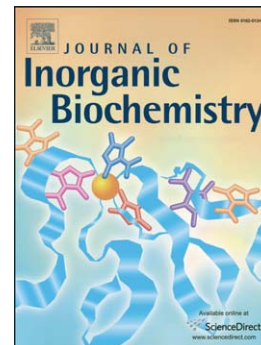# Accepted Manuscript

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Influence of Components of Tumour Microenvironment on the Response of HCT-116 Colorectal Cancer to the Ruthenium-based Drug NAMI-A

Alberta Bergamo[a], Chiara Pelillo[a], Angela Chambery[b], Gianni Sava[a,c]

[a]Callerio Foundation Onlus, via A. Fleming 22-31, 34127 Trieste, Italy

[b]Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, Second University of Naples, via Vivaldi 43, 81100 Caserta, Italy

[c]Department of Life Sciences, University of Trieste, via A. Fleming 22, 34127 Trieste, Italy.

Corresponding author:

Alberta Bergamo
Callerio Foundation Onlus
Via A. Fleming 22
34127 Trieste, Italy
e-mail: a.bergamo@callerio.org
tel: +390405588639

Abstract

Solid tumours are constituted of tumour cells, healthy cells recruited from the host tissues and soluble factors released by both these cell types. The present investigation examines the capacity of co-cultures between the HCEC colon epithelial cells and the HCT-116 colorectal cancer cells (mimicking the primary site of tumour growth) and between IHH hepatocytes and the HCT-116 colorectal cancer cells (metastatic site) to influence the effects of NAMI-A (imidazolium *trans*-imidazoledimethylsulphoxidetetrachloro ruthenate) on the tumour cells themselves. The growth of HCT-116 cells is significantly influenced when the cancer cells are sown on a monolayer of HCEC. The release of soluble factors by the healthy cells promotes, in HCT-116 colorectal cancer cells, the transcription of genes involved in growth, invasion and migration. NAMI-A is not cytotoxic to HCT-116 cells grown on plastics or co-cultured with HCEC or IHH cells, and maintains its ability to control the cell pseudo-metastatic ability, mimicked by the migration in the scratch test. The effects of NAMI-A on HCT-116 migration are supported by its inhibition of the transcription of the ABL-2, ATF-3 and RND-1 genes. In conclusion the study highlights the need of test systems more complex than a single cancer cell culture to study an anticancer drug *in vitro* and reinforces the hypothesis that NAMI-A targets the ability of the cancer cell to interact with the tumour microenvironment and with the signals that support its metastatic ability.

Key words: colorectal cancer, microenvironment, ruthenium, metastasis.

1. INTRODUCTION

In the last 15 years, the search for anti-tumour drugs has been largely shifted from direct targeting of the cell replicative mechanism to the overexpressed molecules to which is attributed the responsibility for the tumour malignancy [1]. Similarly, the research for new chemical entities has been oriented to match with this new fashion with a series of novel compounds to which it has often been attributed the adjective of "biological" drugs because of their similitude with endogenous compounds [2]. This strategy of anti-tumour chemotherapy has produced monoclonal antibodies against receptors overexpressed by cancer cells and small molecules, mainly, but not only, inhibitors of tyrosine kinases representing the pathways responsible for the malignancy of the tumour cells [3-5]. This new approach accounts for certain improvements of the anticancer chemotherapy [6], but the initial enthusiasm has been reduced because of the onset of tumour resistance and because of the tumour cell heterogeneity with often only few cells in a tumour that have responded to therapy [7-10].

In the area of cancer chemotherapy, the role of metal-based drugs is still occupied by the few platinum-based drugs that have been worldwide (cisplatin, carboplatin and oxaliplatin) or locally (nedaplatin, lobaplatin and heptaplatin) approved for use in man [11]. These drugs, although acting on tumour cells with the so-called "conventional mechanism", because their efficacy is ascribed to their ability to bind to DNA [12,13], still offer a good reference for the pharmacological approach to numerous malignancies and are included in the therapeutic schemes of some important neoplasms such as non-small cell lung cancer (NSCLC) [14]. Recent studies open

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the hypothesis that also the activity of platinum drugs may depend on the characteristic pathways of the tumour cells, and in this way it can be explained the relative sensitivity of certain tumours [15-21]. However, tumour growth in the primary and metastatic sites depends also from the relationships that cancer cells hire with the healthy cells surrounding the tumour mass. The presence of a number of healthy cells in the tumour mass is believed to help the growth of the tumour [22,23], and is suspected it can interfere with the activity of the anti-tumour drugs [24-27]. This interference may be even greater with the "targeted drugs" for which any external influence on the expression/activity of the pathways of malignancy may represent an obstacle to their efficacy.

Among the metal-based drugs, the imidazolium *trans*-imidazoledimethylsulphoxide-tetrachloro ruthenate (NAMI-A) has shown important differences of the mechanism of anti-tumour action. It shows only poor capacity to bind to DNA [28], also because of its poor ability to get into the cells [29] while causing a pronounced reduction of the metastatic capacity of the tumours [30-32]. The selectivity of NAMI-A for the tumour metastases has been attributed to its ability to control the angiogenic potential of the tumour [33,34], and to control the integrin-dependent interaction of the tumour with its growth microenvironment [35-37].

With the present investigation we have studied the capacity of the healthy cells surrounding the colorectal cancer cells, in the primary site or in the liver metastases, to influence the effects of NAMI-A on the tumour cells themselves. The study has been therefore carried out with co-cultures mimicking the primary site of tumour growth (co-culture between the HCEC epithelial cells and the HCT-116 colorectal

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cancer cells) and of the liver metastatic site (co-culture between the IHH hepatocytes

and the HCT-116 colorectal cancer cells).

2. MATERIALS AND METHODS

2.1 CELL CULTURES

The human adenocarcinoma colon cancer cell line HCT-116 was kindly supplied by the group of Dr. C. Gaiddon, University of Strasbourg, France. The cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM, Euroclone Ltd., UK) supplemented with 10% foetal bovine serum (FBS, Gibco-Invitrogen Corp., UK), 2 mM L-glutamine (Euroclone), 100 IU/ml and 100 μg/ml streptomycin solution (Euroclone).

The human immortalized hepatocytes cell line IHH was kindly supplied by the group of Prof. C. Tiribelli, CSF, University of Trieste, Italy. The cell line was cultured in 1:1 DMEM/HAM's F12 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin solution, 10^{-4} M dexamethasone (Sigma-Aldrich, MO, USA), 10^{-8} M pancreatic bovine insulin (Sigma-Aldrich), 1 M Hepes Buffer (Sigma-Aldrich).

The human colon epithelial cell line HCEC was kindly supplied by Dr. P. Steinberg, Institute for Food Toxicology and Analytical Chemistry, University of Veterinary Medicine Hannover, Germany. The cell line was cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin solution, 1 mM sodium pyruvate (Sigma-Aldrich), 0.5 mM ethanolamine (Sigma-Aldrich), 0.5 μM o-phosphoethanolamine (Sigma-Aldrich), 0.3% bovine serum albumin (BSA, Sigma-Aldrich), 20 mM Hepes Buffer (Sigma-Aldrich).

The cell lines were grown in an incubator with 5% CO_2 and 100% relative humidity at 37°C. Once 90% of confluence was reached, the cell lines were detached with

trypsin/EDTA solution (0.05% trypsin, 0.02% EDTA in Phosphate Buffered Saline PBS, Sigma-Aldrich). Cell viability was determined by the trypan blue dye exclusion test.

2.2 IHH AND HCEC CONDITIONED MEDIUM PREPARATION

The IHH conditioned medium (IHH-CM) was obtained culturing IHH cells at approximately 70-80% of confluence in their standard medium for 24 h, afterwards it was collected and stored at -20°C until use. The same procedure was followed to prepare the HCEC conditioned medium (HCEC-CM).

2.3 CO-CULTURE MODEL

The co-cultures have been set up by growing the HCT-116 colorectal cancer cells on a monolayer of epithelial colon cells HCEC, or of hepatocytes IHH. With a view to set up a co-culture with the three cell line together, in this experimental setting cells were grown in the complete medium formulated for the IHH cell line, the most demanding cells among the three cell lines used. In each co-culture the two cell lines were stained with different fluorescent dyes. The two cell lines that must constitute the layer on which to grow the HCT-116 colorectal cancer cells, i.e. HCEC and IHH, were stained with Calcein AM (Molecular Probes, Invitrogen, Paisley, UK). Briefly, cells were detached, sown in complete medium in a 96-well plate ($1x10^4$/well) and left adhere for 24 h at 37°C and 5% CO_2. Then, the culture medium was substituted with a solution of 15 μM Calcein AM, dissolved in complete medium, for 30 min at 37°C and 5% CO_2. After a careful wash fresh culture medium was added. Meanwhile, HCT-116 cells were detached, re-suspended in PBS containing Fast DiI (1,1'-

dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) for 15 min at 37°C and 5% CO_2. At the end of the incubation time, cell suspension was centrifuged, carefully washed to eliminate the excess of the dye, re-suspended in complete medium, and sown ($1x10^4$/well) over the monolayer of HCEC or of IHH cells. After 24 h cells were treated with 100 μM NAMI-A, diluted in complete medium starting from a 10^{-2} M mother solution in sterile water, for 72 h at 37°C and 5% CO_2. At the end, the medium containing the ruthenium compound was removed, the cells carefully washed with PBS, and 100 μl of fresh PBS added to each well. Fluorescence was then read with a fluorimeter FluoroCount™ Packard® Bell at λex = 485 nm and λem = 520 nm for Calcein AM, λex = 544 nm and λem = 580 nm for Fast DiI.

2.4 GENE EXPRESSION BY qRT-PCR

The differential expression of ABL-2, ATF-3, NR4A1, RND-1, SIK-1, and STK-11 genes in HCT-116 cells grown in HCEC-CM and in IHH-CM was analysed by real time reverse-transcription PCR (qRT-PCR). Cells ($4x10^5$) were sown in 25 cm^2 flasks, on reaching 90% of confluence, cells were exposed for 24 h to HCEC-CM or to IHH-CM, as controls the same media, but non conditioned, were used (HCEC-M and IHH-M). The method was similarly applied to measure the expression of the same genes after treatment of HCT-116 cells with NAMI-A. In this case, cells at 90% of confluence were treated with 10 μM NAMI-A, dissolved in complete medium, for 1 h or for 24 h.

For both experimental settings at the end of the incubation time cells were detached and re-suspended in TRIzol® (Invitrogen™). Total RNA was isolated following the phenol/chloroform extraction procedure. RNA was reverse-transcribed using qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's protocol. Real time PCR analyses were conducted on a CFX system mounted on a C1000 thermal cycler (Bio-Rad, Hercules, CA, USA) using SsoAdvanced™ SYBR Green® Supermix (Bio-Rad) following manufacturer's protocol. The following thermal profile was used: an initial 30 sec denaturation step at 95°C, followed by 40 cycles at 95°C for 15 sec and at 55°C for 30 sec. Amplification products were analysed using a 65°C/95°C melting curve. The MIT Prime3 online protocol was used to design the primer sequences reported in Table 1. Primers were designed to avoid the non-specific amplification of other human transcripts by an *in silico* analysis via BLASTn against the annotated human genome (Ensambl release 68) [38]. Primer specificity was further assessed by a melting curve analysis of RT-PCR products. Primers were purchased from Invitrogen™. Gene expression levels were calculated using the comparative ΔΔCt method and normalized using the RPL5 gene as the internal control [39]. The results are provided as the mean and standard deviation of three technical replicates.

Table 1. Primers used for qRT-PCR

Gene	Forward	Reverse
ABL-2	5'-CTGTAATGACGACGGTGGTG-3'	5'-ACTGGCTGTGGGTTTACCTG-3'
ATF-3	5'-GTGGTACCCAGGCTTTAGCA-3'	5'-ACAGTAGCCAGCGTCCTTGT-3'
NR4A1	5'-AGTGCAGAAAAACGCCAAGT-3'	5'-CGGACAACTTCCTTCACCAT-3'
RND-1	5'AGCGACTCGGATGCAGTATT-3'	5'-GTCTGTCTTGCAGCCAATGA-3'
SIK-1	5'-TCCCCTGAAATCAAAACCTG-3'	5'-TGAAGTCACCTCTGCCCTCT-3'
STK-11	5'-GAGCTGATGTCGGTGGGTAT-3'	5'-CTTCACCTTGCCGTAAGAGC-3'

2.5 WOUND HEALING ASSAY

The assay was conducted according to the protocol described by Liang et al. [40]. Briefly, HCT-116 cells were sown in complete medium in 24-well plates (4×10^4 cells/well) and grown at 37°C and 5% CO_2 until 90% of confluence. Cells were then serum starved and incubated for 24 h in a medium (HCT-116-M-FBS+BSA) containing 0.1% Bovine Serum Albumin (BSA, Sigma-Aldrich). The next day cells were treated with the medium conditioned by the hepatocytes IHH (IHH-CM), with vascular endothelial growth factor (VEGF 0.9 ng/ml, 1.8 ng/ml, 3.6 ng/ml, Merck Millipore, Temecula, CA, USA) and/or with monocyte chemoattractant protein 1 (MCP-1 0.07 ng/ml, 0.7 ng/ml, 7 ng/ml, Invitrogen™, Life Technologies). Solutions of VEGF and MCP-1 were prepared in HCT-116-M-FBS+BSA starting from a mother solution 9 µg/ml VEGF in 0.1% trifluoroacetic acid, and 25 µg/ml MCP-1 in sterile water. The effects of the ruthenium compound were studied by combining IHH-CM, VEGF and MCP-1 treatment with a contemporary treatment with 1, 10 and 100 µM NAMI-A. Using a yellow pipette tip a straight scratch was made in the cell monolayer. Cells were then incubated for 48 h at 37°C and 5% CO_2. Treatment solutions were removed and cells fixed with 1.1% glutaraldeyde for 15 min. After a careful wash the plates were air-died and cells observed at light microscopy equipped with a Leica DC300 acquisition system.

2.6 TARGETED SECRETOME ANALYSIS OF THE IHH-CM PROFILING BY MULTIPLEXED IMMUNOASSAY

The targeted quantitative analysis of secreted cytokines and chemokines in IHH-CM was performed by using the Bio-Plex multiplex system (Bio-Rad, Milan, Italy) based on xMAP technology that makes use of magnetic beads coated with specific antibodies raised against target analytes. Microspheres are internally labeled with red and infrared fluorophores. Each bead is bound to a specific antibody, thus allowing the simultaneous detection of multiple analytes within one sample. Following reaction of coupled beads with target analytes within IHH-CM, a biotinylated antibody is added for the detection, which is then finalized by adding phycoerytrin-conjugated streptavidin. All steps were performed according to manufacturer's instructions. IHH-CM was analyzed with this technology determining simultaneously the concentration of interleukin IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, IP10, Eotaxin, Granulocyte-colony stimulating factor (G-CSF), Granulocyte-macrophage colony stimulating factor (GM-CSF), Interferon gamma (IFN)γ, Monocyte chemoattractant protein 1 (MCP-1/CCL2), Macrophage inflammatory protein 1-alpha and beta (MIP-1α and MIP-1β), RANTES, Tumor necrosis factor alpha (TNFα), Platelet-derived growth factor-BB (PDGF-BB), Vascular endothelial growth factor (VEGF), Basic fibroblast growth factor (bFGF). Data were acquired using a Bio-Plex MAGPIX Multiplex Reader system equipped with a Bio-Plex Manager software v 6.1 (BioRad). All washing steps were performed on the Bio-Plex magnetic wash station (BioRad). Measurements were performed on IHH-CM samples (50 µL) using the Bio-Plex Pro Human Cytokine 27-plex assay kit (Cat. N° M50-0KCAF0Y, BioRad) according to the manufacturer's protocol. An aliquot of DMEM/HAM's F12 medium (50 µL) was also analysed as negative control. Standard curves optimization and the calculation of analyte concentrations were performed by

using the Bio-Plex Manager software. Data were expressed as mean +/- standard deviation (SD). A two-tailed t-test was used to test the significance between the IHH-CM and the negative control medium by using the GraphPad Prism software v 5.0 (La Jolla, CA USA). Expression data were also imported into the excel software for further analyses.

2.7 STATISTICAL ANALYSIS

Results are processed using InstatGraph3 software and presented as mean $\pm$ S.D.. The group means are compared using a two-Way Analysis of Variance (ANOVA) followed by t-test, or Tukey-Kramer post-test and are considered significant when $p<0.05$.

3. RESULTS

3.1 INFLUENCE OF THE CO-CULTURE WITH EPITHELIAL COLON CELLS (HCEC) AND WITH HEPATOCYTES (IHH) ON THE HCT-116 CELL RESPONSE TO NAMI-A

HCT-116 colorectal cancer cells, cultured on a monolayer of epithelial colon cells (HCEC), gain a significant growth advantage compared to the same cells grown directly on plastics ($p < 0.01$) (Figure 1). Conversely, this effect is not noticed if the monolayer beneath HCT-116 is made up of IHH hepatocytes. The treatment of HCT-116 colorectal cells grown on plastics with 100 μM NAMI-A does not cause a reduction of cell viability, whereas the response of HCT-116 cells to NAMI-A, in a condition of co-culture, depends on the nature of the second cell line. The presence of the IHH hepatocytes confers to the HCT-116 cells a more marked resistance to the ruthenium compound, giving a significant growth advantage compared to the same cells treated with NAMI-A on plastics ($p < 0.05$). The co-culture with HCEC epithelial colon cells does not affect the HCT-116 cell response to NAMI-A (Figure 1).

3.2 NAMI-A TREATMENT CONTRASTS THE INCREASE OF HCT-116 GENE EXPRESSION INDUCED BY HCEC-CM AND IHH-CM

The exposure of HCT-116 cells to the medium conditioned with either the epithelial colon cells (HCEC-CM) and the hepatocytes (IHH-CM) increases the expression of genes involved in processes of invasion and metastasis, and cellular growth in comparison to the same cells kept in the respective non-conditioned media (Figure 2A). HCEC-CM causes a meaningful increase of all the tested genes, particularly relevant for RND-1 and SIK-1. The soluble factors secreted by IHH hepatocytes in

the IHH-CM lead to a similar increase of gene expression, although quantitatively less marked than that induced with the HCEC-CM. Contrariwise, NAMI-A treatment leaves unchanged or even reduces the expression of the same genes (Figure 2B). The reduction is more marked and significant after a prolonged treatment of 24 h of the cells with the ruthenium compound.

3.3 NAMI-A PARTIALLY SUPPRESSES HCT-116 CELL MIGRATION STIMULATED BY THE IHH-CM

The soluble factors secreted in the medium by the IHH hepatocytes induce the HCT-116 cells to migrate from the edge of the wound and to partially cover the artificially drawn groove (Figure 3, right picture). In the same time frame (48 h) the control cells exposed to the non-conditioned IHH medium (Figure 3 middle panel) show a modest migration in comparison to the control at time=0 (Figure 3, left panel), immediately after having drawn the furrow.

The treatment of HCT-116 cells with 1 and 10 μM NAMI-A slowed-dawn the migration of colorectal cancer cells grown with the IHH medium (Figure 4, left panels). NAMI-A is less effective if cell migration is stimulated with the same medium conditioned by the hepatocytes (IHH-CM) (Figure 4, right panels). Interestingly, in this latter case, NAMI-A is more active at the lower dose tested (1 μM).

3.4 THE COMPOSITION OF THE IHH SECRETOME

To investigate the profile of cytokines and growth factors secreted by the IHH hepatocytes, a multiplexed immunoassay-based platform was used. Unconditioned media were in parallel analysed as a reference sample for comparative purposes.

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We found that several soluble factors, including both pro-inflammatory and anti-inflammatory cytokines, chemokines and growth factors, are highly enriched in the IHH conditioned media (Table 2). In particular, we focused our attention on the MCP-1 chemokine and the VEGF growth factor for which the highest concentrations are measured (700 and 1600 pg/ml, respectively).

3.5 NAMI-A CURBS THE MIGRATION OF HCT-116 CELLS INDUCED BY VEGF AND/OR MCP-1

The addition of increasing concentrations of VEGF and MCP-1 alone, or of their combination, to cultures of HCT-116 colorectal cancer cells increases the migration ability of these cells, especially when MCP-1 and VEGF are used at their highest concentrations (Figure 5). The treatment of HCT-116 cells with 1 and 100 μM NAMI-A reduces the migration induced by VEGF and MCP-1, either after the use of each substance alone or with their simultaneous presence (Figure 6).

4. DISCUSSION

The new innovative approaches to cancer chemotherapy, besides the direct targeting of the cancer cell, take into consideration also the tumour microenvironment where the neoplasm is growing [26,41]. This new view implies a role for the microenvironment for either the growth of the tumour and for the response of the tumour cells to the anticancer therapies [23,25,27,42]. In short, it might be said that tumour cells recruit host cells and soluble factors to help themselves to grow and to be protected against chemicals used for their destruction. Then, it is not surprising that many targeted therapies are only marginally effective or rapidly become ineffective even when they are expected to be highly cytotoxic to cancer cells because of the selective targeting of a pathway crucial to cell survival [24,43,44]. Recent evidences with the platinum drugs suggest that also the metal-based chemicals are sensitive to the microenvironment of tumour growth [45,46]. Platinum drugs are the references for the bioinorganic chemistry in the development of novel compounds and among these the ruthenium-based drug NAMI-A has shown the most innovative pharmacological properties, consisting of the selective targeting of metastases [30-32]. The actual knowledge suggests that the targets of NAMI-A are cell components such as the cell surface receptors responsible for the processes of invasion and migration [35-37].

The present study adds new information on this peculiar mechanism of action and points out that host cells and their components, released in the microenvironment of tumour growth, may influence the efficacy of NAMI-A to control cancer invasion and migration.

The HCT-116 colon carcinoma cells grow faster when they are sown on a monolayer of the healthy counterpart HCEC colon epithelial cells, suggesting that the use of simple monolayers of cancer cells is only partially representative of cancer growth. Similarly, the pharmacological response to drugs tested on simple monolayers or on complex environments, constituted of tumour microenvironment cells and their soluble factors, gives substantially different results [47-49]. NAMI-A has never shown direct cytotoxicity towards cells cultured *in vitro* and also in the present study its cytotoxicity for the HCT-116 cells is null. The same result is obtained when the HCT-116 cells are grown over a monolayer of healthy colon epithelial cells. When the same study is done with the HCT-116 cells grown over a monolayer of healthy hepatocytes (mimicking the metastatic site), the cancer cells become even less sensitive to the potential cytotoxic effects of NAMI-A. It should be noted that the pharmacological effects of NAMI-A on cancer cells *in vitro* have always been much more evident on the invasion and migration processes than on cell viability [50,51], and these properties are confirmed also in the present study. IHH hepatocytes stimulate the adhesion of HCT-116 cells to fibronectin, an important extracellular matrix component of the liver, suggesting that this mechanism could help the formation of colorectal cancer liver metastases on which NAMI-A is active [52]. Here we show how IHH hepatocytes also stimulate the migration of HTC-116 cells. IHH cells release a number of soluble factors in the medium in which they grow, including both pro-inflammatory and anti-inflammatory cytokines, chemokines and growth factors. Among these factors, the highest concentrations were determined for MCP-1 and VEGF. Both these factors are known to be involved in cancer growth and dissemination [53-55]. Accordingly, we found that both the single and simultaneous

addition of VEGF and MCP-1 to cultures of HCT-116 cells stimulates their migration ability. Interestingly, we also found that the treatment of HCT-116 cells with NAMI-A reduces the migration induced by VEGF and MCP-1, either used alone or in combination. These findings open new perspectives for understanding the NAMI-A peculiar properties of selective targeting of metastases by affecting invasion and dissemination processes mediated by growth factors and chemokines. When the whole "secretome" is used to induce HCT-116 cell migration, the effects of NAMI-A are significantly reduced, suggesting that the presence of an array of soluble factors released by the IHH hepatocytes synergically and negatively influence the anti-metastatic effects of the ruthenium-based drug. Similarly to past experiences, also in this experiment the highest activity of NAMI-A is evident at the lowest dosage tested [37]. This concentration-response trend is typical of the "enzyme-substrate" and/or "antigen-antibody" interactions and suggests the action of NAMI-A is the result of a pharmacological interaction with a specific target (likely an integrin) rather than the merely consequence of a non-selective cytotoxicity.

These results support the role of the microenvironment, including cells and soluble factors, in the modulation of the pharmacological response of cancer cells exposed to NAMI-A. This modulation partially reduces the efficacy of NAMI-A even though the overall effect of the drug is still measurable. Nevertheless, the results of the present study stress the role of the type of model, and of its complexity, used to test the pharmacological effect of a drug *in vitro*. The fact that NAMI-A, for which the weak effects on cell cultures *in vitro* are known, is significantly influenced by the tumour complexity may suggest why many drugs fail *in vivo* although they showed good activity on simple cancer cell cultures *in vitro*.

The relationship between cancer cells and healthy cells of the microenvironment of tumour growth (whether primary or metastatic site) is also supported by the effects of the media conditioned by HCEC or by IHH cells on the expression of genes of the colorectal cancer cells. The transcription of genes such as ATF-3 and RND-1, involved in processes of invasion and metastasis [56-58], and of NR4A1, SIK-1 and STK-11, responsible for growth processes [59,60], is significantly induced by the conditioned media obtained from cultures of HCEC or IHH cells. Interestingly, HCEC conditioned medium stimulates more relevant changes of all the tested genes except NR4A1, which expression is more potently induced by IHH conditioned medium. A plausible hypothesis about this finding relies on the non-genomic activities of NR4A1. In fact, NR4A1 contributes to the proteasomal degradation of β-catenin in colon cancer cells *in vitro* and *in vivo* [61,62]. We could expect that the greater induction of NR4A1 expression by the hepatocyte conditioned medium, respect to the medium conditioned by HCEC colon epithelial cells, favours the β-catenin degradation in the liver. The consequences of the β-catenin pathway attenuation are a reduction of cell migration and an increase of cell adhesion, thus stressing two features crucial for the setup and the consolidation of colorectal tumour metastasis. NAMI-A appears to be able to completely control the expression of the genes induced by HCEC-CM and IHH-CM in the HCT-116 cells with a result qualitatively opposite to that observed with the conditioned media. The functional consequences of the activity of NAMI-A in that scenario have been investigated *in vitro* using a device called "Plastic Mouse", where both healthy epithelial cells and hepatocytes simultaneously condition the colorectal cancer cells. It is composed of two chambers, connected through a channel permitting the circulation of tumour cells. The first chamber is constituted by a

monolayer of epithelial colon cells HCEC on which the HCT-116 colorectal cancer cells are growing. The second chamber is set up with a monolayer of IHH hepatocytes. The two chambers represent the primary tumour and the liver, respectively. The movement of fluorescence-labelled HCT-116 colorectal cancer cells from one well to the other is followed in static and dynamic conditions (i.e. with a dynamic circulation of the medium, heated by resistors, and completed by a computer assisted control of gases and medium injections). The results of this study indicate that tumour cells migrate from the "primary tumour" to the "liver metastatic site", and that they adhere to the hepatocytes. In the presence of 10 μM NAMI-A we could measure an increase of HCT-116 colorectal cancer cells remaining on the monolayer of HCEC (+53%), a decrease of the number of circulating colorectal cancer cells (-43%), and an even more pronounced decrease (-91%) of the number of HCT-116 cells adhering onto the monolayer of IHH hepatocytes in the second well [data from Callerio Foundation]. These findings correlate with the results of the studies of the effects of the treatment of cancer cells *in vitro* (and *in vivo*) prior to their implant *in vivo* in syngeneic animals to observe their growth at primary site of implant, and the metastasis formation [63,64]. In these studies, the tumour cells exposed to the ruthenium-based drug significantly loose the capacity to metastasize to the lung after subcutaneous or intramuscular implantation while maintaining their ability to grow in the primary site of tumour implantation. This is in good agreement with the results of the present paper showing the reduction by NAMI-A of the expression of genes related to the metastatic process, such as RND1, ABL-2, and ATF3. In addition, they reinforce the hypothesis that NAMI-A has a preferential

activity on the metastasis genes, leaving unaffected the tumorigenic genes involved in the proliferation of the tumour cells at their primary site of growth.

In conclusion, the present study highlights the problem of the *in vitro* evaluation of the potential anticancer activity of a new chemical, suggesting the need of the use of more complex systems involving more components in addition to the target cancer cell. Also the ruthenium-based drug NAMI-A is sensible to the characteristics of the test system *in vitro* even though, probably because of its poor if not null capacity to penetrate the cell membranes and to get into the cell [29] the overall pseudo-anti-metastatic effects are maintained. In particular, the overall study, and particularly that on the gene expression of the cells exposed to NAMI-A, supports the published data that showed its capacity to reduce the ability of the tumour cell to metastasize without any loss of its capacity to grow, reinforcing the hypothesis that this drug targets the ability of the cancer cell to positively interact with the tumour microenvironment and with the signals that support its metastatic ability.

Declaration of interest

The authors declare that they have no conflict of interest.

Authorship contributions:

The conception and design of the study: A.B., C.P., G.S.

Acquisition of data, analysis and interpretation: C.P., A.C., A.B.

Drafting the article and revising it critically for important intellectual content: A.B., G.S., A.C.

Final approval of the version to be submitted: A.B., A.C., C.P., G.S.

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6. LEGEND TO FIGURES

Figure 1. Influence of the co-culture with epithelial colon cells HCEC and with hepatocytes IHH on the HCT-116 cell response to NAMI-A. HCT-116 cells were sown on a monolayer of HCEC or of IHH and treated with 100 μM NAMI-A for 72 h. At the end the fluorescence associated to cells (HCT-116 stained with Fast DiI λex/em = 544/580, HCEC and IHH stained with Calcein AM λex/em = 485/520) was measured with a fluorimeter. Data are the variations of the percentage ratio calculated respect the relevant controls. Tukey-Kramer post-test: ** p<0.01 vs monoculture; ° p<0.05 vs NAMI-A untreated HCT-116.

Figure 2. Gene expression variation of HCT-116 exposed to HCEC-CM and IHH-CM (A), or treated with NAMI-A (B). HCT-116 cells were exposed to HCEC-CM and IHH-CM for 24 h (A), or treated with 10 μM NAMI-A for 1 h or 24 h (B). Total RNA was isolated, reverse transcripted and the levels of the genes were detected by qRT-PCR. Data are given as mean ± S.D. of the percentage ratio treated/untreated. Unpaired t-test: * p<0.05, ** p<0.01, *** p<0.001 vs relevant controls.

Figure 3. IHH-CM stimulates the migration of HCT-116 cells. HCT-116 cells were exposed to the medium conditioned by hepatocytes (IHH-CM) or not (IHH-M) for 48 h after drawing a scratch in the cell monolayer. CTRL represents the controls at time = 0, right after the drawing of the scratch.

Figure 4. NAMI-A effects on HCT-116 cell migration stimulated by IHH-CM. HCT-116 cells were treated with 1, 10, and 100 μM NAMI-A in IHH-M or IHH-CM for 48 h after drawing a scratch in the cell monolayer.

Figure 5. Modulation of VEGF, MCP-1 and of their combination of HCT-116 cell migration. HCT-116 cells were treated with 0.9, 1.8, and 3.6 ng/ml VEGF, 0.07, 0.7, and 7 ng/ml MCP-1 or with their combination for 48 h after drawing a scratch in the cell monolayer.

Figure 6. NAMI-A treatment affects the HCT-116 cell migration induced by VEGF, MCP-1 and by their combination. HCT-116 cell were treated with 1, and 100 μM NAMI-A and contemporary with 3.6 ng/ml VEGF, 0.7 ng/ml MCP-1 or with their combination for 48 h after drawing a scratch in the cell monolayer.

Table 2. Expression levels of cytokines, chemokines and growth factors detected in IHH unconditioned (IHH-M) and conditioned (IHH-CM) media

		Concentration [pg/ml]	
		IHH-M	**IHH-CM**
Pro-inflammatory cytokines	IFN-γ	-	8.50 ± 0.94***
	IL-1β	0.09 ± 0.01	0.35 ± 0.02**
	IL-9	1.50 ± 0.08	3.00 ± 0.09***
	IL-8	-	150.00 ± 9.40***
	IL-12 p70	-	78.00 ± 4.88***
	IL-5	0.06 ± 0.01	0.10 ± 0.01*
	TNF-α	2.20 ± 0.01	4.80 ± 0.56**
	IL-15	32.00 ± 1.00	48.00 ± 1.41***
	IL-17A	16.00 ± 1.00	17.00 ± 0.09
Anti-inflammatory cytokines	IL-1ra	-	9.00 ± 0.70***
	IL-4	0.05 ± 0.03	0.22 ± 0.01**
	IL-10	4.00 ± 0.01	12.00 ± 0.71***
	IL-13	0.12 ± 0.01	0.68 ± 0.05***
Cytokines with dual role	IL-6	-	56.00 ± 3.50***
	IL-2	10.50 ± 0.10	9.00 ± 0.62

Chemokines	Eotaxin	5.90 ± 0.01	4.50 ± 0.16**
	IP-10	2.30 ± 0.01	13.00 ± 0.04***
	RANTES	11.00 ± 0.10	12.00 ± 0.38**
	MIP-1α	0.34 ± 0.01	0.50 ± 0.01**
	MIP-1β	1.90 ± 0.06	2.20 ± 0.06*
	MCP-1	-	700.00 ± 18.00***
Growth factors	b-FGF	14.00 ± 1.00	20.00 ± 1.11**
	IL-7	0.01 ± 0.01	0.90 ± 0.12***
	GM-CSF	140.00 ± 3.00	160.00 ± 4.60*
	G-CSF	4.00 ± 0.50	11.00 ± 1.38**
	PDGFbb	2.40 ± 0.13	4.80 ± 0.27***
	VEGF	-	1600.00 ± 84.00***

CM was simultaneously screened for determining the cytokines concentration by interpolation on properly generated standard curves. All measurements were performed in triplicate. Data are reported as means±SD. * p-value<0.05; ** p-value<0.01; *** p-value<0.001 IHH-CM *versus* IHH-M.

Figure 1
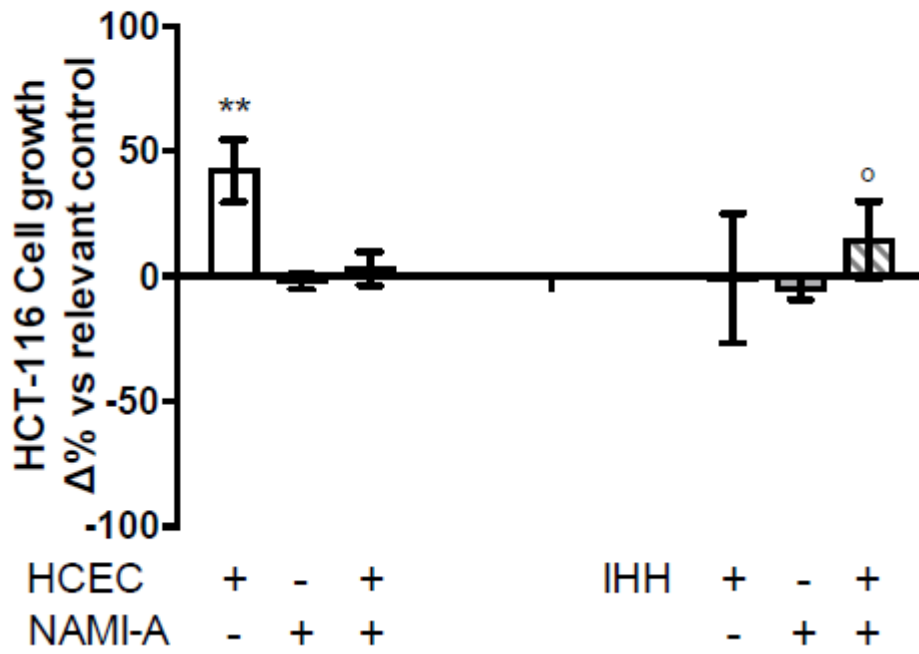
Fig. 1
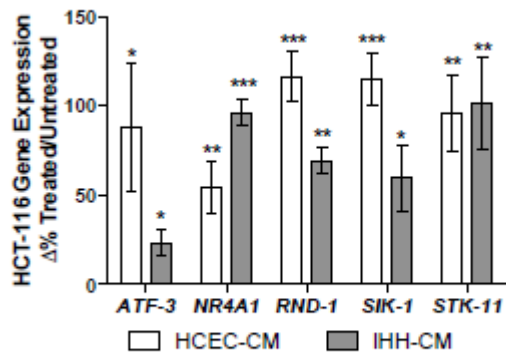
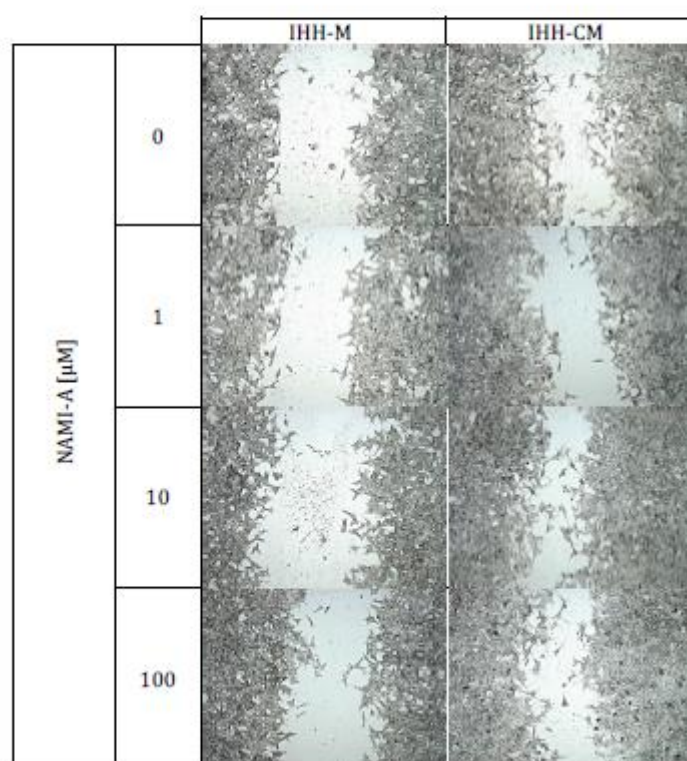**Figure 2A**

Figure 2B
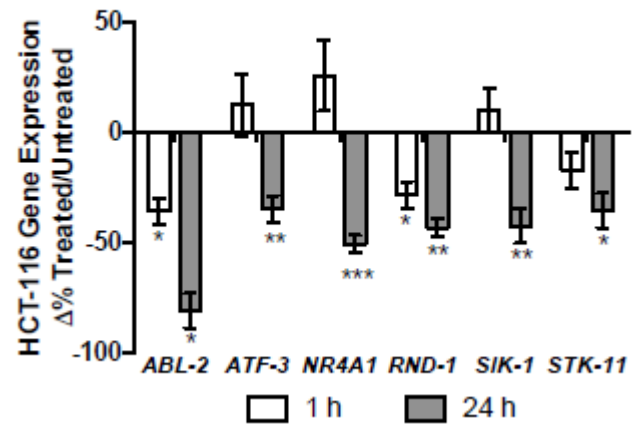
Fig. 2

Figure 3
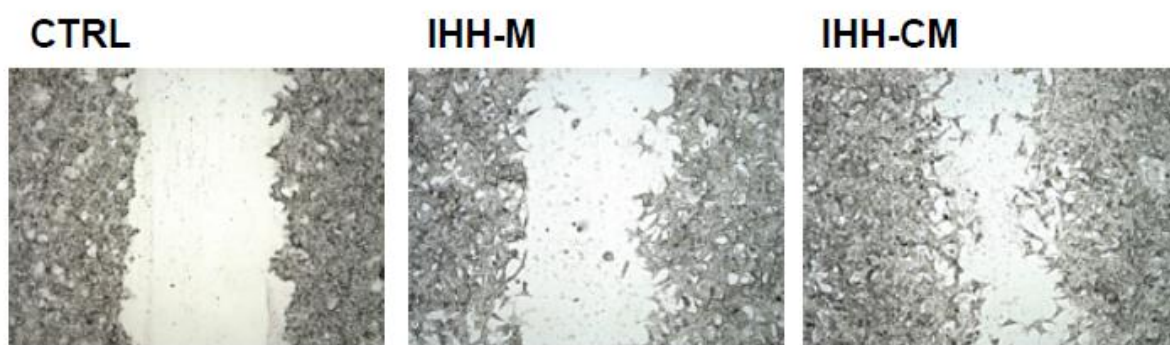
Fig. 3

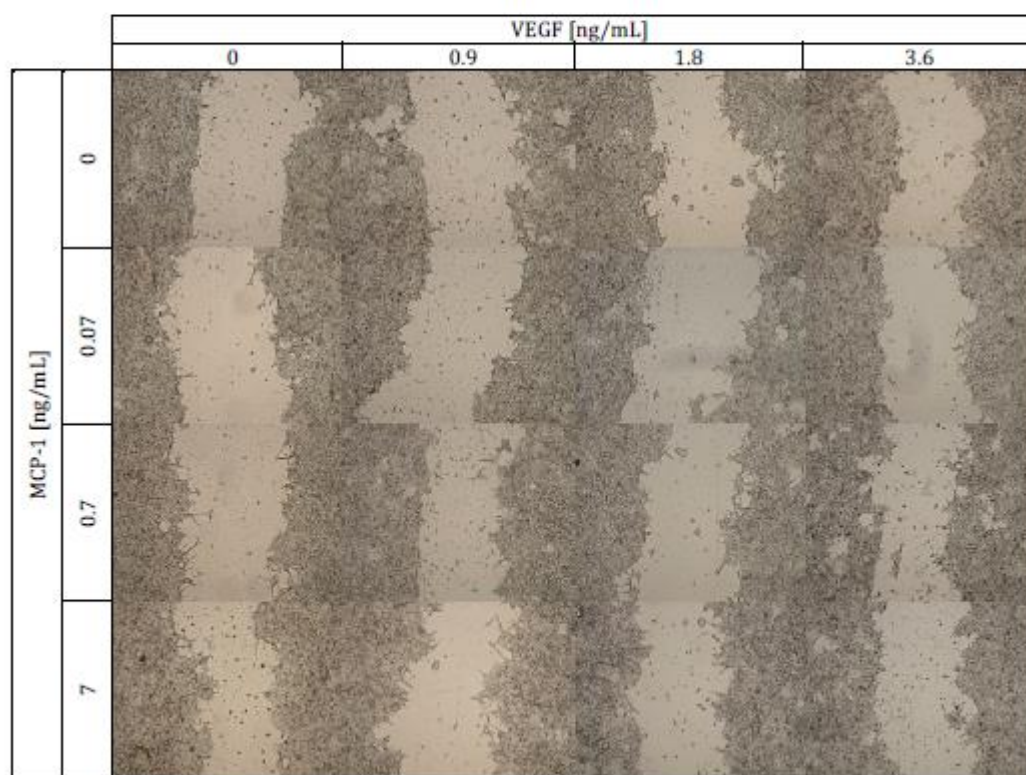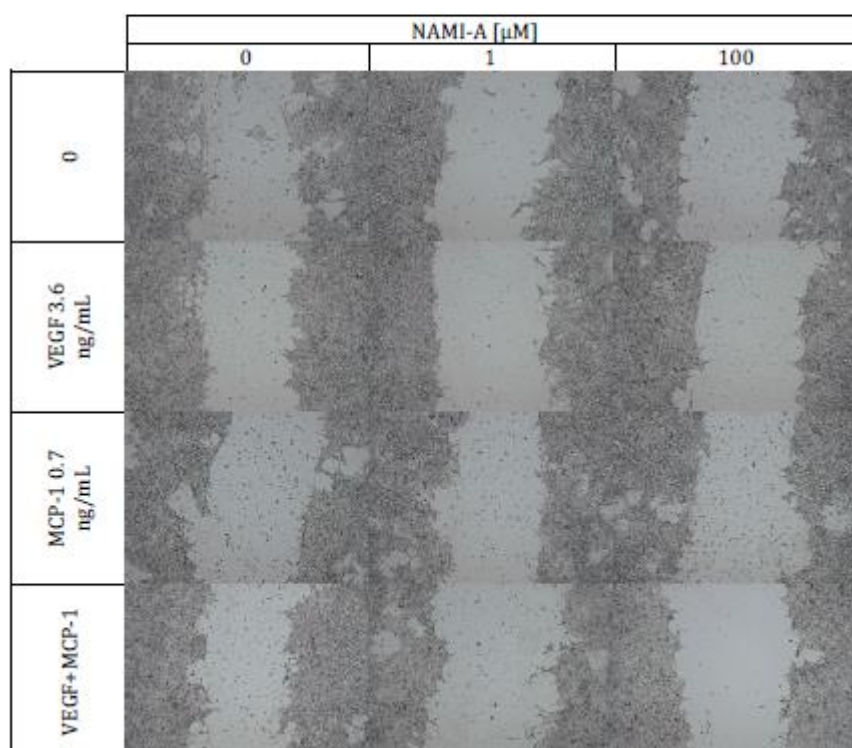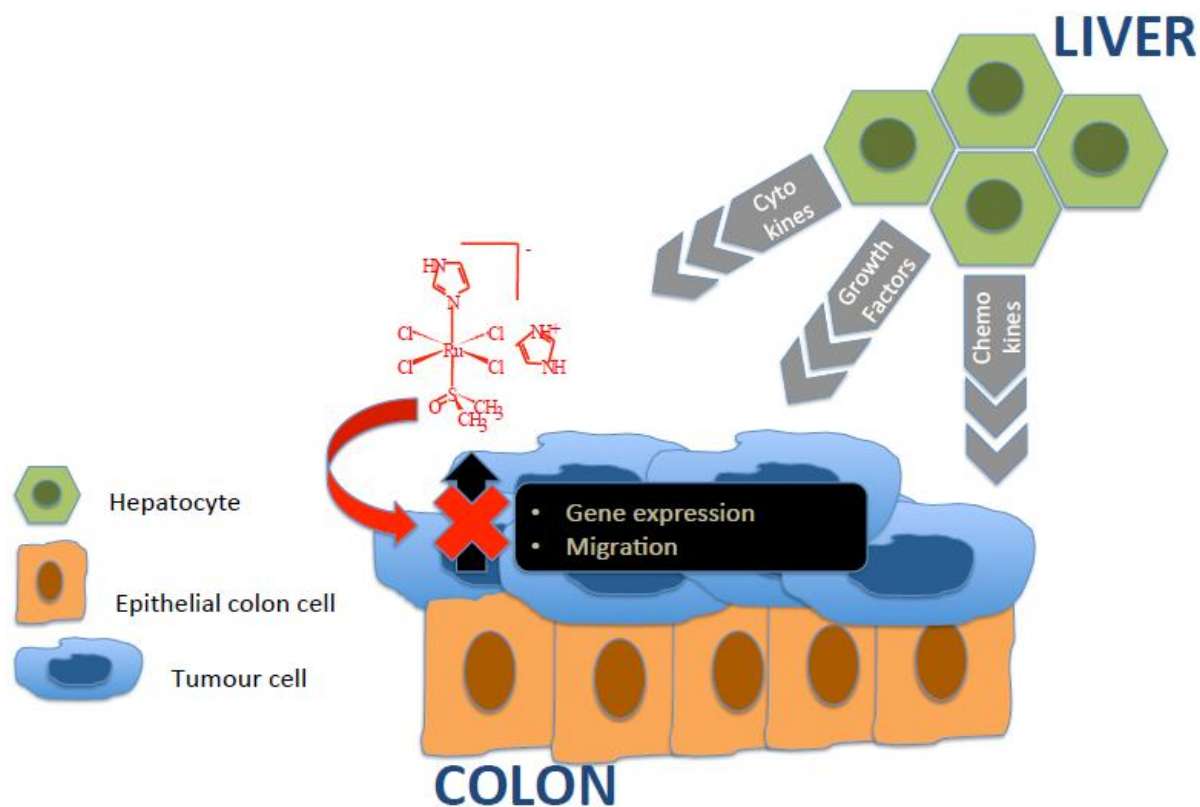Figure 4

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Figure 6

Fig. 6

Graphical abstract

Graphical abstract (synopsis)

Healthy epithelial colon cells and hepatocyte release soluble factors thus promoting the transcription of genes of the tumour cells involved in growth, invasion and migration. NAMI-A (imidazolium *trans*-imidazoledimethylsulphoxidetetrachloro ruthenate) inhibits the transcription of the same genes and the migration process itself, targeting the interactions between cancer cells and the microenvironment.

Highlights

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- Healthy epithelial colon cells influence the growth of colorectal cancer cells.
- Soluble factors from the healthy microenvironment cells modulate gene expression.
- The drug NAMI-A is imidazolium *trans*-imidazoledimethylsulfoxidetetrachloro ruthenate.
- NAMI-A counteracts the effects of the microenvironment on gene expression.
- Gene expression control by NAMI-A supports its inhibition of tumour cell migration.