Three-dimensional patient-derived breast cancer assays to study the efficacy of natural and synthetic products combined with standard chemotherapy

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1. Abstract:

Despite of remarkable advances in prevention, diagnosis and treatment, cancer prevalence is still increasing worldwide. In the last 10 years, cancer cases increased by 33%. For women, the most common and the leading cause of cancer deaths is breast cancer. Triple negative breast cancers (TNBC) are a heterogeneous group of aggressive tumours that display higher rates of relapse and shorter overall survival, compared to other breast cancer subtypes. They represent approximately 15% of all breast cancers, lack expression of both estrogen (ER) and progesterone receptors (PR) and do not exhibit amplification of the human epidermal growth factor receptor 2 (HER2) gene. Accordingly, endocrine and HER2-targeted therapies are not suitable to treat TNBC patients. In the absence of well-defined molecular targets, standard chemotherapy is extensively used to treat metastatic TNBC. However, its safety and toxicity to normal tissues remain primary concerns. Therefore, the proponent's field of expertise, the search for natural and synthetic products, with fewer side effects, that which may be used either as an alternative, or as an enhancer for chemotherapy, is of great interest and one of the biggest challenges in cancer therapy. In order to test the efficacy of these compounds, new preclinical models that incorporate heterogeneous cell populations found in human tumours are needed. Transition of cells between tumour xenografts and *in vitro* models involves direct transfer into traditional 2D tissue culture, which results in growth of a highly-selected subpopulation of cells and does not capture tumour heterogeneity and complexity. The laboratory of Dr. Anna Grabowska developed a novel 3D *ex vivo* preclinical model utilizing tumour derived ECM and incorporating patient-derived tumour-associated stromal cells which allows profiling of humanized close-to-patient xenografts at early passage. In this sense, this project proposes to investigate the treatment efficacy of natural and synthetic products associated with standard chemotherapy using the model described. Previous results obtained at Dr. Grabowska's laboratory by the principal applicant demonstrated that the compounds to be tested were efficient to inhibit 3D cell proliferation for TNBC cell lines, giving us confidence for proceeding now with further tests in PDXs. The two laboratories involved in this proposal have different, yet complementary, approaches in cancer research: while Dr. Cominetti works mostly with traditional *in vitro* 2D assays and *in vivo* syngeneic orthotopic mouse models, Dr. Grabowska uses *in vitro* and *in vivo* PDXs. Therefore, the work wherein proposed would bring these strategies together, adding confidence to the possible anticancer effects of the compounds under analysis. Results obtained may be used in a combinatory therapy leading to more effective treatment options for TNBC.

2. Goals and objectives:

This study aims to investigate the treatment efficacy of natural and synthetic products developed in the Cominetti lab, alone or associated with standard

chemotherapy, in three-dimensional (3D) patient-derived breast cancer assays using patient derived xenografts (PDXs), established in the Grabowska lab.

3. Background and justification:

In 2015, there were 17.5 million cancer cases worldwide and 8.7 million deaths. In the last 10 years, cancer cases increased by 33%. For women, the most common and the leading cause of cancer-related deaths is breast cancer [1]. Roughly, one in eight to ten women will develop breast cancer during their lifetime. However, mortality from breast cancer in developed countries has decreased, which is mostly attributable to early detection and efficient systemic therapies [2].

Triple negative breast cancer (TNBC) is a heterogeneous group of aggressive tumours that displays higher rates of relapse and shorter overall survival, compared to other breast cancer subtypes. They represent approximately 15% of all breast cancers, lack expression of both estrogen (ER) and progesterone receptors (PR) and do not exhibit amplification of the human epidermal growth factor receptor 2 (HER2) gene. Accordingly, TNBC patients do not respond to endocrine and HER2-targeted therapies (i.e. Trastuzumab) [2].

In the absence of well-defined molecular targets, standard chemotherapy is extensively used to treat metastatic TNBC and other cancer types, even though its safety and toxicity to normal tissues remain primary concerns [2]. Moreover, the development of chemoresistance is a major obstacle to the effective treatment of many tumour types, including TNBC [3]. Therefore, the search for natural and synthetic products, with fewer side effects, that may be used either as an alternative to, or as an enhancer for chemotherapy, is of great interest [4].

Among the natural products proposed to be used in this study is gingerol, derived from ginger. Ginger (*Zingiber officinale* Roscoe) is widely used worldwide as food, spice and herb and it is emerging as a novel multitarget nontoxic approach for cancer management [5]. Gingerols are the major pungent constituents found in the oleoresin from fresh ginger rhizome, where [6]-gingerol is the most abundant. Structurally, gingerols are differentiated by the length of their alkyl chains with [6]-, [8]- and [10]-gingerol having 10, 12 and 14 carbons in their unbranched alkyl chains, respectively [6].

Several studies have reported anti-tumour effects of gingerols, including anti-proliferative, pro-apoptotic and inhibition of cell migration/invasion. However, most studies have focused on [6]-gingerol due to its greater abundance and availability of purified material [7]. Less is known about the anti-tumour/anti-metastatic properties of the less abundant [10]-gingerol. Studies in colon cancer models have reported that the cytotoxic effects of [10]-gingerol are associated with induction of intracellular Ca^{2+} accumulation [8] and apoptosis, via activation of mitogen-activated protein kinases [9]. Our previous work described an efficient procedure for the isolation of [10]-gingerol by reverse phase HPLC and demonstrated its superior anti-proliferative activity against MDA-MB-231 breast cancer cells compared to [6]- and [8]-gingerols [10]. Whether [10]-gingerol exerts similar inhibitory responses in other breast cancer cells is under investigation by our research group.

Rosenberg and colleagues in 1964 were responsible for the discovery of cisplatin, that posteriorly proved to be an effective complex for the treatment of a range of cancer types, including breast cancer [11]. Despite the success of platinum-based drugs, their continued use is greatly limited by severe dose limiting side effects and intrinsic or acquired drug resistance [12]. Due to the mentioned limitations of cisplatin,

researchers have focused on the development other metal-based compounds, such as ruthenium (Ru) complexes.

There are some hypotheses to explain the low toxicity and the antitumour and antimetastatic properties of Ru compounds. Ru accumulates preferentially in the tumours rather than in normal tissues, possibly due to its ability to mimic iron binding to transferrin receptors [13, 14]. It has been proposed that tumours contain high amounts of transferrin receptors, allowing Ru complexes to be actively transported into neoplastic tissues that require higher iron requirement [15]. Once bound to the transferrin receptor, Ru would be internalized by the tumour [16]. In addition, Ru complexes could be considered pro-drugs. Ru remains in its relatively inactive Ru(III) oxidation state until it reaches the tumour site, where a lower oxygen content and higher acidity leads to the reduction to the more reactive Ru(II) [17]. This reaction causes selective tumour targeting by direct cytotoxic activity toward hypoxic tumours [18]. Furthermore, due to their slow ligand exchange kinetics, they only reach general inertness in the range of minutes to days, instead of microseconds to seconds (as per seen for other drugs), preventing rapid equilibration reactions [19]. Finally, Ru complexes have unique DNA binding patterns due to their special octahedral structure and ligand geometries [20]. These features would allow them to inhibit DNA replication, promote mutagenesis, induce SOS repair, bind to nuclear DNA and reduce RNA synthesis, which are all consistent with their reported antitumour effects [14].

Currently, one Ru complex, NKP-1339 (sodium trans-[tetrachloridobis(1H-indazole)ruthenate(III), is ongoing clinical trials. NKP-1339 has entered clinical trials as the more soluble alternative to the indazolium compound KP1019 [21] and shows promising results in solid tumours, such as non-small cell lung cancer, colorectal carcinoma, and most distinctively in gastrointestinal neuroendocrine tumours [22].

Despite the potential of new chemotherapy using natural and synthetic products, the vast majority of antitumour therapeutic agents that enter human clinical trials fails to succeed for clinical use [23], indicating that current preclinical tumour models do not represent the reality found in human disease [24]. Cell lines, grown in 2D *in vitro* culture and *in vivo* as subcutaneous tumours are the most widely used tumour paradigm in research; however, these models have been shown to be poorly predictive of clinical efficacy [25]. Xenografts of such lines typically form rapidly growing, undifferentiated tumours, but lack the architecture, complexity and biological phenotype of the tumours they are meant to represent. On the other hand, the so-called patient derived xenografts, directly established from human tumours, do resemble more accurately the tumours they came from [26-29].

Preclinical models incorporating heterogeneous cell populations found in human tumours are only just beginning to emerge [30]. In these models, the influence of paracrine factors produced by tumour-recruited stroma and immune infiltrate on disease progression is guaranteed. Particularly, heterogeneous 3D culture systems, incorporating close-to-patient cells can be generated from patient-derived xenografts (PDX) [31].

Traditionally, transition of cells from tumour xenografts to *in vitro* models involves direct transfer into 2D tissue culture, which results in growth of a highly-selected subpopulation of cells that ultimately does not capture tumour heterogeneity. The laboratory of Dr. Grabowska developed a 3D *ex vivo* assay that is consistently capable of growing tumour cells with minimal loss from low passage PDX lines, such that they are rapidly amenable to pharmacologic assay [31, 32]. This was based on a 3-D Tumour Growth Assay (3D-TGA) [33], which comprises a low stiffness laminin-rich extracellular matrix (lr-ECM) [34] to embed tumour cells admixed with stromal cells, to

provide the paracrine signaling present in the TME of solid tumours [35, 36] but aimed to further align the biochemical and biophysical properties of the assay to patient's tumour microenvironment (TME). In summary, they established a novel preclinical model utilizing tumour derived ECM and incorporating patient-derived tumour-associated stromal cells which allows profiling of humanized close-to-patient xenografts at early passage [31].

Utilizing the model described, this project aims to investigate the efficacy of natural and synthetic products, developed in the Cominetti lab which will be tested alone or in combination with standard chemotherapy. Thus, this collaboration comprises an innovative and challenging study, and the results obtained would greatly contribute to the development of more effective treatment options for TNBC.

4. Details of the project activities to be developed:

Specimens and establishment of PDXs

Fresh surgical material from tumour resections at Nottingham University Hospitals NHS Trust, will be collected with informed patient consent and National Research Ethics Service (NRES) approval. Samples of tumour tissue will be dissected, formalin-fixed and paraffin embedded (FFPE) for immunohistochemistry. A small amount of finely minced tumour tissue will be enzymatically disaggregated (as below for xenograft tissue) and plated into 6-well tissue culture plates in DMEM, 10% FBS, 2 mmol/L glucose (Sigma) to establish fibroblasts; fibroblasts will be banked at early passage and used in the 3D assay at less than passage 5. The majority of the finely minced and disaggregated tumour will be mixed with bone marrow-derived mesenchymal stem cells (MSC) ($5x10^5$/mouse) (ScienCell) and resuspended in ice-cold Matrigel (200 µL/mouse; BD Biosciences). Animal procedures will be carried out under

UK Home Office Licence (PPL 40/3559) by qualified persons holding UK Home Office Personal Licences in accordance with the 3R's framework for humane animal research. Tumour samples were grafted subcutaneously into sex-matched MF-1 nude or NOD/SCID mice (Harlan). Initial grafting will be referred to as passage 0 (P0). Upon growth, tumours will be surgically removed under anesthesia, minced, and passaged on into further donor mice (P1, with the addition of MSCs and Matrigel as above) or taken for banking and 3D-TGA assays.

Even though the take rate for breast cancer PDXs at Grabowska's lab is around 40-50%, which is higher than the general rate reported in the literature, if we don't have the chance to establish new ones (tissue availability, take rate, etc), the group has already generated a number of breast cancer PDXs, including some triple negatives, ready for us to use (Table 1). Table 1 summarizes main clinical characteristics of BC-PDXs including whether metastasis to lymph node (LN) or to other sites (Br: brain, Bo: bone, Li: liver, Lu: lung) is known to have occurred in the patient. NST: no special type, ILC: invasive lobular carcinoma, IDC: invasive ductal carcinoma, DCIS: ductal carcinoma in situ, nk: not known, +: positive, -: negative.

Table 1. Panel of established breast cancer patient-derived xenografts (BC-PDXs).

BC-PDX	Histology	Receptor status			LN status	Other mets
		ER	PR	HER2		
BR8UoN*	Ductal/NST	-	-	-	+	Br
BR11UoN	BASAL	-	-	+/-	-	nk
BR12UoN	Ductal / NST	-	-	-	-	nk
BR15UoN	Ductal / NST	-	-	+/-	+	nk
BR17UoN	Ductal / NST	+	+	+/-	+	nk
BR18UoN	nk	+	nk	-	-	nk
BR22UoN	nk	+	+	-	nk	nk
BR28UoN	nk	+	+	-	nk	nk
BR29UoN	nk	+	-	-	-	nk
BR32UoN	nk	+	+	nk	-	nk
BR33UoN	nk	+	+	-	-	nk

Disaggregation of xenograft tumours

Finely minced tumour will be disaggregated using type II collagenase (100 U/mL; Invitrogen) and dispase (2.4 U/mL; Invitrogen) in HBSS (Sigma) at 37ºC under constant rotation. Cells will be removed at 1 to 2 hourly intervals until the tumour be completely disaggregated. Cell number and viability will be determined using trypan blue exclusion and analyzed by flow cytometry for expression of EpCam.

The 3D-TGA

Cells will be resuspended in ice-cold Cultrex basement membrane extract (BME) (3 mg/mL; Trevigen) diluted in modified RPMI-1640 (Life Technologies; phenol red free with 6 mmol/L D-glucose and pH6.8) and plated at $2.5x10^4$ tumour cells ± $8.33x10^3$ patient-derived CAFs/MSCs (Sciencell) per well (100µL) into low-adherent, black-walled, clear-bottom, 96-well plates (BrandTech) pre-warmed to 37ºC. CAFs derived from patient LU6 will be used in all assays for NSCLC specimens and MSCs for all other tumour types. The desired compound ([10]-gingerol, Ru complexes, standard drug) will be serially diluted in modified RPMI-1640 and 50µL added in triplicate wells of the TGA on day 3. For the 384-well plate 3D-TGAs, a quarter of the 96-well plate cell number and volumes and six replicate wells will be used. For combinations, [10]-gingerol and doxorubicin or a Ru-complex and cisplatin will be premixed and serially diluted together before adding to the assay. Drug exposure will last 96 hours before final endpoint readings. The Alamar Blue assay [Invitrogen; 10% (v/v), 37ºC for 1 hour] will be used to monitor cell growth daily, using a fluorescent plate Reader (Flex Station II, Molecular Devices). Drug sensitivity will be calculated as a percentage of matched untreated control and IC_{50} curves were determined using GraphPad Prism 5 (GraphPad Software Inc.). Combination of drugs will be at constant

ratios to make them amenable to synergy testing using the Chou-Talalay method [37] and CalcuSyn Software (Biosoft).

Immunohistochemistry

Immunohistochemistry and hematoxylin and eosin (H&E) staining will be performed on 5-mm tissue sections of FFPE tissue using standard techniques and the manufacturer's recommendations for the following primary antibodies: anti-human E-Cadherin (DAKO Clone NCH-38) and anti-human Vimentin (DAKO, Clone V9).

In vivo *efficacy testing*

Established PDXs from donor mice will be surgically removed under anesthesia and tissue minced to passage on into mice for efficacy evaluation (passage 4–10, with the addition of MSCs and Matrigel described previously). Procedures will be carried out under UK Home Office License (PPL 70/7317). When tumours reach 150 to 200 mm^3, mice will be randomized to treatment and control groups (4 groups: vehicle, standard chemotherapy, natural/synthetic compound and combination) , and treatment will be initiated using concentrations to be determined from *in vitro* assays, or matched vehicle control. Tumour size will be measured using calipers (length and width) three times weekly, and tumour volumes will be calculated by the formula: volume = (length + $[width]^2$)/2) [31]. Power calculations, based on size of effect observed *in vitro* and known variation for individual models will be used to determine group sizes.

5. Scientific/academic gains

Scientific gains of this collaborative project, for Cominetti's group, are initially related to the opportunity of testing our compounds in patient derived xenografts and 3D-TGAs derived from them, which ultimately means a more close-to-patient experimental setting. These are new and necessary steps to add confidence to the results

obtained so far. On the other hand, access and knowledge-gain about syngeneic orthotopic models, where tumours grow in immunocompetent mice, is of great interest for Grabowska's group. Therefore, this collaborative work would also allow the exchange of experience about *in vivo* models, once we work with syngeneic orthotopic models at our laboratory, while Dr. Grabowska's lab has experience in patient derived xenografts. While PDXs consist in heterogeneous freshly isolated human tumours growing in immunocompromised mice, syngeneic models are characterized by tumours growing in their species of origin in an intact immune context both have advantages and limitations but collectively are widely accepted and maybe the two most representative cancer models *in vivo*.

The important experience already acquired by the Dr. Grabowska's laboratory, along with our knowledge regarding to the biological effects of the substances to be tested, will greatly improve the expertise of both groups. All these aspects will ultimately result in higher impact publications and or patents, which is a reflection of combined efforts from dedicated scientists with different backgrounds and complementary fields of expertise.

6. Activities to sustain collaboration

Exchange of students

We aim to establish a long-term collaboration between the two groups. Once the experiments described in this proposal were accomplished, post-graduation students could be responsible for further investigating either the underlying mechanisms by which these substances promoted anticancer effects in TNBC PDXs, or the effects in other cancer types, for example. Currently, there are three highly-motivated PhD students working on some of the compounds contemplated in this proposal that would be interested in spending up to a year at Dr. Grabowska's lab.

UK Newton Funding

Brazil is one of the countries the UK is partnering with under the Newton Fund. The Newton Fund's aim is to develop science and innovation partnerships that promote the economic development and welfare of collaborating countries. Thus, there are likely to be opportunities to apply for some of these grants and/or fellowships in the near future.

7. Expected results and performance indicators

Considering we proposed to test compounds which have already demonstrated anticancer effects in traditional 2D *in vitro* assays and syngeneic orthotopic mouse models of breast cancer, it is expected that the use of [10]-gingerol, as well as Ru complexes, alone or in combination with chemotherapeutic drugs, in the proposed 3D-TGA and PDXs models, would confirm the findings we have so far: antiproliferative, anticancer, antitumour and antimetastatic properties of the mentioned substances. The outcome of this study could be the development of a new, effective and safer therapeutic approach to treat TNBC. Similar anticancer effects in different models are highly appreciated and almost compulsory for high impact factor journals, and if successful, this grant would take some of our ongoing projects to this level.

8. Description of the infrastructure and financial resources available

Laboratory of Biology of Aging (Federal University of Sao Carlos):

Since 2010, the Laboratory of Biology of Aging (LABEN) has been dedicated to the study of the effects of natural and synthetic products on tumour cells. The lab head, Dr. Marcia Cominetti received a Young Researcher grant from FAPESP (2008/56758-0) entitled "High throughput screening of natural products with antitumour activity in culture of mammal cells". This study gave rise to the initial results obtained with [10]-

gingerol, which later resulted in a series of publications as well as a patent deposited on September/2015, entitled: “Pharmaceutical composition comprising [10]-gingerol and its use as an antitumour and antimetastatic molecule” (BR1020150240937). Currently, there are two ongoing grants in the laboratory, one from FAPESP (2015/24940-8, R$ 142.300,00, U$ 11.500,00) entitled “Evaluation of the effectiveness of structural changes of [10]-gingerol in combination with chemotherapeutic doxorubicin for the treatment of breast cancer: *in vitro* and *in vivo* studies” and another from CNPq (401506/2016-9, R$ 60.000,00) entitled “Proof of concept and pre-clinical studies using [10]-gingerol combined with doxorubicin for the treatment of triple negative breast cancer”. Additionally, our group is also part of the Thematic Project, from FAPESP (2013/00798-2, R$ 706.596,60; U$ 301.710,78) entitled “Extracellular matrix in aging, exercise and in the tumour microenvironment”.

LABEN’s infrastructure accounts with different equipment and facilities, including[1]:

1) Flow cytometer Becton Dickinson (Accuri C6), U$ 65,000.00
2) Chemoluminescent reader system Bio-Rad Chemidoc-MP U$ 30.000,00;
3) Freezer -80ºC Thermo Forma™ 88000 Series U$ 20.261,00

Other equipment available as a multiuser FAPESP project[2]:

1) Real time PCR thermocycler Bio-Rad
2) Fluorymeter SpectraMax i3 Molecular Devices
3) Slide scanner panoramic desk
4) ImageXpress Micro XLS Wide field High-Content Analysis System

[1] Only equipment over U$ 20,000.00 was described.
[2] Equipment over U$ 20,000.00 available from the Laboratory of Biochemistry and Molecular Biology, resulting from a multiuser FAPESP project (2013/00798-2).

5) TMA Tissue Microarray system
6) Dual-energy X-ray absorptiometry

<u>Division of Cancer and Stem Cells (University of Nottingham)</u>:

1) IVIS Spectrum In-vivo Imaging system
2) Centrifuge - High speed/Floor standing
3) Confocal microscope Leica SP8 2 laser confocal microscope with small animal physiology stage
4) Deconvolution microscope Nikon deconvolution microscope
5) Licor Odyssey multiwavelength fluorescent plate reader
6) Hypoxia chamber 3M / Ruskin
7) Tissue processor bench standing tissue processor
8) qPCR Viia7 qPCR machine
9) qPCR ABI 7500 Fast real time PCR system and Dell laptop
10) qPCR Mx3005P qPCR System stratgene (Agilent)
11) Plate reader and Dell desktop Light scanner HRM plate reader Idaho technology
12) Flourometer QFX-Fluorometer/Denovix
13) Cell counter Automated BIO-RAD cell counter
14) Light Cabinet/Gel image capture MultiImage
15) Fast Prep MP Fast prep
16) BMG Omega plate reader Fluorescent / Bioluminescent plate reader

Dr. Grabowska' group also have access to flow cytometry (http://www.nottingham.ac.uk/life-sciences/facilities/flow-cytometry-facility/) and Next Gen sequencing (http://www.nottingham.ac.uk/deepseq/).

The following in grants are also available:

1) NCI 60 cell-line panel and additional bank of 150 human and animal cell-lines.
2) Ethical permission and dedicated tissue culture facilities to isolate multiple cell types from patients' cancer specimens and associated normal tissue.
3) Immuno-histology laboratory with a tissue processing and image analysis unit.
4) 2- and 3-dimensional biophotonic fluorescent/luminescent imaging systems (Caliper Life Sciences, IVIS SPECTRUM)

9. Schedule of exchange missions

Proposed exchange missions are described in table 2.

Table 2. Schedule of exchange missions.

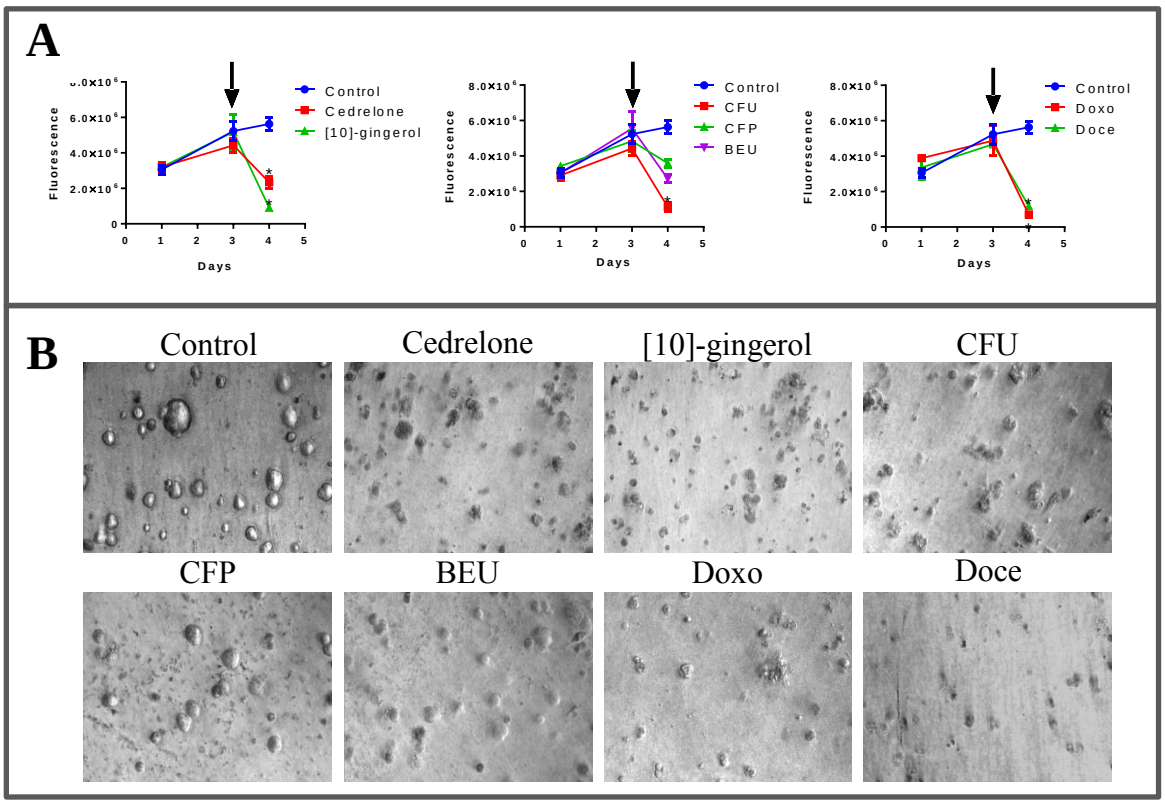
Researcher	University of destiny	Time	Proposed activities
Dr. Rebeka Tomasin	University of Nottingham	September-November/2017	Development of the experiments
Dr. Anna Grabowska	Federal University of Sao Carlos	January 2018	Review of data and planning of future work/knowledge exchange
Dr. Ana Carolina B. M. Martin	University of Nottingham	March-May/2018	Development of the experiments
Dr. Marcia Cominetti	University of Nottingham	June/2018	Results presentation, discussion, final papers review and submission

10. History of joint work

From April to July/2015, Prof. Dr. Marcia Cominetti was selected by Nottingham University for a Visiting Fellowship, when she was able to stay at Dr. Grabowska laboratory. During this time, Dr. Cominetti tested different natural and synthetic products to verify their ability to inhibit cell proliferation in a 3D *in vitro* model. A summary of the main results, indicating that [10]-gingerol, cedrelone and CFU (Ru complex) are promising substances to be taken to further investigation (3D-

TGA from PDXs and PDXs themselves), as they could inhibit TNBC cell proliferation in 3D, in the same extent as standard chemotherapy (doxorubicin and docetaxel) (Figure 1).

Figure 1. (A) Effects of the compounds tested on the proliferation of MDA-MB-231 cells co-cultured with mesenchymal stem cells, measured with Alamar Blue. **(B)** Representative images of 3D cultures after treatment visualized under an inverted microscope at 100× amplification. Arrows indicate the day on which the compounds were added to the 3D cultures. CFU, CFP and BEU are ruthenium complexes. Doxo = doxorubicin, Doce = docetaxel. Assay were repeated three times in triplicate and data are represented as mean ± SEM (*p < 0.001, 2-way ANOVA, Tukey post-test).



For these assays, MDA-MB-231 cells ($2.5x10^4$/50µL) were co-cultured with mesenchymal stem cells (3:1), counted and diluted in TGA medium. A solution of BME (Basement Membrane Extract, Cultrex®, 6mg/mL) in TGA medium was added to the cells at 4ºC. This BME solution, when on ice, is a liquid solution, and at 37°C it becomes a gel, creating the 3D environment. The mixture of cells and BME was then plated at 37°C with the aid of a plate heater and the cultures were maintained at 37°C in

a humidified incubator for 3 days to allow spheroids to be formed. Next, the desired compounds were added at a concentration five times higher than the IC_{50}, previously determined via 2D viability assays. Morphological effects thereof were evaluated by optical microscopy (Nikon Eclipse MA200) and cell proliferation was accessed using Alamar Blue (Invitrogen).

More recently, a second encounter occurred in September 2016, when Dr. Anna and other members of her laboratory came to Brazil for the meeting "IV Symposium on Oncobiology", organized by Dr. Franklin Rumjanek, at Federal University of Rio de Janeiro. We also attended the meeting and thus had the opportunity to discuss once again our results and the mutual desire to keep and consolidate the collaboration previously established. If granted, this collaborative project would seal a long and successful partnership between LABEN/UFSCar and Grabowska's lab/University of Nottingham.

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