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A high-throughput screening of a chemical compound library in ovarian cancer stem cells

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Abstract:

Background: Epithelial ovarian cancer has a poor prognosis, mostly due to its late diagnosis and to the development of drug resistance after a first platinum-based regimen. The presence of a specific population of "cancer stem cells" could be responsible of the relapse of the tumor, and of the development of resistance to therapy. For this reason, it would be important to specifically target this subpopulation of tumor cells in order to increase the response to therapy.

Method: We screened a chemical compound library assembled during the COST CM1106 action to search for compound classes active in targeting ovarian stem cells. We here report the results of the high-throughput screening assay in two ovarian cancer stem cells and the differentiated cells derived from them.

Results and conclusion: Interestingly there were compounds active only on stem cells, only on differentiated cells and compounds active on both cell populations. Even if these data need to be validated in *ad hoc* dose response cytotoxic experiments, the ongoing analysis of the compound structures will open up to mechanistic drug studies to select compounds able to improve the prognosis of ovarian cancer patients.

Keywords: Cancer stem cell, chemical compounds library, oncology screening, high-throughput screening, ovarian cancer, therapy resistance.

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1. INTRODUCTION

Epithelial ovarian carcinoma (EOC) is the sixth most common cancer in both European and North America women and the leading cause of death from gynaecological malignancies [1, 2]. The lack of effective screening tests accounts for an advanced disease diagnosis. In addition, after an initial response to chemotherapy (generally a combination of platinum salts and taxanes), patients relapse with a chemo-resistant disease. Resistance to therapy has multifactorial causes [3]. Among them, the existence of a cancer stem cells (CSCs) population was put forward some years ago [4, 5]. The "cancer stem cell" hypothesis states that tumours are hierarchically organized as their normal tissues counterpart, and that their long term maintenance is attributable to the ability of CSCs to self-renew indefinitely [5]. The cumulating data suggest that CSCs or cells with stem-like properties are much more resistant to chemotherapy than cells comprising the bulky tumour [6]. Moreover, it has been shown that transcriptional signatures associated to CSCs is predictive of poor overall patients survival, and experimental and clinical evidence suggests that CSCs survive to commonly used anticancer treatments (both cytotoxic and targeted therapy), implying that these cells are possibly responsible for disease recurrence and treatment resistance [7-9]. For these reasons the identification and the targeting of CSCs has been an important research area in oncology.

An unambiguous phenotype for ovarian CSC is still lacking, even if some hypothesis on the origin of ovarian cancer has been recently proposed. Indeed, recent studies suggest that the most high-grade serous ovarian cancer (~80%) originates in the ovarian fimbriae [10, 11]. Specifically, the hypothesis states that a stem cell in the fimbria could accumulate DNA damage, which may result in the development of a "p53 signature", followed by development of a serous tubal intraepithelial carcinoma (STIC). These STIC lesions may shed from papillary tufts, and may implant on the surface of the ovary. The exposure to a stromal niche and autocrine signals may induce the development of high-grade serous carcinoma (HGSC) carcinogenesis and metastasis. It has been reported that the fimbria stem cells are characterized as c-Kit+, tubulin β4+, paired box 8+, and CD44+ cells, and are able to form spheres containing all the different fimbriae cells (ciliated, secretory and basally located cells) [11]. The first description of stem cells in ovarian cancer was reported in the ascites of an ovarian patient, derived from a single clone which could grow as spheres in culture, and could be sequentially propagated in tumours over several generations [12]. The characterization of ovarian CSCs was also studied by the detection of specific markers (i.e. CD133, CD117, CD24, and CD44), by the presence of the side population (SP, a particular phenotype that depends on the ability of the cells to extrude drugs by membrane pumps), or by the ALDH activity [13-16]. Alvero *et al*., were able to isolate CD44+ cells from primary cell lines, from tumour and ovarian ascites, and these could produce tumours in mice [17]. Gao et al reported the CD24 as a putative CSC marker in ovarian cancer, as few as 5,000 $CD24^+$ cells were able to form tumours in nude mice [18]. We could isolate two ovarian cancer stem-like cells (#83 and #110) from fresh tumour samples, that were able to grow as spheres in culture [19]. Specifically, we demonstrated that these cells were able to form tumour when as few as one cell was injected in immune-deficient nude mice, they were able to self-renew, and to differentiate *in vitro*. We showed that these cells were more resistant to drugs usually used in the treatment of ovarian cancer (such as, cisplatin, paclitaxel), and to other anti-tumour drugs (such as, etoposide), than their more differentiated counterpart *in vitro*. Moreover, these spheres cultures present a mesenchymal phenotype, and recently different groups, including ours, have published that genes involved in epithelial-mesenchymal transition (EMT) were associated with overall or progression free survival, suggesting also contribution of EMT to the resistance mechanisms [19].

With this background, in order to find potential new agents in ovarian cancer, we screened the chemical library gathered in the frame of the COST action CM1106 (http://www.cost.eu/COST_Actions/cmst/CM1106) on the survival of our two ovarian cancer cell lines (#83-SC and #110-SC) and in the differentiated cells derived from them (#83-DC and #110-DC).

2. EXPERIMENTAL

Cell cultures. Low adherence cell conditions. #83 and #110 were grow in low adherence flasks (Corning) under stem-cell conditions as reported [19] serum-free DMEM/F12 medium supplemented with 5 µg/mL insulin (Sigma), 20 ng/mL human recombinant epidermal growth factor (EGF, Peprotech), 10 ng/mL basic fibroblastic growth factor (bFGF, Peprotech) and B27 Supplement (Gibco).

Differentiated cell conditions. Cells from dissociated spheres were cultured in differentiating conditions (RPMI/F12 medium 1:1 supplemented with 10% fetal bovine serum) for one week. These conditions have been demonstrated to induce cell differentiation [19]. After one

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week of culture, differentiated cells and those derived from dissociated spheres were plated in 384 well plates at a concentration of 12,500 cells/ml.

Chemical library. The chemical compound library was comprised by 576 compounds dissolved in DMSO and stored at -20°C in pre-prepared aliquots ready to use. It included synthetic compounds and natural products extracted from marine organisms and from plants, as well as reference compounds and not yet published compounds. The library is characterized by an extreme diversity: from glucosides, nucleosides to pseudo peptides.

Drug treatment. 96 hours after seeding, cells were treated at the dose of 20μM. The high-throughput screening was performed with an automated liquid handling system (JANUS^{TM}, PerkinElmer), connected to a WinPREP for Janus software, with which it was possible to set up ad hoc programs for the screening (e.g. seeding and drug treatment). Cell survival was analyzed by the MTS assay system (Promega) 72 hrs after treatment. MTS reagent (5 μL) was added to each well and after a constant incubation time for all the plates absorbance was acquired using a plate reader (Infinite M200, TECAN). Each sample (control and treated) was done triplicate. Beyond the chemical compounds included in the library, cells were treated also with cisplatin, as an internal positive control of cell toxicity in each plate. Cisplatin was found to be more active in differentiated cells than in stem cells (3% versus 25% of %Ctr), as already reported [19].

Data analysis. Survival data were elaborated and were considered active if they induced a maximum of 20% cell survival compounds over control/untreated cells (%CTR), calculated as: [Abs treated/Abs no-treated cells*100]). Positive compound (cisplatinum at the IC80 dose of 32μM) were included in the screening platform. Vehicle control was included in the screening (DMSO at the same concentration used in the screening). The *t-test* analysis revealed a statistical significance with a p value< 0.0001 for all the active compounds. The power of the high-throughput screening was evaluated by calculating the Z-score value, as previously described [20]. The average Z' score for the overall screening was 0.69.

3. RESULTS and DISCUSSIONS

Under the COST Action CM1106 a library of 1200 compounds coming from different research groups was assembled. The library is made up of synthetic and natural compounds, whose structure and mechanism of action is under evaluation (data not shown). We used 576 of them for our screening. The compounds were tested in the two ovarian enriched stem cell cultures #83 and #110, and in their differentiated counterpart. This setting has already been demonstrated to possibly discriminate compounds active (%Ctr ≤20% and 10 %) on stem cells and on the more differentiated counterpart [19] (Supplementary Table **1**).

A number of compounds was found to be very active in both #83 and #110 cells (Fig. **1**). In particular, 29 and 34 compounds out of 576 were found to be cytotoxic (Ctr%≤ 10 and 20%) in both SC and DC of #83 and #110 cell lines, respectively 4.5% and 6.0% (Fig. **1**, panel A, Supplementary Tables **2** and **3**). Interestingly, a number of compounds were found to be active in both SCs (n=33) or in both DCs (n=44) of the #83 and #110 cell lines (Fig. **1**, panel B, Supplementary Tables **4** and **5**), suggesting the possibility to specifically target the former cellular subtype.

In addition, among the compounds contained in the CM1106 library, we observed that some were targeting only the stem-like cells (Table **1**) and some only the differentiated cells (Table **2**), while other compounds were active on both cell states (Table **3**). The chemical structures of all the active compounds are reported in Fig. (**2**).

Table 1. Compounds found to be active only in SC cells.

#	Compound	#83 SC	#110 SC	Putative mechanism of action
1	SAHA	16,00	-5,08	HDAC inhibitor
2	NJW5	-13,65	-12,57	Sirtuin inhibitor
3	CSA4	16,82	6,51	Tubulin destabilizer
4	RN 246	17,73	10,30	HDAC inhibitor
5	RN 422	4,41	-6,17	HDAC inhibitor
6	Sunitinib	9,80	2,61	Tyrosine kinase
7	JK20 (R004)	14,07	6,23	Tubulin destabilizer
8	KO191 (R005)	19,53	0,45	Tubulin destabilizer
9	BB20	-8,39	-7,08	Unknown
10	Chaetocyne	-3,25	-2,63	HMT ihnibitor

The %Ctr value for each compound is reported.

Among the most active compounds targeting stem cells, we found compounds with different mechanism of action. Sirtuins are NAD(+)-dependent class III histone deacetylases regulating important metabolic pathways in prokaryotes and eukaryotes and are involved in many biological processes, including cancer [21]. NJW5 is a sirtuin inhibitor whose discovery and validation of SIRT2 inhibitors was based on tenovin-6 by a ^{1}H-NMR method to assess deacetylase activity [22, 23]. We observed that compounds RN246 (%Ctr of 17.7% and 10.3% in #83 and #110 SC, respectively) and RN422 (%Ctr of 4.41% and -6.2% in #83 and #110 SC, respectively) displayed good activity, while in both the DCs we observed a very low cytotoxic activity. The syntheses of these compounds have been recently published [24, 25]. The more efficient compounds able to kill 90% (%Ctr≤10%) of the stem like cells were the tyrosine kinases inhibitors: sunitinib (platelet derived growth factor receptor- PDGFR, vascular endothelial growth factor receptor-VEGFR), erlotinib (against the EGFR kinase) and dasatinib (against the bcrl/abl kinase), while we observed no activity with imatinib (against ABL, KIT, PDGFR kinases). This differential activity is difficult to explain and could be due to the different role of the kinases in the growth of SC and requires further work.

Table **2** shows the compounds specifically active on differentiated cells. Again, clinically used cytotoxic drugs were preferentially active on DC (taxol and camptothecin). Perezone (compound VR7), a CDC25 phosphatase inhibitor is a natural compound recently isolated from the soft coral *Pseudopterogorgia rigida*, and was found more active on DC than on SC. Phosphatases represent a group of proteins involved in different pathological processes, including cancer [26], and recent evidence suggest that their inhibition could have an antitumor effect [27, 28].

Table **3** summarizes the compounds that were active on both SC and DC cells. Again, most of these compounds are new compounds that are under characterization.

Table 2. Compounds found to be active in only DC cells.

#	Compound	#83 DC	#110 DC	Putative mechanism of action
11	PB-NODB	-8,07	-7,82	HDAC inhibitor
12	LOM 621	-4,08	-2,35	Unknown
13	RC 960	-5,50	-2,13	HDAC inhibitor
14	RC 714	-3,57	-4,30	HDAC inhibitor
15	Taxol	18,03	7,15	Tubulin destabilizer
16	Camptothecin	15,06	14,34	Topoisomerase I inhibitor
17	BB15	14,25	15,42	Unknown
18	VR7	-9,50	-2,86	CDC25 inhibitor
19	VR10	19,51	12,86	Unknown
20	ADTA240	-3,96	0,61	Unknown
21	ADMR231	8,02	11,54	Unknown
22	Entinostat	14,04	1,31	HDAC inhibitor
23	ELTE-CSA2	15,94	10,30	Unknown
24	MTA-BSz-1	-11,97	-3,48	Unknown
25	MTA-BSz-3	-12,77	-10,48	Unknown
26	MTA-BSz-4	-12,14	-8,58	Unknown
27	ELTE-MZs-3	13,50	0,23	Unknown
28	ELTE-MZs-8	16,29	7,26	Unknown
29	ELTE-MZs-10	2,56	7,08	Unknown

The %Ctr value for each compound is reported.

Table 3. Compounds found to be active in both #83 and #110 SC and DC cells.

#	Compound	#83 SC	#83 DC	#110 SC	#110 DC	Putative mechanism of action
30	RB241	-8,53	6,99	-15,06	-0,56	Base analogue
31	5 Aza-Cytidine	11,04	10,54	3,01	13,73	DNA demethylating agents
32	AB-040	-7,39	-7,19	-10,06	-9,16	Base analogue
33	Tenovin 6	-1,79	2,46	-1,00	-2,28	Sirt inhibitor
34	PA-BU	-1,20	-5,85	-5,76	-6,22	Apoptosis inducer
35	6MeOH	15,44	-4,76	-5,44	-5,46	Apoptosis inducer
36	RS3301	17,20	18,41	4,71	1,19	Tubulin destabilizer
37	RS3883	19,10	17,63	10,43	15,87	Tubulin destabilizer
38	COLChicine	15,78	17,49	6,08	3,70	Tubulin destabilizer
39	LOM 612	-2,18	-4,08	-6,96	-2,88	Foxo inhibitor
40	RC 173	15,79	-1,80	-1,37	1,31	Atypical retinoid
41	Thio-colchicine	10,07	9,64	17,07	5,95	Tubulin inhibitor
42	Podophyllotoxin	19,74	5,30	10,74	-0,42	Antimitotic
43	LY-83.583	-10,84	-11,08	-5,22	-15,07	Guanyl cyclase inhibitor
44	BB3	14,09	14,98	12,42	-1,08	Apoptosis inducer
45	BB6	6,39	-9,08	-6,10	-5,51	Apoptosis inducer
46	BB7	-10,05	-4,70	-5,63	-11,92	Unknown
47	MIC1	-1,53	-2,88	5,03	5,25	Unknown
48	MTA-BSz-2	7,48	-10,18	15,40	-5,13	Unknown
49	MTA-BSz-5	6,91	-10,90	9,31	-2,03	Unknown
50	ELTE-MZs-2	18,19	-5,63	15,35	-3,07	Unknown

The %Ctr value for each compound is reported.

The screening of this library in our experimental setting allowed us to find that some compound families were active on stem cells, while others were indistinctly active on both states. Apart from the cytotoxic agents and tyrosine kinases inhibitors, drugs modulating the gene expression have been clearly found to be active on both cell types. Epigenetic alterations through modulation of the level of acetylation and methylation of DNA have been shown to exert antitumor effect. Recently, combinations of 5-AzaC or 5-AzaDC with HDACi have been approved by FDA and the European Medicines Agency (EMA) for treatment of hematologic malignancies [29]. It was reported that not only the combination 5-AzaC plus butyrate targets CSCs [30], but that the same combination markedly reduced CSC abundance and increased the overall survival in a mouse model, differentially regulating genes that are involved in tumor growth [31]. Many of the compounds we found active were epigenetic compounds and potentially active in both stem cells and differentiated cells foreseeing the idea that to treat cancer we likely need compounds with different mechanism of action (epigenetic and cytotoxic), and able to kill tumor cells with different biological state (stem cell versus differentiated).

The fact that compounds with different specificity were found corroborates the notion that tumors are made up of different subset of cells with not only specific biological properties but also with different pharmacological sensitivities [32]. We found compounds active on each cell type or active of both. In our opinion, the most interesting are both the ones acting on SC, that we envisage need be used in conjunction with compounds active on bulky tumor cells, and the ones acting on both DC and SC.

4. CONCLUSION

We successfully screened part of the chemical compound library (576 compounds) gathered in the frame of the COST action CM1106 using the recently isolated ovarian cancer stem cell enriched cultures and the differentiated non-tumorigenic cells derived from them. We found compounds active only against cancer stem cells, only against differentiated cells and compounds active against both SC and DC cells. From the available information the active drugs belong to different chemical classes, and their mechanisms of action on stem cells are under study.

These data need to be validated in *ad hoc* dose-response cytotoxic experiments, mechanistic studies, and the specificity of the cytotoxic activity investigated by using a larger panel of cancer cell lines. Analysis of the compound structures is underway opening up to mechanistic drug studies to possibly select compounds able to improve the prognosis of ovarian cancer patients.

ABBREVIATIONS

EOC: Epithelial Ovarian Cancer
CSCs: Cancer Stem Cells
STIC: Serous tubal in situ carcinoma
HGSC: High-Grade Serous Cancer
ALDH: Aldehyde Dehydrogenase
EMT: Epithelial-Mesenchymal Transition

CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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SUPPLEMENTARY MATERIALS

Supplementary Tables:

Supplementary Table 1. List of all the tested compounds.

Supplementary Table 2. Compounds found to be active in both #83 cell lines (SC and DC).

Supplementary Table 3. Compounds found to be active in both #110 cell lines (SC and DC).

Supplementary Table 4. Compounds found to be active in both SCs (#83 and #110).

Supplementary Table 5. Compounds found to be active in both DCs (#83 and #110).

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