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Bando 2019 - Programma 5 per mille anno 2019
Investigator Grant (IG)

TRANSLATIONAL RESEARCH

LILT will support research projects in the field of cancer aimed at improving cancer diagnosis and treatment. Particularly considered will be those translational research projects that promise short-medium term effects in clinical practice, concerning new diagnostic methodologies and new therapies. Multicentric studies with national coordination, aimed at validating new diagnostic methods, diagnostic, prognostic and predictive tumor markers, able to improve the clinical management of cancer patients are potentially eligible for funding. Specific research projects on new oncological therapeutic approaches are also eligible for LILT funding as IG. For this type of grants it is necessary to demonstrate solid preliminary experimental data supported by a rigorous biological rationale.

1. Principal investigator's full name and qualification:
   Gianluca Bossi, Senior Researcher

(Please include: CV in European format with list of publications; IF end Hi-index)

2. Proposal title: **IDENTIFICATION OF NOVEL THERAPEUTIC OPPORTUNITIES FOR METASTATIC COLORECTAL CANCER PATIENTS.**

3. Primary area of Relevance **Oncology 1 – Basic Translational**

4. Relevance for the National Health System **Development of new therapies easily translatable in clinic**

5. Institution **Istituti fisioterapici ospitalieri IRCCS - Regina Elena National Cancer Institute** address **Via Elio Chianesi 53,** phone **+(39) 06 52666995** e-mail **gianluca.bossi@ifo.gov.it**

6. Authorized Administrative Official **Ripa di Meana Francesco** address **Via Elio Chianesi, 53** phone **(+39) 06 52662702** e-mail **dirgen@ifo.gov.it**

7. Proponent's signature

8. Authorized Administrative Official's signature.....

DIREZIONE GENERALE I.F.O. ROMA

9. Place and date Rome, 11/02/2020

SELF EVALUATION FORM

1. Investigator's full name: (PI) **Gianluca Bossi**
2. Total papers **47** IF **277,855**
3. Total papers (last 10 years) **31** IF **186,568**
4. Total Papers as first/last author or corresponding author **20**
5. Total H-index **23** *(scopus)*

PROPOSAL MAIN BODY

1. Proposal title.

IDENTIFICATION OF NOVEL THERAPEUTIC OPPORTUNITIES FOR METASTATIC COLORECTAL CANCER PATIENTS.

2. Abstract

Background. Colon cancer (CRC) is one of the most common, lethal diseases worldwide. The World Health Organization reported over 800000 people died from CRC in 2019. While 5-year survival rates remain high for patients diagnosed in the early stages of disease, a stage IV diagnosis reduces survival to near 10%. This low survival rate highlights the lack of clinical advances for late stage CRC, making the identification of novel molecular targets imperative to develop more selective and efficient therapeutic strategies.

Rationale of the study. MKK3 targeting might constitute a novel potential and promising therapeutic approach to boost the efficiency of anti-cancer therapies in CRC patients. We propose to validate MKK3 as a therapeutic target dealing with two integrated tasks that will contribute to: TASK1) validate MKK3 as potential therapeutic target in patient-derived CRC models; TASK2) assess the therapeutic potential of MKK3-Knockdown Mimicking Compounds (MKDMCs) in CRC models.

Preliminary results. According to our published and preliminary data: i) MKK3 is highly expressed in advanced stages of CRC; ii) its expression correlates with poor prognosis; iii) its depletion univocally exerts anti-tumor effects and boosts therapy efficacy in both in vitro and in vivo models; iii) effects of its targeting could be reproduced by already available and tested drugs, which we termed MKDMCs (MKK3 Knockdown Mimicking Compounds).

Experimental Design. The proposed tasks will be carried out in parallel: TASK1) to validate the appropriateness of MKK3 as a therapeutic target with highly representative models of CRC selected by a collection of patient-derived organoids (PDOs) and xenografts (PDXs) to test the effect of MKK3 targeting alone and in combination with selected agents both in vitro and in vivo; TASK2) to assess efficacy of MKK3 Knockdown Mimicking Compounds (MKDMCs), in vitro and in vivo, thus providing easily translatable drugs to be perspectively applied for the therapy of CRC.

Translational value of the research and the expected impact on the NHS. The proposed research will validate MKK3 as a new therapeutic target, provide detailed insights into anti-tumor effects triggered upon MKK3 targeting and, most importantly, re-purpose already tested and readily exploitable therapeutics to safe and efficient MKK3 targeting in CRC. Overall, results from this project will allow the design of effective clinical trials exploiting MKK3 targeting therapy in CRC, and ultimately bringing this new therapy to CRC patients: bench to bedside.

3. Introduction

An estimated 1,361,000 people are diagnosed with CRC annually and approximately 694,000 people die from CRC annually; 3,544,000 individuals are living with CRC and patients with stage IV at diagnosis revealed a survival rate near to 10%. Conventional and targeted therapies are already available, but development of resistance is a common event [*PMID:26557002*] and the identification of new molecular targets is imperative to design novel and more effective therapeutic strategies.

MKK3 is a member of the dual-specificity protein kinase group (MKK) that belongs to the mitogen-activated protein kinase-signaling pathway. MKK3 is activated by MKKK proteins through Ser-189 and Thr-193 phosphorylation and is a specific activator of p38MAPK proteins through Thr180 and Tyr182 phosphorylation. The role of p38MAPK signaling in cancer is still heavily debated, being reported as tumor suppressor or favoring tumor growth in different cell-contexts [*PMI:27446920*], controversy likely explained by the pleiotropic nature of the p38MAPK signaling pathway that relies on isoform-specific activation of p38 family members by either MKK3 and MKK6 [*PMID:20004242*]. Thus, anticancer approaches inhibiting p38MAPK could not meet expectations due to signal compensation by upstream regulators, while MKK3 might constitute a novel potential and promising therapeutic target to likely inhibit specifically the tumor promoting p38MAPK signaling leaving unaffected the antitumor cascade. Accordingly, novel emerging literature suggests MKK3 involvement in tumor malignancy in liver [*PMID:26824501; PMID:28792132; PMID:30720101*], NSCLC [*PMID:26958086*], breast cancer [*PMID:27181679; PMID:29180066*], pancreatic cancer [*PMID:29346059; PMID:31075266*], CRC [*PMID:29637628*], melanoma [*PMID:31257538*], and cell migration and invasion [*PMID:30770795*].

4. background and rationale

We previously identified the MKK3 as mutant (mut) p53 gain-of-function (GOF) target gene [**1**] and putative candidate therapeutic target [**2**]. MKK3 silencing by RNA interference (RNAi) affects cell proliferation and survival in mut [2], wild-type (wt), and p53-null cancer lines by promoting autophagy and cell death [**3**]. MKK3 knockdown (KD) does not affect proliferation and survival of primary non-transformed cells [**3**], credentialing MKK3 as a potential tumor-specific required factor [**4**]. Through more dedicated studies we confirmed with a panel of authenticated MSS and MSI CRC lines and primary colonocytes that MKK3 targeting exerts detrimental effects in cancer but not healthy cultures [**5,6**]. We therefore identified a novel pro-survival signaling pathway MKK3/p38delta MAPK/ERCC1 in CRC lines, which is further activated by 5-fluorouracil (5-FU), a cornerstone drug in CRC treatments [**5**]. In accordance with other studies, linking MKK3 to cisplatin (CDDP) resistance in lung cancer and head and neck squamous cell carcinoma lines [*PMID:22164285*], MKK3 targeting boosts tumor cell response to anticancer drugs in both wt and mutp53 bearing cancer cells allowing drug-dose-reduction in vitro and in vivo [**5,6**]. Of interest, some MKK3 inhibitors have recently been developed [*PMID:26704264*], however, they still need to be validated in pre-clinical settings and most of them fail in specificity. Moreover, these drugs are developed to inhibit the MKK3 kinase activity leaving unaffected the recently identified kinase-independent activity, such as the remarkable interaction and stabilization of c-Myc [*PMID:2862811820*]. Thus, MKK3 might be endowed with relevant uncovered functions involved in the interaction with other important tumor-involved mediators, which could be affected by the MKK3 silencing or by the identification of MKK3 KD mimicking compounds (MKDMCs).

5. experimental design.

TASK 1. VALIDATE MKK3 AS POTENTIAL THERAPEUTIC TARGET IN PATIENT-DERIVED CRC MODELS:

1A) Explore the sensitivity of CRC Patient derived Organoids (PDOs) to MKK3 targeting in vitro.

1B) Assess the effects of MKK3 depletion in highly relevant patient-derived models in vivo (PDXs).

TASK 2. ASSESS THE THERAPEUTIC POTENTIAL OF MKK3-KNOCKDOWN MIMICKING COMPOUNDS (MKDMCs) IN PATIENT-DERIVED CRC MODELS:

2A) Screening putative MKDMCs in PDOs in vitro.

2B) Validate the antitumor efficacy of putative MKDMCs in PDXs in vivo.

6. further details on the overall methods that will be used in this project

Experimental Design TASK 1:

1A. We propose to validate MKK3 as therapeutic target with preclinical CRC population-based studies enabling to recapitulate the wide heterogeneity of human malignancy that occurs among individuals on a population basis. Thus, thanks to the collaboration of Prof. Trusolino (University of Turin) we will select subsets of patient-derived organoids (PDO) and xenografts (PDXs) from a large platform based on established n.800 PDXs and n.200 PDOs from metastatic colorectal cancer (mCRC), and 350 PDXs from primary tumors. Molecular annotation includes whole exome sequencing for 129 mCRC PDXs with targeted next-generation DNA sequencing for 116 cancer genes; global RNAseq and global methylomics analyses in 380 mCRCs and 124 primary tumours; low-pass copy number data for 140 serially passaged mCRC tumours (fresh surgical sample, early implant, late implant). Accordingly, based on preliminary data (Fig.1,2), we will investigate MKK3 targeting effects in n. 7 KRAS/NRAS mutant, n. 5 BRAF mutant and n.8 KRAS/NRAS/BRAF wild type PDOs of metastatic CRC patients who received adjuvant therapy after removal of the primary tumor and before metastasectomy. Adopted panel of PDOs will be engineered with well-established lentiviral-based TET-inducible RNAi system vectors encoding two different shRNA sequences specific to MKK3 (sh/MKK3 1.1 and 1.2) and relative controls (sh/scr1.1 and 1,2), already available in laboratory [2-6] and following standard procedures. Efficiency in MKK3 depletion will be verified by Western Blot (WB) and Real-Time PCR analysis upon 96h of induction with doxycycline (DOX). Effects on cell proliferation will be assessed (time dependently) by MTT and long-term time-laps by IncuCyte Live-Cell Analysis, an automated image acquisition and analysis system available at IRE. Effects on cell-cycle and cell-death monitored by Propidium Iodide and Annexin-V/7-AAD staining, respectively, and Flow-cytometric analysis. Autophagy will be investigated by WB for p62/SQSTM, LC3I/LC3II, PI3K, pAKT, AKT, mTOR, g-H2AX, ATM, ATR, BCL-2, BCL-XL, ERCC1, actin and GAPDH as reported [3,5]. According to preliminary data (Fig.2) we will explore the MKK3/p38MAPK pathway in PDOs upon 5-FU exposure. Increasing drug dose and time exposure (6h, 24h and 48h) will assess 5-FU-IC50 for each PDOs by MTT. We will assess by WB effects on phosphoMKK3, MKK3, phosphop38MAPK, p38MAPK isoforms and ERCC1 levels upon exposure to 5-FU-IC50 for each PDOs, as well as, upstream (TAK1, ASK1, TAO1/2/3) and downstream (MK2, MSK1, ATF2, HSP27, AP-1) mediators. We will also investigate activation of ATM/ATR kinases as well as phosphatases (PP2CA, PP2CB, PTP, MKP, PP5). According to Fig. 2H-I [5], by p38 MAPK isoform specific immunoprecipitation (IP) and phosphop38 MAPK WB we will explore activation of each p38MAPK isoform upon 5-FU. Similarly, with sh/scr and sh/MKK3 engineered PDOs we will explore MKK3 contribution in 5-FU response. Based on achieved results, commercially available chemicals and Stealth RNAi [5] will be adopted to assess the roles of the above identified p38MAPK isoform in mediating MKK3 signaling in untreated and 5-FU treated PDOs. We will further investigate the MKK3/p38MAPK pathway activation in response to other chemotherapeutics currently

in clinical CRC practice as irinotecan (IRI) and oxaliplatin (OXA) and pending results we will consider co-treatments mimicking patient's therapy (FOLFOX/FOLFIRI). When required, confirmatory analyses will be carried out in a subset of PDOs with lentiviral-based TET-inducible CRISPR/Cas9 gene editing, already available in laboratory.

1B. We will investigate the effects of targeting MKK3 in PDX models generated from at least 2 different PDOs for each mutational setting. For each selected PDO, the sh/scr and sh/MKK3 generated sublines (TASK1A) will be implanted subcutaneously (s.c.) in nude mice and effects of MKK3 targeting, alone and in combination with 5-FU (50mg/kg) assessed. Briefly: upon establishment of palpable tumor, MKK3 depletion will be attained via DOX administration (2g/lt) in drinking water and 5-FU treatment delivered by i.p. as reported [2,3,5]. Tumor growth will be monitored via caliper measurement twice a week. Upon reaching specific enpoints (e.g. tumor volume exceeding 1,5 cm3), all mice will be euthanized and tumors collected for molecular analysis to assess effects of the MKK3/p38MAPK pathway modulation by WB, as in TASK1A, and histologic and IHC analysis performed to explore cell proliferation (Ki67, p27), angiogenesis (CD31), apoptosis (TUNEL assay), and autophagy (LC3II localization, p62). Pending results in TASK1A, other tested chemotherapeutics will be considered.

Cohorts of 8 mice/group will be adopted to attain significance (sh/scr, sh/scr+drug, sh/MKK3, and sh/MKK3+drug), and pending results experiment will be repeated.

Experimental Design TASK 2:

2A. According to Fig.3 we will firstly validate AT-9283 and other three already tested as putative MKDMCs (data not shown) in PDOs carrying different BRAF/KRAS gene status. Effects on proliferation and survival will be validated by MTT; molecularly will be investigated whether selected MKDMCs recapitulate MKK3 dependent gene signature by Real Time PCR analysis (Fig 3C), as well as reproducing effects such as autophagy induction and cell death by WB (as in AIM1). We will also assess whether selected MKDMCs boost response to 5-FU and/or other tested chemotherapeutics (as in TASK1). Based on preliminary results in in CRC lines (Fig. 2K, 2L) we will investigate similarly, the effects of commercially available p38 MAPK inhibitors for mediating MKK3 depletion dependent effects. Temporarily, we will explore for other putative MKDMCs by pre-testing in subset of CRC lines (Fig.2A): top 10 ranking drugs will be tested with 3 different dosages (literature/LINCS indicated concentration, and 5-fold higher and lower) and effects assessed as in Fig.3. Pending results the top 5 best responding MKDMCs will be investigated in PDOs.

2B. Pending results in 2A, we will test the efficacy of our two best performing MKDMCs in PDX model. Thus, at least 4 primary PDXs (at least 1 KRAS mutant) will be implanted s.c. as in TASK1 and, upon tumor establishment, treatments of selected drugs performed according to manufacturer suggested schedule: at least three different dosages for each drug will be tested (5 mice / group). For therapy testing, selected MKDMCs will be delivered into mice as following groups (vehicle, 5-FU, MKDMC, 5-FU+MKDMC). Therapy efficacy will be assessed for each PDX as in TASK1B. Cohorts of 8 mice/group will be used

Statistical analysis. Experiments in vitro: repeated 3 times in triplicates, and significance assessed by Student's t, Mann-Whitney or ANOVA tests. Experiments in vivo: Student's t-test for growth and Wilcoxon Rank Sum test for survival will assess significance.

7. work carried out and preliminary results

Query of GEO profiles for CRC patients correlated low MKK3 levels with better survival (Fig.1A). Exploring IRE CRC cohort of n.185 patients: high MKK3 levels correlated with advanced CRC stages (Fig.1B), lymph-nodal invasion (Fig.1C) and clinically relevant mutations (KRAS) by NGS (Fig.1D-E) and IHC (Fig.1F-G). In CRC lines (Fig.2A), MKK3

depletion triggers autophagy only in KRASwt cells (Fig.2B-C) whereas increases 5-FU efficacy in all CRC lines (Fig.2D) with no effect on primary colonocyte (Fig.2B-C and E); 5-FU activated MKK3 (Fig.2F-G) triggers p38delta MAPK / ERCC1 signaling (Fig.2G-I); p38delta MAPK targeting recapitulates MKK3 depletion effects molecularly (Fig.2J) and functionally in vitro (Fig.2K) and in vivo (Fig.2L). Down-regulated gene signatures upon MKK3 depletion in HT29 cross-referenced with TCGA CRC database revealed significant prognostic relevance (Fig.3A) that queried on LINCS NIH database identified n.34 putative MKDMCs (score < -98) (Fig.3A) ranked according to LINCS score, clinical/preclinical testing, FDA approval, and predicted IC50. Primary tests found that compound A (AT-9283): i) exerts high killing in CRC lines and relative ineffective in primary colonocytes (Fig.3B); ii) recapitulates MKK3 dependent gene signature (Fig.3C); iii) affects MKK3/p38 MAPK signaling activation (Fig.3D) inducing authophagy (Fig.3E-F).

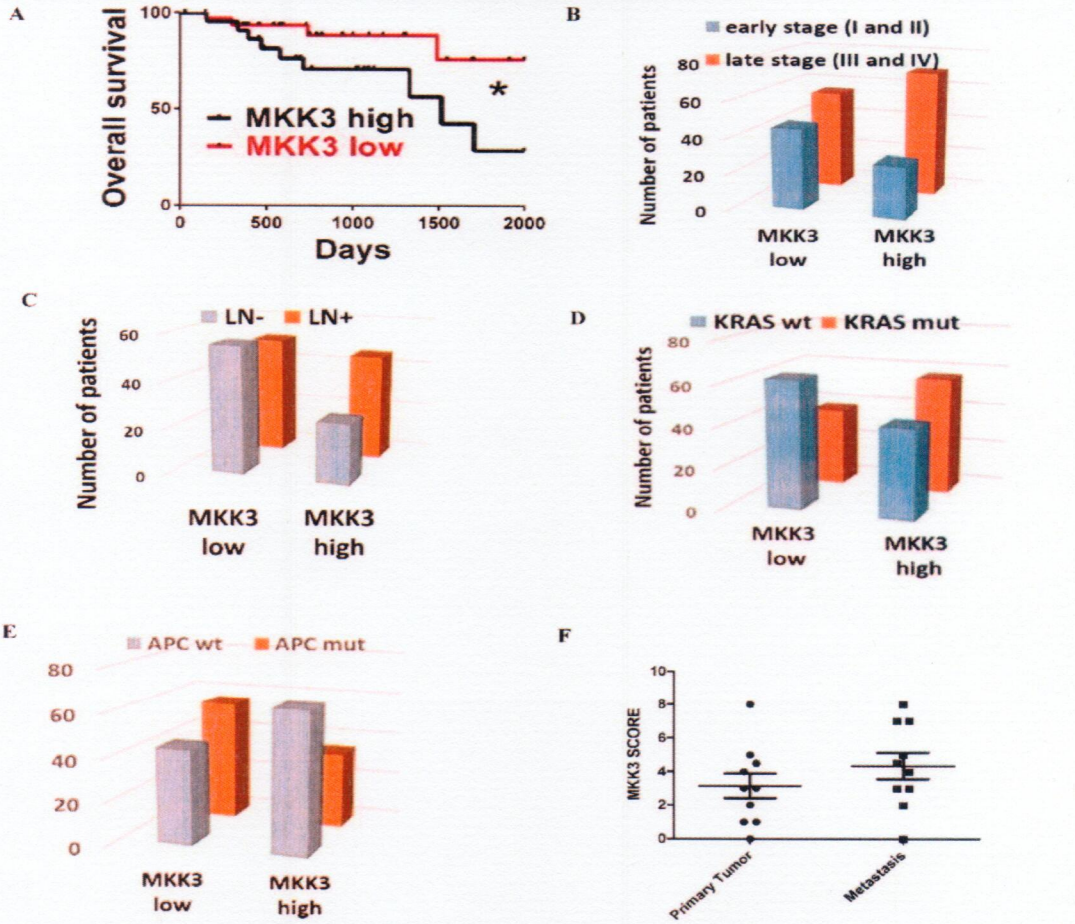
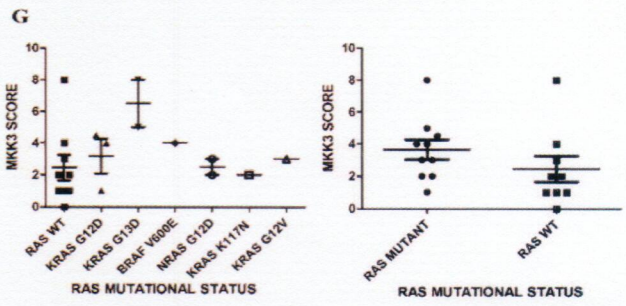


Figure 1. MKK3 prognostic relevance in CRC. A. *Overall Survival for TCGA database of CRC patients stratified for MKK3 expression* ***B.*** *MKK3 positivity by TMA of IRE CRC cohorin stratified in Early and Late stage and N0 and N1 score* ***(C)****;* ***D, E.*** *Stratification of MKK3 positivity according to KRAS status* ***(D)*** *and APC status* ***(E)*** *determined by NGS analyses of 50 cancer related genes;* ***F,G.*** *primary analyses performed on independent CRC cohort from S. Andrea ospital scoring for MKK3 score positivity by IHC on in paired primary tumors (n. 10 cases) and metastases (n.10 cases)* ***(F)*** *and KRASmut status (n. 10 cases) and KRASwt (n.10 cases)* ***(G)****.*



A

Cell line	KRAS status	BRAF status	TP53 status	Tissue of origin
CCD-841	wt	wt	wt	Normal colon
CCD-18CO	wt	wt	wt	Normal colon
Colo-205	wt	V600E	del27	Metastasis (Ascites)
HT29	wt	V600E	R273H	Primary Tumor
SW480	G12V	wt	R273H/P309S	Primary Tumor
SW620	G12V	wt	R273H/P309S	Metastasis (LN)
HCT-116	G13D	wt	wt	Primary Tumor

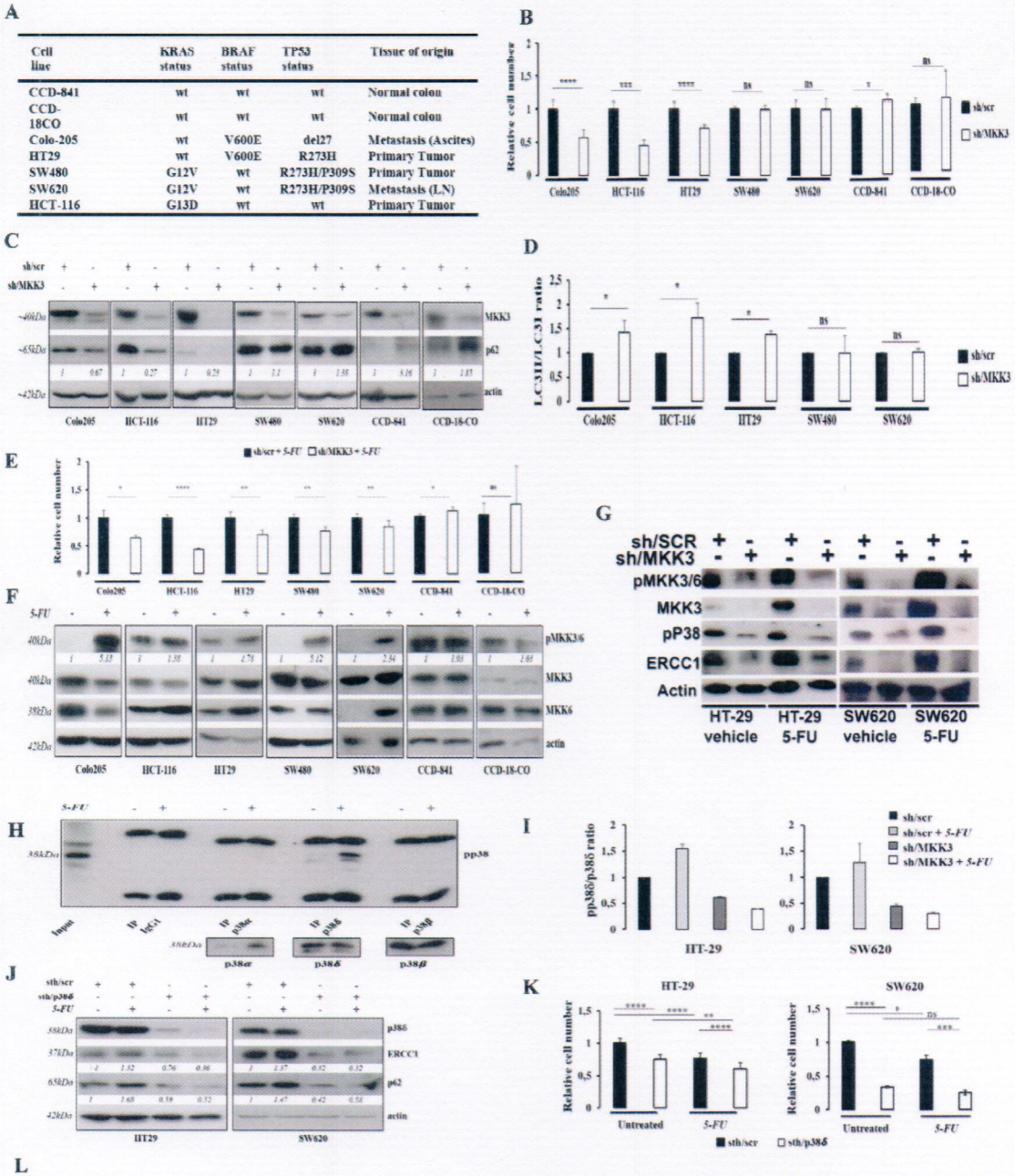


Figure 2. MKK3 KD effects in CRC lines in vitro, in vivo. *A. Panel of authenticated CRC lines and primary colonocytes;* ***B.*** *effects on cell growth upon MKK3 depletion.* ***C and D.*** *Induction of autophagy in MKK3 depleted cells;* ***E.*** *Effects of MKK3 depletion on 5-FU efficacy;* ***F and G.*** *Alterations in the MKK3/p38/ERCC1 pathway upon 5-FU exposure and MKK3 depletion;* ***H and I.*** *p38delta isoform activation upon 5-FU in CRC lines;* ***J.*** *Molecular effects of p38delta targeting;* ***K.*** *Functional effect of p38delta targeting in vitro and in vivo* ***(L)****;*

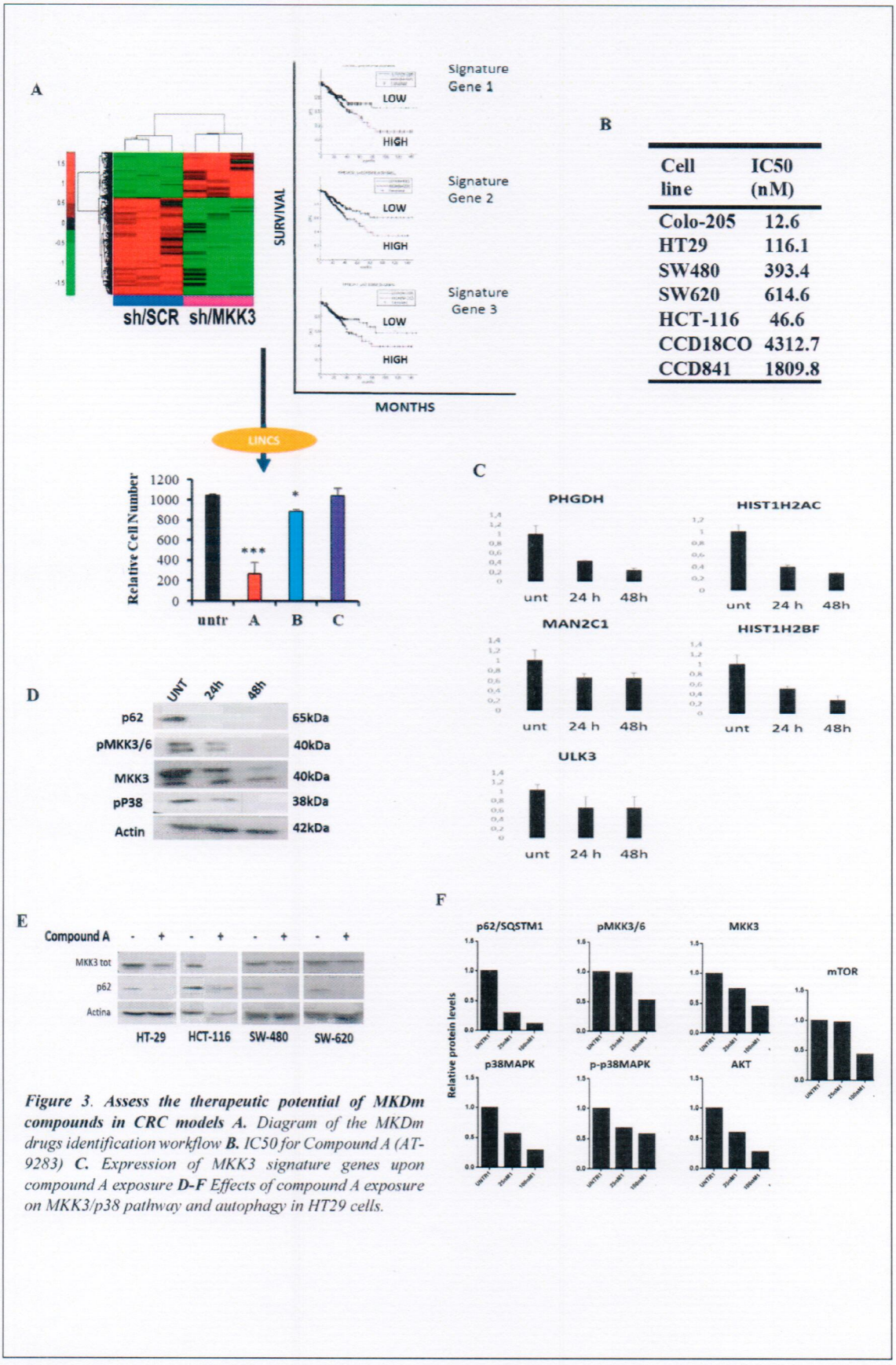


Figure 3. Assess the therapeutic potential of MKDm compounds in CRC models A. *Diagram of the MKDm drugs identification workflow* ***B.*** *IC50 for Compound A (AT-9283)* ***C.*** *Expression of MKK3 signature genes upon compound A exposure* ***D-F*** *Effects of compound A exposure on MKK3/p38 pathway and autophagy in HT29 cells.*

8. expected results and relevant corresponding milestones.

We expect MKK3 to be confirmed as a potent target to boost 5-FU effects in more relevant CRC models in vitro and in vivo, validating its significance in CRC therapy. Molecularly, we expect to confirm in PDOs the MKK3 KD to exert anti-tumor effect via autophagy only in KRAS wild-type context, suggesting exclusively cotreatments (MKK3 KD + 5FU) as a possible strategy in KRAS mutant patients. MKK3/p38delta MAPK signalling activation might emerge as a new putative therapeutic target. Finally, we expect to prove the efficacy of MKDMCs thus bringing new, easily translatable, therapeutic agents in the therapy of CRC patients, and development of patenting for identification of validated MKK3 drug inhibitors.

Milestones 12 month

- Engineering of PDOs cultures with inducible MKK3 RNAi model;
- In vitro and in vivo assessment of MKK3 targeting in PDOs and PDX respectively;
- Assessment of MKDMC (AT-9283) effects on PDOs;
- screening for efficacy of putative MKDMCs in CRC lines;

Milestones 24 month

- Molecular dissection of MKK3 and p38 isoform(s) specific involvement in anti-tumor effects in PDOs;
- In vitro assessment of MKDMCs drugs in PDOs;
- In vivo validation of MKDMCs drugs in PDXs;

8. References and relevant publications by the research group, already available

1. Bossi G, et al., Cell Cycle. 2008 Jun 15;7(12):1870-9, PMID: 18594199
2. Gurtner A et al. J Biol Chem. 2010;285(19):14160-14169. PMID 20223820
3. Baldari S et al. Cell Death Dis. 2015;6:e1621. PMID 25633290
4. Bossi G. Aging (Albany NY). 2016 Jan;8(1):1-2. PMID: 26805700
5. Stramucci L, et al. Cell Death Dis. 2019 Nov 6;10(11):842. PMID: 31695024
6. Stramucci L, & Bossi G. J Exp Clin Cancer Res. 2019 Dec 27;38(1):504. PMID: 31881903

PERSONNEL INVOLVED IN THE RESEARCH

Name and date of birth	Role on Project	Fellowship required	Effort on project (%)	Present position
Gianluca Bossi (09/11/1967)	Principal Investigator	no	70	Senior Researcher, Department of Diagnostic Research and Technological Innovation, IRCCS - Regina Elena National Cancer Institute
Angelina Pranteda (20/07/1992)	Internal Collaborator	no	80	PhD student, Department of Diagnostic Research and Technological Innovation, IRCCS - Regina Elena National Cancer Institute
Valentina Piastra (05/03/1993)	Internal Collaborator	YES	100	Graduate Student, Department of Diagnostic Research and Technological Innovation, IRCCS - Regina Elena National Cancer Institute
Edoardo Pescarmona (21/11/1958)	Internal Collaborator	no	30	Director of the Department of Pathology of the IRCCS

				Regina Elena National Cancer Institute
Livio Trusolino	External Collaborator	no	30	Full professor, Department of Oncology, University of Turin

DESCRIPTION OF THE WORK FOR EVERY UNIT OF PERSONNEL

1. **Dr. Gianluca Bossi:** Principal Investigator. Coordination throughout the project (TASK1-2). (man/year effort 70%).
   1- Generation of inducible MKK3 silencing in PDOs (Task 1A);
   2- Assess the MKK3 depletion effects in PDX (Task 1A);
   3- In vivo assessment of MKDMCs efficacies (Task 2A);
   4- In vivo assessment of MKDMCs antitumor efficacy in PDX (TASK 2B).
2. **Dr. Angelina Pranteda,** Internal collaborator, PhD student, (man/year effort 80%).
   1- Assess the MKK3 depletion effects in PDOs (Task 1A);
   2- Validate AT-9283 and other putative MKMCs in PDOs (Task 2A);
   3- Biochemical and molecular analysis of MKK3 targeting in PDX (TASK1B)
   4- Biochemical and molecular analysis of MKMCs efficacy in PDX (TASK2B)
3. **Dr. Valentina Piastra,** Internal collaborator, Graduate, (man/year effort 100%).
   1- Validate AT-9283 in PDOs (Task 2A);
   2- Assess efficacy of selected putative MKDMCs in CRC lines (Task 2A);
   3- Biochemical and molecular analysis of MKK3 targeting in PDX (TASK1B)
   4- Biochemical and molecular analysis of MKDMCs efficacy in PDX (TASK2B)
4. **Dr. Edoardo Pescarmona,** Internal collaborator (man/year effort 30%).
   As Director of the Department of Pathology of the IRCCS Regina Elena National Cancer Institute, he will provide all the required support to the project with the histopathological examination of generated PDXs generated described overall project (TASK1, TASK2)
5. **Dr. Livio Trusolino,** External collaborator (man/year effort 30%).
   He will provide the CRC PDOs and the know-how required to carry out the characterization of MKK3 targeting and the MKDMCs efficacy in vitro (PDOs) and in vivo (PDX) proposed in TASK1 and TASK2 respectively.

Budget Form /year

1.	research costs	40000 E
2.	Instruments	-
3.	Indirect costs	10000 E
4.		**Sub-total 50000 E**
5.	Overheads	4500 E *(9.0%)*
6.	Fellowships	16000 E
7.		**Total 70500 E**

Justifications

Itemized research costs

• » *Consumables and supplies:* will cover costs for culture medium, antibiotics, reagents for CRC organoids (PDO) culture (BME, B-27 SUPPLEMENT, N-2 SUPPLEMENT, N-ACETYLCYSTEINE, EGF), plastic ware, MTT-kit, antibodies for biochemical studies, protein and DNA marker sizes, pre-casting gel for WB, Taq polymerase, dNTP, Trizol, RNASE-Out, M-MLV reverse transcriptase, protein A, Real Time PCR reagents, Transfection kits, drugs, molecular biology reagents. Anesthetic, formalin/PBS 10%, reagents and antibodies for IHC staining, Balb/c athymic nude mice purchasing.

• » *Services:* will cover costs for molecular biology, primers ordering for PCR and Q-PCR, oligonucleotides, shRNA cloning, and Stealth siRNA.

• » *Maintenance contracts:* will cover costs for animal facility and animal care services at the

Animal Technology Station of the University of Rome "Tor Vergata, partially supported by Institution;
• » *Publication costs:* Will cover costs for n.2 publications of results that will be achieved.
Indirect costs. Will cover costs for core-facilities personnel required for IHC analyses of tumors generated from PDXs.
Overheads. Will cover costs for 9,0% of the Overheads.
Fellowship. Salary for Dr. Valentina Piastra involved in the overall project (16000,00 euros per year).

EXISTING/PENDING SUPPORT N/A

SUGGESTED REVIEWERS (MAX 3)

- **Ana Cuenda,** Department of Immunology and Oncology, Centro Nacional de Biotecnología/CSIC, Madrid, Spain, acuenda@cnb.csic.es;
- **René Bernards,** National Cancer Institute (NKI), Amsterdam, NL, r.bernards@nki.nl
- **Rodrigo Dienstmann,** Vall d'Hebron Institute of Oncology (VHIO), Barcelona, ES, rdienstmann@vhio.net

BIOETHICAL REQUIREMENT

1. Human experimentation **NOT** – please provide clearance from the competent ethical committee as addendum A

2. Animal experimentation **YES** – please include a statement as addendum B specifying which regulations the proposed research meets

Declaration
I shall confirm to the Declaration of Helsinki in its latest version.
I shall also apply the Bioethics Convention of the Council of Europe.
In implementing the proposed research, I shall adhere most strictly to all existing ethical and safety provisions applicable.
Before start of the research, I shall obtain clearance from the competent ethical committee in case of involvement of human subjects in the research and /or in case of other ethical implications.
I shall conform with all regulations protecting the animals used for research purpose.

Date: **10/02/2020** Name of PI **Gianluca Bossi** signature

Principal investigator's signature

Authorized Administrative Official's signature.....

Date, 11/02/2020

DIREZIONE GENERALE I.F.O. ROMA

Si autorizza al trattamento dei dati ai sensi del d.lgs. 196/2003