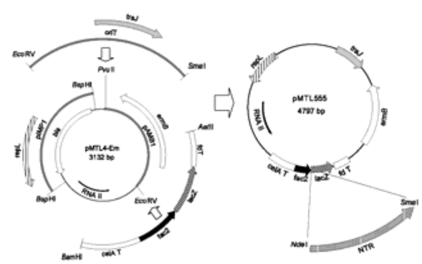
Assessment of CB1954 cytotoxicity. Cytotoxicity of CB 1954 was assessed in 96-well plates by incubating cells with CB 1954 alone (3.9-500 M), prodrug+NAD(P)H (250 M), or prodrug, cofactor and enzyme (4 g well^{-1}) for 3 h in serum free DMEM. The medium was replaced and the cells grown for 72-96 h post-treatment in DMEM+10% FCS until control (untreated) cells had achieved confluence. The cells were fixed, stained with SRB and the plates read at 492 nm. Results are expressed as % of control (untreated cells). Key: , prodrug alone; , prodrug +NADH; , prodrug+NADPH; , HinNTR+NAPDH+Prodrug; , NfnB+NADH+prodrug, and; , YwrO+NADPH+prodrug.

- produced only the 4HX derivative from CB1954

- K_m = 690 μM and k_{cat} = 56.2 s^{-1}

Improving levels of recombinant bacteria in the tumor

Transfer of recombinant constructs to *C. sporogenes*

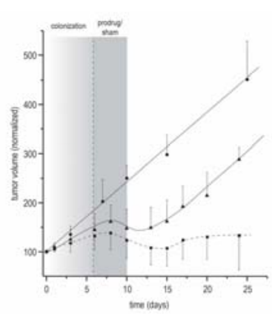


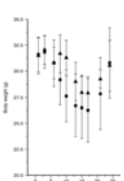
Method to transform clostridia: conjugation

- Donor strain: *E. coli* HB101 (R702, shuttle vector containing oriT site = mobilizable vector)
- Acceptor strain: *C. sporogenes*

Quantitative in vivo evaluation

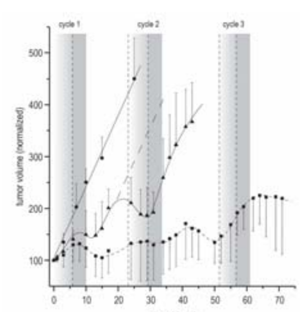
one treatment cycle





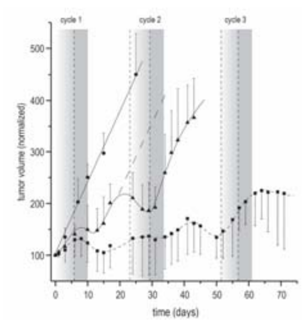
Quantitative in vivo evaluation

multiple treatment cycles



Theys et al, BJC, 2006

Quantitative in vivo evaluation
multiple treatment cycles

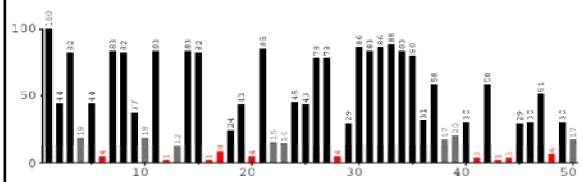


Two major hurdles to clinical trial

1. Poor NTR expression
2. Gene has to be in the chromosome

Theys et al, BJC, 2006

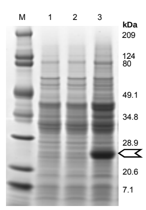
1. More effective expression
Synthetic Gene: Clostridial Codons



- analysis of gene indicated the presence of numerous infrequently used codons
- gene re-sythesised using preferred clostridial codons

1. More effective expression
Synthetic Gene: Clostridial Codons + Increased Plasmid Stability

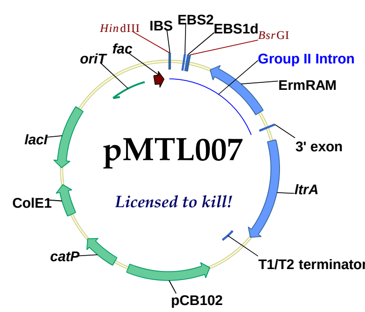
- Plasmids introduced in *C. sporogenes* by conjugation from *E. coli* donors
- Levels of recombinant NTR in lysates measured after 8 hours (OD_{600} 1.5) growth by enzyme assay and densiometric scanning of Comassie stained SDS Gels



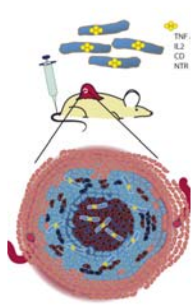
SDS-PAGE analysis of effect of plasmid stability on *Hin*NTR over-expressing c *C. sporogenes* NCIMB 10696 clones.

2. Gene integration:
ClosTron Gene Knock-out System

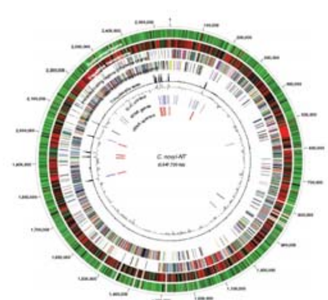
The University of Nottingham



Prokaryotic vectors as tumor-specific delivery system



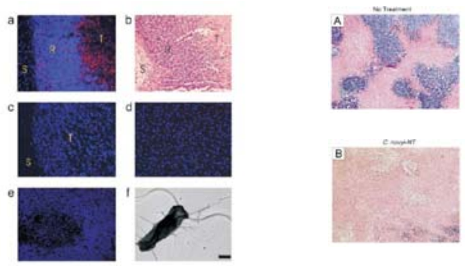
Clostridium novyi-NT



Dang et al., 2001; Bettegowda et al., 2003; Agrawal et al., 2004; Cheong et al., 2006; Bettegowda et al., 2006

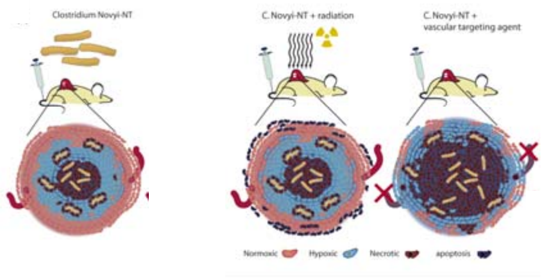
Clostridium novyi-NT

COBALT "Combination Bacteriolytic Therapy"

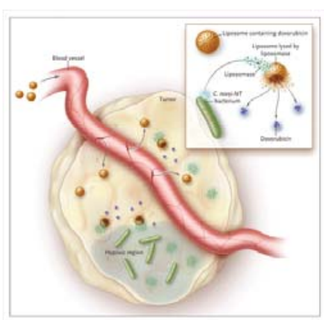


Dang et al., 2001; Bettegowda et al., 2003; Agrawal et al., 2004; Cheong et al., 2006; Bettegowda et al., 2006

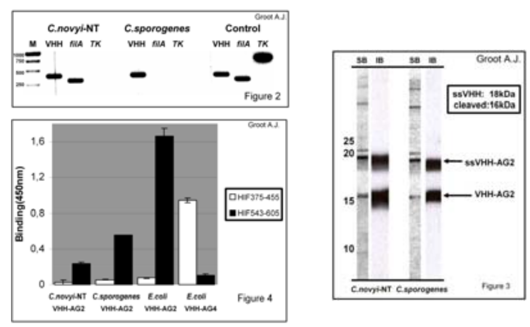
Prokaryotic vectors as tumor-specific delivery system



C. novyi-NT: liposomase



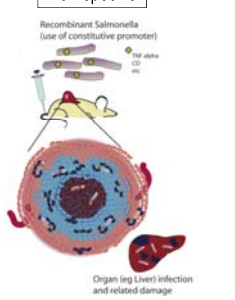
C. novyi-NT: VHH



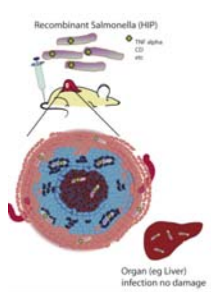
Asferd Mengesha*; Arjan J Groot*; Elsken van der Wall; Paul J van Diest, Jan Theys, Marc Vooijs. Functional antibodies produced by oncolytic Clostridia. Biochem Biophys Res Commun. 2007 Dec 28;364(4):985-9.

Attenuated *Salmonella* as tumor-specific delivery system

Non-specific



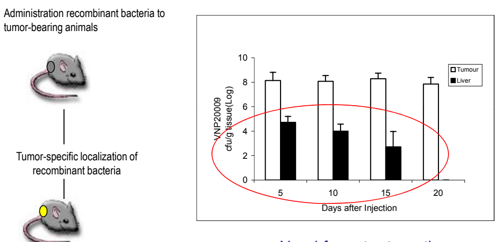
Specific



Attenuated *Salmonella*: 2nd generation

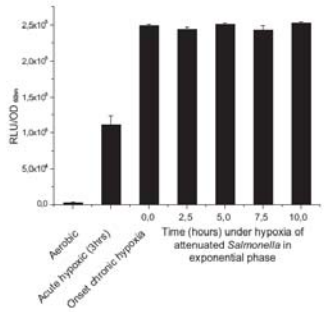
Administration recombinant bacteria to tumor-bearing animals

Tumor-specific localization of recombinant bacteria



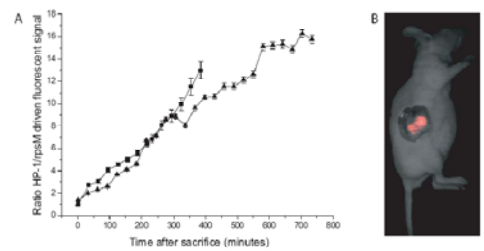
⇒ Need for extra targeting:
hypoxia-inducible promoter (HIP)

Hip-1 promoter drives hypoxia specific gene expression



Mengesha et al., CB&T, 2006

In vivo validation of HIP-1 promoter activity.



Bacterial vectors versus replication competent viral vectors

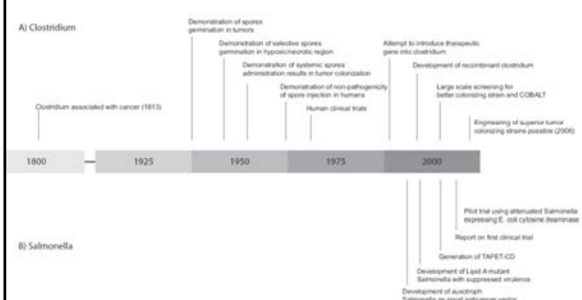
Table 2 – Bacterial vectors versus replication competent viral vectors

Advantages	Oncolytic viral vectors	Modified bacteria
Safety	+	+++
Easy and economic production	+	++
Convenient to store	0/+	+++
Easy administration	+	+++
Systemic delivery	0/+	+++
Extracellular growth	0	+++
Transduce tumour cells to kill	+++	0
Effect on stromal cells	0	+++
Gene integration	+/++	0
Mutagenesis	+/++	0
Clear out of the circulation	0	+++
Potential long-term side effect	++/+++	+
'Cure' tumours	0/+	++

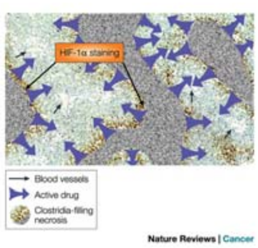
0, no effect; +, small effect; ++, medium effect; +++, significant effect.

Wei et al., EJC, 2007

Timeline

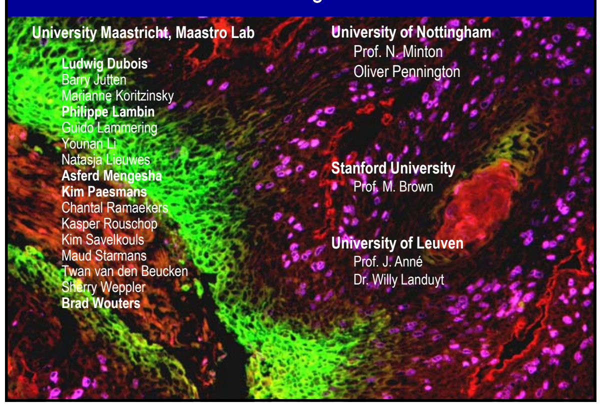


Concluding remarks



- Highly tumor specific
- Safe
- Best colonizing strains can be genetically engineered
- Variety of therapeutic genes can be expressed
- In vivo data are promising
- Increased therapeutic ratio can be expected in combination with e.g. vascular targeting, radiotherapy

Acknowledgements



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