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Live Biotherapeutic MRx0518 as a modulator of immune responses in intestinal tissue and breast tumor microenvironment

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4D pharma plc is a pharmaceutical company focused on developing Live Biotherapeutic products (LBPs) derived from the human gut microbiome. LBPs are a regulated, emerging and disruptive new class of medicines, which have the potential to transform the way in which we treat many diseases. 4D pharma currently has clinical stage programs in cancer, asthma, irritable bowel syndrome (IBS) and Crohn's disease, and a strong pipeline of pre-clinical programs including immuno-oncology, CNS and autoimmune diseases.

Oncodesign is a biopharmaceutical company dedicated to precision medicine. With its unique experience acquired by working with more than 800 clients along with its comprehensive technological platforms combining state-of-the-art medicinal chemistry, pharmacology, bioanalysis, pharmaco-imaging and artificial intelligence, **Oncodesign** orients, drives & executes the development of drug candidates from hit findings up to early clinical phases in oncology and autoimmune/inflammatory diseases**.**

Introduction

The human gut microbiota plays an essential role in modulating both intestinal and systemic immunity. Its importance in regulating anti-tumorigenic responses is now being explored, supported by evidence that functional components of the gut microbiome influence response to cancer therapy, including but not limited to immunotherapies.

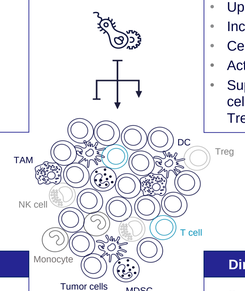
We previously demonstrated that the single strain Live Biotherapeutic, *Enterococcus gallinarum* MRx0518, had strong immunostimulatory properties *in vitro*, and was a TLR5 and NF-κB activator.

We further investigated whether this bacterial strain displayed anti-tumor efficacy in a murine mammary carcinoma model.

LBPs and the tumor microenvironment

Innate immunity
- APC recruitment and activation
- NK cell recruitment
- Induction of pro-inflammatory cytokines
- PRR activation

HDAC inhibition
- Upregulation of MHC proteins
- Increased antigen expression
- Cell death
- Activation of cytotoxic cells
- Suppression of regulatory cells, such as MDSCs and Tregs



Adaptive immunity
- Increased T cell trafficking
- Increased activation of effector cells (CTL and Th1 cells)
- Reduced Treg differentiation

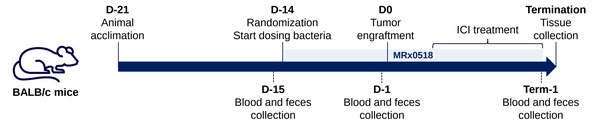
Direct tumor destruction
- Cell cycle arrest
- Apoptosis
- Cytotoxic compounds
- Oncometabolites
- Release of tumor antigens

MRx0518

- Strain of *Enterococcus gallinarum*
- Gram-positive, motile, anaerobic bacteria
- Oral Live Biotherapeutic
- Originally isolated from the gut microbiome of a healthy human donor
- Selected for immunostimulatory host response profile, relevant for immuno-oncology
- Immune stimulation primarily through action of bacterial flagellin on TLR5

Experimental design: EMT6 model

Experimental timeline



Analysis

Tumor
Tumor volume
Transcriptomics analysis using the PanCancer IO 360 NanoString panel

Gut
Transcriptomics analysis using the PanCancer IO 360 NanoString panel

Blood
Cytokines measurement by MagPix multiplex

Feces
16Sv3-4 PCR / Illumina MiSeq sequencing (2x250 bp)

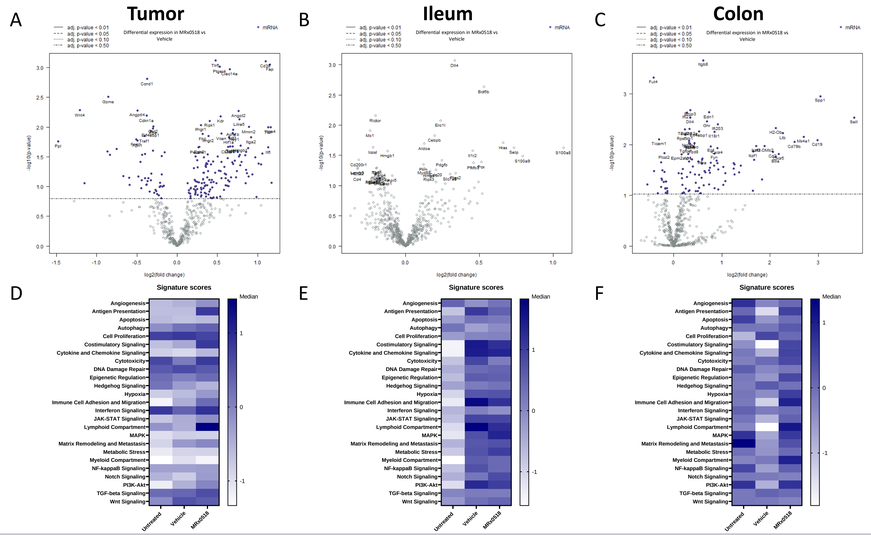
Experimental groups

Group	Dose	Route	Treatment schedule	Tumor induction
Untreated	N/A	N/A	N/A	1 x 10^6 EMT6 cells at D0
Vehicle	N/A	PO	QD From D-14	
MRx0518	2 x 10^8 CFU	PO	QD From D-14	
Anti-CTLA-4	10 mg/kg	IP	BIW From D10	

Results: Transcriptional analysis

MRx0518 significantly modulates gene expression in the tumor and the colon

MRx0518 therapeutic activity was associated with upregulation of genes involved in immune cell adhesion and migration, such as *Cd38* and *Clec14a*, and increased *Tlr5* (innate immune receptor) expression in the tumor. At the intestinal level, lymphoid compartment and cell adhesion pathways were upregulated in the colon, in particular *Spp1*, *Cd19*, *Ms4a1* (CD20) and *Sell* (CD62L) genes.



Transcripts analysis of the tumoral (A), ileal (B) and colonic (C) tissue in the mouse model of breast cancer (EMT6) was conducted using the PanCancer IO 360 NanoString gene expression panel.

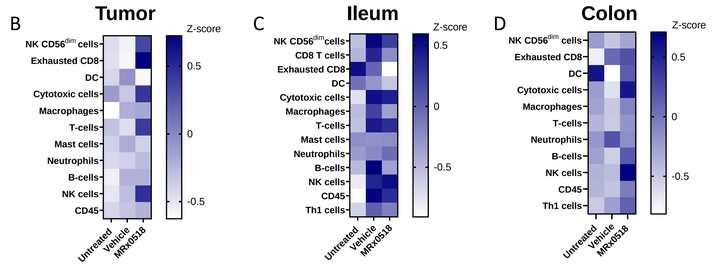
KEGG database was used to annotate the transcripts isolated from tumoral (D), ileal (E), and colonic (F) tissues and to conduct pathway identification.

MRx0518 modulates immune cell populations

Immune cell populations including NK, T cells and cytotoxic cells were increased by MRx0518 treatment in both colonic tissue and the tumor microenvironment.

A

Cell type	Marker genes
NK CD56^{dim} cells	*Il21r, Kir3dl1, Kir3dl2*
Exhausted CD8	*Cd244, Eomes, Lag3, Ptger4*
DC	*Ccl2, Cd209e, Hsd11b1*
Cytotoxic cells	*Ctsw, Gzma, Gzmb, Klrb1, Klrd1, Klrk1, Nkg7, Prf1*
Macrophages	*Cd163, Cd68, Cd84, Ms4a4a*
T-cells	*Cd3d, Cd3e, Cd3g, Cd6, Sh2d1a, Trat1*
Mast cells	*Cpa3, Hdc, Ms4a2*
Neutrophils	*Ceacam3, Csf3r, Fcgr4, Fpr1*
B-cells	*Blk, Cd19, Fcrlb, Ms4a1, Pnoc, Spib, Tcl1, Tnfrsf17*
NK cells	*Ncr1, Xcl1*
CD45	*Ptprc*
CD8 T cells	*Cd8a, Cd8b1*
Th1 cells	*Tbx21*
Treg	*Foxp3*



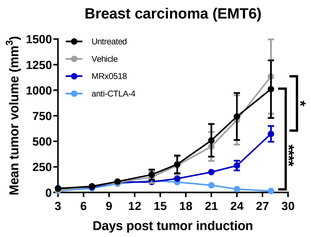
Genes previously shown to be characteristic of various immune cell populations (Table A) were used to measure the abundance of populations for cell type profiling. Heat maps represent tumor (B), ileum (C) and colon (D) immune cell population abundance for untreated, vehicle-treated and MRx0518-treated animals in the EMT6 model.

Results: Tumor growth

MRx0518 monotherapy induces a reduction in EMT6 murine tumor burden

- From D-14, mice received vehicle or 2x10^8 CFU MRx0518 daily until termination.
- On D0, mice were engrafted with EMT6 tumor cells subcutaneously.
- Anti-CTLA-4 (10 mg/kg, IP, BIW) was used as positive control.
- Tumor length and width as well as body weight were measured 2-3 times a week. No side effect of the treatment was observed.

Group	Tumor volume at D28 in mm^3		T/C (%) at D28	
	Mean ± s.e.m	Median	vs Untreated	vs Vehicle
Untreated	1010.88 ± 282.98	925.18	100.00	94.01
Vehicle	1134.43 ± 362.03	984.15	106.37	100.00
MRx0518	572.93 ± 77.61	511.49	55.29	51.97
Anti-CTLA-4	14.33 ± 8.57	0.00	0.00	0.00



Conclusions

MRx0518 *in vivo* Efficacy
- Reduces tumor growth in EMT6 model (also LLC and RENCA models, data not shown)
- Increases tumor and intestine immune infiltration: NKs, T cells and cytotoxic cells
- Increases TLR5 expression within the tumor tissue

MRx0518 Mechanism of Action
- Modulation of both the gut immune system and the tumor microenvironment
- Flagellin TLR5 agonism (**Lauté-Caly *et al.* Sci Rep 2019**)

MRx0518 Clinical development

- Phase I/II, open-label, combination with pembrolizumab in anti-PD-(L)1 secondary resistant NSCLC, RCC, melanoma, bladder
  - Early signals of activity and clinical response, no serious drug-related adverse events
  - Part A enrollment complete
- Phase I, neoadjuvant monotherapy study in treatment-naïve patients awaiting surgical resection of solid tumors; enrolling
- Phase I, pancreatic cancer in combination with radiotherapy; enrolling