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ORIGINAL ARTICLE

Selective efficacy and safety of first-line and non-front-line antineoplastic agent combinations determined with individualized assays on colorectal cancer primary cultures

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Summary

Purpose: *To evaluate the efficacy of first-line and not-conventional antineoplastic drug combinations in colorectal adenocarcinoma primary cultures (CRAC-PCs).*

Methods: *The efficacy and safety of 21 drug combinations (DCs) were evaluated using a simplified adenosine triphosphate-based chemotherapy response assay (sATP-CRA). The efficacy of each DC was reported as the percentage of cell death (PCD) produced on each of 12 CRAC-PCs, and the safety of each DC was evaluated as a safety window (SW). The SW was calculated as the quotient of PCD-CRAC-PC/PCD-hMSC-TA (human mesenchymal stem cells derived from adipose tissue). Nine DCs contained 5-fluorouracil and oxaliplatin, and 1-3 non-front-line drugs (NFLDs [carboplatin, doxorubicin, cisplatin, aspirin, or 3,3'-diindolylmethane]). The other 11 DCs only contained 2-4 NFLDs.*

Results: *The efficacy and safety each DC were highly variable and depended on each CRAC-PC and DC. The usefulness of DCs was considered as a combination of PCD >20 and an SW >0.6: 13 /21 DCs (62%) met the requirements of efficacy and safety on 7/12 CRAC-PCs (58.3%).*

Conclusions: *The resistance to 5-fluorouracil/oxaliplatin of CRAC-PCs and the usefulness of seven new DCs strongly suggest the convenience of performing ex vivo individualized assays to evaluate DCs and implement new and more useful treatments, instead of submitting patients to standardized chemotherapies in a blinded manner. Approaches such as this and properly evaluated in clinical assays could increase the life expectancy of patients with cancer and improve their quality of life.*

Key words: *Alternative anti-neoplasic treatment, colorectal cancer, ex vivo anticancer drug evaluation, individual response to anti-cancer drugs, primary colorectal culture, simplified ATP-CRA*

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer mortality worldwide. Conventional therapeutic schemes are iatrogenic, and, in the majority of cases, advanced CRC eventually results in metastatic disease and death within 5 years after treatment initiation [1]. In advanced CRC, the treatment consists of surgical removal of the primary tumor and chemotherapy. The conventional chemotherapeutic schemes most used worldwide are FOLFOX6 [comprising 5-fluorouracil (5FU),

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oxaliplatin (Oxa) and leucovorin] [2], XELOX (comprising capecitabine and Oxa) [2], Xeloda (comprising only capecitabine) [3] and FOLFIRI (comprising a combination of 5FU, irinotecan, and leucovorin) [4]. However, with any of the regimens above, drug failure occurs in ~90% of metastatic CRC cases [5]. This failure is attributed to the drug resistance of the tumors [6]. Previously, our research group identified [7] non-front-line chemotherapeutic agents that produced improved results when compared with standardized chemotherapeutic schemes. These are predominantly used as adjuvant therapies to first-line drugs. Carboplatin (Carbo), doxorubicin, (Doxo), cisplatin (Cispl), aspirin (Asp), and 3,3'-diindolylmethane (DIM) are of particular interest, since none of these compounds has yet been used as anti-CRC agents; instead, they are currently being used to treat other types of cancer successfully [8]. For example, Carbo has been prescribed to treat at least 18 different types of cancer, including those of the male and female reproductive and urinary systems, acute myeloid leukemia, refractory osteosarcoma, and small cell cancer of the lung (webMD.com, 2018). Doxo has been employed as chemotherapy for 13 different types of cancer, including breast cancer, lymphomas, ovarian and lung cancer, and leukemias [9]. Cispl forms part of the treatment regimens for ovarian, bladder, and testicular cancer chemotherapies [9]. Asp has been used since ancient times to treat numerous disorders where inflammation is involved, including fever, headache, rheumatic fever, rheumatoid arthritis, pericarditis, and Kawasaki disease. Also, low doses of Asp have been demonstrated to reduce the risk of heart attack or stroke [10], and the most interesting feature of Asp may prevent CRC [11,12]. Concerning DIM, it is well known that a diet rich in cruciferous vegetables, such as cabbage, broccoli, and cauliflower, can reduce the risk of several types of human cancer [13,14]. Indole-3-carbinol and DIM have been proposed as cancer preventive agents. Each of these compounds may be orally administered safely to humans in repeated doses, reaching concentration peaks in blood plasma that are sufficient to enable the treatment of different types of cancer [15-17].

Currently, improvements in medical science have enabled the development of physical [18,19] and *ex vivo* methods [20] to evaluate the sensitivity of malignant tumors to chemotherapeutic drugs, which act as valuable predictors of success or failure of an anticancer drug or a mixture of drugs.

About the present study, *ex vivo* chemosensitivity assays particularly attracted our attention, since these methods provide an excellent alternative to assist the oncologist in selecting optimal treatments based on individual tumor responses, as opposed to using standardized regimens. Among these bioassays, the adenosine triphosphate (ATP)-based chemotherapy response assay (ATP-CRA) is being widely employed given its ease of use, and since only small tumor samples are required [21].

The safety window (SW) is another important aspect to take into account since, ideally, an anti-cancer medication should have high toxicity towards the malignant cells, and yet exert no toxicity towards healthy cells, i.e., the toxicity of an ideal anti-cancer medicine should be specifically targeted to malignant tumors. Therefore, to test the safety of anti-cancer drugs, our research group has previously used human mesenchymal stem cells derived from adipose tissue (hMSC-TA cells). These cells are healthy, relatively easy to procure and preserve, and proliferate rapidly; furthermore, the fact that they are of human origin is a further advantage, since the results obtained with these cells are more reliable compared with those from other species. Our group has previously demonstrated that ATP-CRA and hMSC-TA cells are excellent tools to determine the efficacy and SW of anticancer drugs, in combination or alone, using as a model the adenocarcinoma cell line, COLO-320DM [7]. The present study follows on logically from the previous one, wherein the efficacy and safety of non-front-line drug combinations (DCs) were investigated, either including or not the first-line drugs used in the FOLFOX regimen. The effects of a variety of DCs were investigated on colorectal adenocarcinoma primary cultures (CRAC-PCs), which contained both malignant- and tumor-associated cells.

Methods

Patients

Twelve patients were included in the present study. They underwent surgical extirpation of their intestinal tumors for therapeutic reasons. The sociodemographic and clinical characteristics of the patients are depicted in Table 1. Six patients were female and the other six male. The age range of the patients was 35-86 years (mean 56; median 58). Two-thirds of tumors were located in the rectum, and one-third were identified in the colon. All the tumors were diagnosed as adenocarcinomas (ACs). The cancer of ten patients (83.3%) was in an advanced stage (either T3 or T4), whereas that of the remaining two patients (16.7%) was in an earlier stage (T2). Only two patients were admitted for radiotherapy before surgery, and immediately following surgery, three patients commenced a course of chemotherapy: Two with Xeloda and one with XELOX6. The other nine patients did not receive chemotherapy or radiotherapy following surgery.

Isolation of the CRAC-PCs

A section of about 1.5 cm^3 of each tumor was collected immediately following its surgical resection. These sections were promptly transported to our cell therapy laboratory (Laboratorio de Terapia Celular Monterrey, Universidad Autónoma de Nuevo León (UANL), Monterrey, Mexico) in a cooler at 4°C. The tumor samples were extensively washed with sterile phosphate-buffered saline (PBS) (Sigma-Aldrich; now a brand of Merck KGaA, Darmstadt, Germany), immersed in 70% ethanol (reactive grade diluted with sterile double distilled water) for 1 min (Sigma-Aldrich; Merck KGaA), cut into tiny pieces (<1 mm^3), and mixed with 100 IU/ml Gibco® Collagenase Type I (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) diluted in 4 ml Gibco® DMEM-F12 culture medium to which was added 100 µg/ml gentamicin and 2.5 µg/ml Gibco® amphotericin B (Invitrogen; Thermo Fisher Scientific, Inc.). The tumor preparations were subsequently incubated at 37°C in an atmosphere of 5% CO_2 with magnetic agitation (120 rev./min) for 2 h. The disaggregated cells were separated from debris, passing the digested tissue through a BD Falcon™ 100 µM mesh cell strainer (BD Biosciences, Bedford, MA, USA), and washed three times with PBS. The cells were resuspended in 1 ml Gibco® Iscove's Modified Dulbecco's medium (IMDM), added with 100 µg/ml Gibco® gentamicin, 2.5 µg/ml Gibco® amphotericin B and 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scentific, Inc.), and cell viability was subsequently determined using the trypan blue exclusion test. The concentration of viable cells was adjusted to 2×10^5 cells/ml with IMDM containing gentamicin, amphotericin B and 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc), and the cells were used immediately afterward.

Table 1. Sociodemographic and clinical findings of patients having colorectal cancer

Findings	*Number of patients (%) (n=12)*
Gender	
Female	6 (50)
Male	6 (50)
Age, years	
30-49	1 (8.4)
50-69	7 (58.3)
70-89	4 (33.3)
Location and histopathological diagnostic	
Rectum adenocarcinoma	9 (75)
Colon adenocarcinoma	3 (25)
Clinical stage[1]	
T2N0M0[1]	2 (16.7)
T3N0M0	3 (25)
T3N1M0	4 (33.2)
T4N0M0	2 (16.7)
T4N1M0	1 (8.4)
Radiotherapy	
Yes	2 (16.7)
No	10 (83.3)
Chemotherapy	
XELOX[2]	1 (8.4)
XELODA[3]	2 (16.7)
None	9 (75)

[1]T2 to T4 means the tumor grade of advance, T2 is the less advanced stage and T4 the most advanced. N0 means that cancer has not taken lymph nodes, N1 cancer took one lymph node; M0, cancer has not made metastases. [2]XELOX, capecitabine plus oxaliplatin. [3]XELODA, capecitabine.

Isolation, proliferation, and characterization of the hMSC-TA cells

hMSC-TA used to determine the SW of each tested DC were isolated, allowed to proliferate and characterized as described previously [7]. Briefly, hMSC-TA were isolated from surplus adipose tissue, which was obtained by liposuction from two men and two women. The samples were digested with Gibco® Collagenase Type I (0.04 g/ml), sedimented, washed with PBS, and resuspended in α-minimum essential medium (α-MEM) (Invitrogen; Thermo Fisher Scientific, Inc.) to which was added 30 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich; Merck KGaA), 50 µg/ml gentamicin, and 2.5 µg/ml amphotericin B (enriched α-MEM). The percentage of viable cells was determined, and the cells were then allowed to proliferate in cell culture flasks containing 5 ml enriched α-MEM. The cells were subsequently frozen in liquid nitrogen. The hMSC-TA cells were characterized by immunocytochemistry, labeling the cells with anti-CD105 monoclonal antibodies. The percentage of hMSC-TA ($CD105^+$) present was calculated according to the mean ± standard deviation of $CD105^+$ and $CD105^-$ cells. Our group has previously shown that, by following the above procedure, the greater part of cell isolates was hMSC-TA [6], and that these cells can differentiate into chondrocytes and osteoblasts [23,24].

Determination of the sensitivity of CRAC-PCs and hMSC-TA cells to the different DCs

The sensitivity of each of the 12 CRAC-PCs was determined using the ATP-CRA [25], simplified for clinical use (sATP-CRA) [7] by employing the Cell Titer-Glo Luminescent Cell Viability Assay kit of the manufacturer's protocol. In brief, each CRAC cell suspension was distributed in a Costar™ 96-well ultralow attachment microplate (Corning, Inc., Corning, NY, USA) in aliquots of 100 µl/well. Aliquots of 100 µl/well of each of the 21 DCs were added into three of the wells containing CRAC cells, as depicted in Table 2. The concentration of each component in the DCs was equal to that which produced half-maximal (50%) cell death (CD_{50}) in cultures of CRAC cells from the COLO-320DM cell line, except DIM, which was insufficiently toxic to attain a CD_{50} value with the COLO-320DM cells. The concentrations used were as follows, expressed as µg/ml: 5-FU, 8.58; Oxa, 0.4; Cispl, 0.53; Carbo, 5.47; Doxo, 1.04; and Asp, 1,548 [6]. The concentration of DIM (108 ng/ml) employed was equal to the maximum concentration that this drug reaches in blood plasma. This data was obtained by administering

Table 2. Classification of CRAC-PCs[1] according to their response to drug combinations

Number of drug combination[2]	*Drug combination*[3]	*CRAC-PCs*		
		Resistant[4]	*Sensitive*[5]	*Very sensitive*[6]
		Number (%)[7]		
1	5FU/Oxa	10 (83.3)	2 (16.7)	
2	5FU/Oxa/Carbo	9 (75.0)	3 (25.0)	
3	5FU/Oxa/Doxo	6 (50.0)	4 (33.3)	2 (16.7)
4	5FU/Oxa/Cis	9 (75.0)	2 (16.7)	1 (8.3)
5	5FU/Oxa/Doxo/Carbo	6 (50.0)	5 (41.7)	1 (8.3)
6	5FU/Oxa/Cis/Carbo	10 (83.3)	2 (16.7)	
7	5FU/Oxa/Doxo/Cis/Carbo	5 (41.7)	6 (50.0)	1 (8.3)
8	5FU/Oxa/Asp	6 (50.0)	6 (50.0)	
9	5FU/Oxa/DIM	11 (91.7)	1 (8.3)	
10	5FU/Oxa/DIM/Asp	10 (83.3)	1 (8.3)	1 (8.3)
11	Doxo/Carbo	6 (50.0)	5 (41.7)	1 (8.3)
12	Carbo/Cis	8 (66.7)	4 (33.3)	
13	Doxo/Carbo/Cis	5 (41.7)	6 (50.0)	1 (8.3)
14	DIM/Asp	9 (75.0)	2 (16.7)	1 (8.3)
15	Doxo/Carbo/DIM	4 (33.3)	7 (58.3)	1 (8.3)
16	Doxo/Carbo/Asp	5 (41.7)	5 (41.7)	2 (16.7)
17	Carbo/Cis/DIM	10 (83.3)	2 (16.7)	
18	Carbo/Cis/Asp	10 (83.3)	2 (16.7)	
19	Carbo/Cis/Asp/DIM	9 (75.0)	3 (25.0)	
20	Carbo/Cis/Doxo/DIM	6 (50.0)	5 (41.7)	1 (8.3)
21	Carbo/Cis/Doxo/Asp	6 (50.0)	5 (41.7)	1 (8.3)

[1]CRAC-PCs: colorectal adenocarcinoma primary cultures. [2]Number arbitrarily given to each drug combination. [3]Abbreviations mean 5FU, 5-fluorouracil; Oxa, oxaliplatin; Cis, cisplatin; Carbo, carboplatin; Doxo, doxorubicin; DIM, 3,3'-diindolylmethane; Asp, aspirin. The concentration of each component in the DCs was equal to that which produced half-maximal (50%) cell death (CD_{50}) in cultures of CRAC cells from the COLO-320DM cell line, except DIM, which was insufficiently toxic to attain a CD_{50} value with the COLO-320DM cells. All drug combinations were assayed against the same 12 CRAC-PCs. The percentage of cell death (PCD) distribution was displayed in Figure 1. [4]PCDs ≤ 25. [5]PCDs >25 to 50. [6]>50. [7]Number and percentage of CRAC-PCs resistant, sensitive or very sensitive to any drug combination. The percentage of each of the above category was calculated concerning the total number of CRAC-PCs assayed (n=12). Blanks correspond to zero CRAC-PCs. The shadowed zone highlights the results obtained with drug combinations containing 5FU/Oxa. The white zone frames the results obtained with drug combinations containing exclusively non-front-line drugs .

a single dose of 300 mg [26]. As negative controls, three wells in each plate were incubated with untreated cells, and three wells contained IMDM medium as a blank, without cells or drugs. As an internal control, 105 pg ATP diluted in 100 µl IMDM medium/well was added into three wells in each microplate, following the protocol provided with the CellTiter-Glo Luminescent cell viability assay kit. Carbo and Oxa were obtained from Asofarma de México (Mexico City, Mexico); Cispl, 5-FU, and Doxo were purchased from Teva Pharmaceutical Industries, Ltd. (Mexico City, Mexico); and Asp and DIM were from Merck KgA. The microplates were incubated at 37°C in an atmosphere of 5% CO_2 for 48 h. Subsequently, the cells were lysed in lysis buffer provided with the CellTiter-Glo kit, which features a luciferin-luciferase system for luminescent cell viability assay. The ATP content in each well was measured with a Veritas microplate luminometer (model 9100-002; Turner BioSystems; Thermo Fisher Scientific, Inc.). Cytotoxicity was evaluated using the following equation: PCD =1-(LT/LNT)×100, where PCD represents the percentage of cell death, LT is the luminescence recorded in each cell culture treated with one DC, and LNT is the luminescence recorded in untreated cell cultures.

Regarding the SW of each DC, this was calculated as the quotient of the PCD of CRAC-PCs / PCD of hMSC-TA cells derived from the adipose tissue. Therefore, the greater the quotient, the wider was the SW.

Statistics

Values are shown as the mean of three independent experiments.

Ethical aspects

The institutional research and ethics committees of the Medical School, UANL, and of the IMSS authorized this study with the registry numbers BI11-004 and R-2012-785-075, respectively. All patients, who donated a sample of their tumors and allowed us to use their sociodemographic and clinical data in anonymity, signed the informed consent form.

Results

CRAC-PC sensitivity and efficacy of the DCs

Figure 1 shows a schematic representation of the individual sensitivities of the CRAC-PCs, according to the PCD determined for each of the 21 DCs evaluated. The PCDs exhibited a high range of values, which was also reflected in the categories corresponding to each classification of drug-sensitivity. The PCDs were categorized as follows: 0-20, resistant; >20-50, sensitive; and >50-80, very sensitive. Five CRAC-PCs were positioned on the limit of resistance (19.8 to 20) to DC-5, -11, -16 and -19 and two CRAC-PCs were situated in the sensitivity borderline (PCD=50) to DC-12 and -16.

Table 2 shows a detailed analysis of these results. Since the same 12 CRAC-PCs were assayed with each of the 21 PCs, a large number of these CRAC-PCs were revealed to be resistant, sensitive or very sensitive to more than one DC. Little more than half of the assays performed (160/252; 63.5%) corresponded to a resistance of the CRAC-PCs to the DCs. Breaking down the above results further, it can be observed that the distribution of CRAC-PCs resistant to DCs containing 5FU/Oxa or 5FU/Oxa plus non-front-line drugs (NFLDs; 82/160, or 51.3%) was slightly lower (2.5) than the DCs featuring exclusively NFLDs (78/160; 48.8%).

The highest number of resistant CRAC-PCs (11/12) corresponded to the DC-9, followed by 10/12 CRAC-PCs, which were resistant to the DCs 1, 6, 10, 17 and 18 (Table 2).

The difference between the specific compositions of these DCs was as follows: DC9 contained 5FU/Oxa/DIM, DC1 included only 5FU/Oxa and DC10 5FU/Oxa/DIM/Asp. On the other hand, DC17 and DC18 included only NFLDs (Carbo/Cis/DIM, and Carbo/Cis/Asp, respectively). By contrast, the lowest number of resistant CRAC-PCs (4/12) was observed in the assays performed with DC15, which contained only NFLDs (Doxo/Carbo/DIM). The intermediate range of CRAC-PCs, resistant to the remaining 13 DCs, was 5-9.

A smaller, but still substantial, number of assays (78/252; 31%) were identified with a sensitivity of CRAC-PCs to one or more DCs, in the range of 1-7 CRAC-PCs out of the total of 12. Those DCs containing only 5FU/Oxa or 5FU/Oxa and NFLDs were active in 32/78 (41%) assays, as identified in 1-6 of the CRAC-PCs. DCs containing exclusively NFLDs demonstrated activity in 46/78 (59%) of

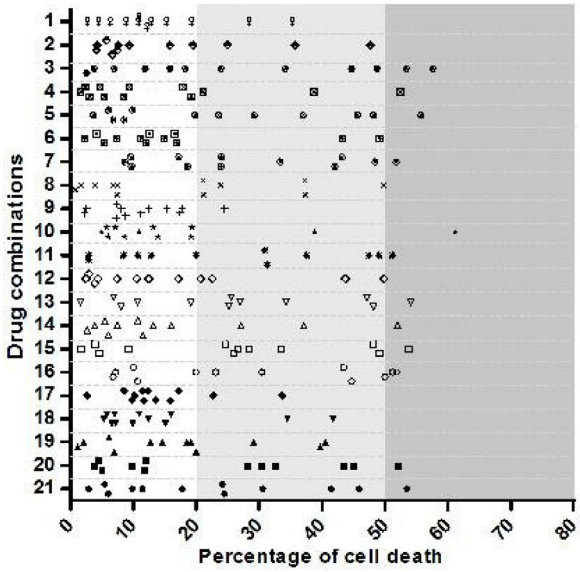

Figure 1. Cytotoxicity of DCs on CRAC-PCs. Numbers in the vertical axes correspond to the DCs depicted in Table 2. Symbols represent the PCD of each of the 12 CRAC-PCs that were subjected to the toxic effect of each DC. The segmented lines frame the lanes corresponding to the PCD symbols of each DC. The identical 12 CRAC-PCs were assayed with every DC. The white, light and dark gray zones classify the CRAC-PCs, respectively, as resistant, sensitive or very sensitive to the assayed DCs. Each symbol corresponds to the average of three determinations. DC, drug combination; CRAC-PC, colorectal adenocarcinoma primary culture; PCD, percentage of cell death.

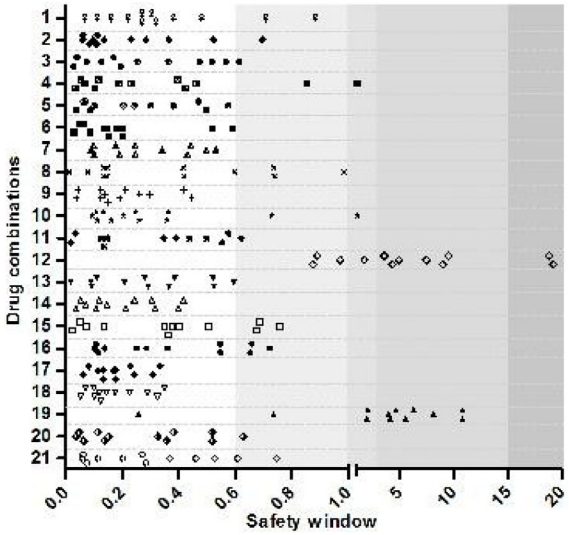

Figure 2. Distribution of the SWs of the different DCs. Numbers in the vertical axes correspond to the DCs depicted in Table 2. Symbols represent the SWs calculated as the quotient of the PCDs of the 12 CRAC-PCs / PCDs of the 12 human mesenchymal stem cells derived from adipose tissue produced by each DC. The quotient values (0 20) corresponding to the SWs are presented in the horizontal axes. The segmented lines frame the lanes corresponding to the SW symbols of each DC. The zones, marked as white, and gray with ascending darkness, correspond to the following categories of SW classification: Closed, narrow, open, broad, and very broad. Each symbol corresponds to the average of three determinations. DC, drug combination; CRAC-PC, colorectal adenocarcinoma primary culture; PCD, percentage of cell death.

the assays. Only 2/78 (2.6%) CRAC-PCs were sensitive to 5FU/Oxa DC (DC1). The number of assays showing CRAC-PCs sensitive to DCs containing exclusively NFLDs was 36% higher compared with those of 5FU/Oxa plus NFLDs. The DC that were associated with the highest number of sensitive CRAC-PCs (7/12; 58.3%) was DC15, and this contained only NFLDs: Doxo/Carbo/DIM. Three DCs (DC-8, -7 and -13) revealed to be active, each on six CRAC-PCs. Of these, DC8 and DC7 contained 5FU/Oxa plus one (Asp) and three (Doxo, Cis, and Carbo) NFLDs, respectively. DC13 included only NFLDs (Doxo, Carbo, and Cis). Considering the opposite regarding the efficacy of the DCs (i.e. the sensitivity of the CRAC-PCs to the smallest number of DCs), two assays revealed that only one CRAC-PC was sensitive to one of the following two DCs: DC9 and DC10, which contained 5FU/Oxa plus DIM and DIM/Asp, respectively. A possible positive effect of DIM or Asp on the sensitivity or resistance of CRAC-PCs appeared to depend on the other components of DCs, 50% of CRAC-PCs were sensitive to 5FU/Oxa and Asp.

The number of very sensitive assays of CRAC-PCs was smaller compared with that of sensitive CRAC-PCs (14/252; 5.6%). Among the DCs containing 5FU/Oxa plus NFLDs, four DCs (DC4, DC5, DC7, DC10) and six DCs containing only NFLDs (DC11, DC13-D15, DC20, and DC21) were very active against one CRAC-PC. DC3 (5FU/Oxa/Doxo) and DC16 (Doxo/Carbo/Asp) were very active against two CRAC-PCs.

SW assessment

Figure 2 shows the distribution of SW values. SWs were classified according to the following five categories: closed, narrow, open, broad, and very broad. A great dispersion of SWs is exhibited in Figure 2. Most data were concentrated in the closed and narrow categories. Table 3 presents the data associated with the classification of the SWs into each of the above categories. Closed SWs embrace a range of quotients, from 0 to 0.6. These quotients indicated that the cytotoxicity of DCs against hMSCs was higher compared with the cytotoxicity of these same DCs against CRAC-PCs. In the closed SWs 20/21 (91%) DCs were involved. From the 252

Table 3. Classification of DCs according to their safety window

Number of drug combination[1]	*Classification of safety window*				
	Closed[2]	*Narrow*[3]	*Open*[4]	*Broad*[5]	*Very broad*[6]
	Number and (%) of CRAC-PCs[7]				
1	10 (83.3)	2 (16.7)			
2	11 (91.6)	1 (8.3)			
3	11 (91.67)	1 (8.3)			
4	10 (83.3)	1 (8.3)	1 (8.3)		
5	12 (100.0)				
6	12 (100.0)				
7	12 (100.0)				
8	9 (75.0)	3 (25.0)			
9	12 (100)				
10	10 (83.3)	1 (8.3)	1 (8.3)		
11	11 (91.6)	1 (16.7)			
12		3 (25.0)	1 (8.3)	6 (50.0)	2 (16.7)
13	12 (100.0)				
14	12 (100.0)				
15	9 (75.0)	3 (25.0)			
16	9 (75.0))	3 (25)			
17	12 (100.0)				
18	12 (100.0)				
19	1 (8.3)	1 (8.3)	2 (16.7)	8 (66.7)	
20	11 (91.6)	1 (8.3)			
21	11 (91.6)	1 (8.3)			

[1]The numbers of this column correspond to the composition of each DC depicted in Table 2. [2]Safety window (SW) of 0-0.6. [3]SW > 0.60-1.0. [4]SW >1.0-2.5. [5]SW >2.5-15. [6]SW >15.0-20. [7]Number and percentage of CRAC-PCs belonging to one of the five SW classifications. The shadowed zone belongs to DCs having 5FU/Oxa or 5FU/Oxa plus non-front-line drugs. The white zone corresponds to DCs exclusively having non-front-line drugs. The blanks denote absence of data.

SWs categorized, 209 (82.9%) were closed SWs. All 12 SWs that were determined with 5FU/Oxa plus NFLDs (DC5, DC6, DC7, and DC9) were classified as closed. Considering the opposite in terms of the lowest number of closed SWs, DC8 was associated with 9 closed SWs. The remaining 5 out of 10 DCs containing 5FU/Oxa were associated with 10 or 11 closed SWs, including to DC1, which contained only 5FU/Oxa and revealed to have 10 closed SWs.

Regarding DCs having NFLDs exclusively, three of these (DC12, DC17 and DC18) were associated with 12/12 closed SWs, three (DC11, DC20 and DC21) with 11, and two with 9 closed SWs. On the other hand, DC12 was not associated with any closed SW and CD19 with only one closed SW.

The narrow SWs (i.e., quotients >0.6-1.0) corresponded to PCD values for hMSCs that were higher compared with the PCD values for CRAC-PCs, although they were lower for hMSCs than were the PCD values resulting from DC1. Narrow SWs (22/252 assays; 8.7%) were exhibited by 13 out of the 21 DCs (61.9%). Among these, six DCs (46.2%) contained 5FU/Oxa or 5FU/Oxa plus NFLDs, and seven DCs (53.8%) comprised only NFLDs (Table 2). The range of narrow SWs was 1-3: Those DCs showing one narrow SW were DC2-DC4, DC10, DC11, and DC19-DC21; DC1 exhibited two narrow SWs; and three narrow SWs were associated with DC12, DC15, and DC16. Concerning the other categories of SW, the following DCs showed one or two open SWs: One open SW, DC4, DC10 and DC12, and two open SWs, DC19. Two DCs exhibited six and eight broad SWs: DC12 and DC19, respectively. Only DC12 showed two very broad SWs.

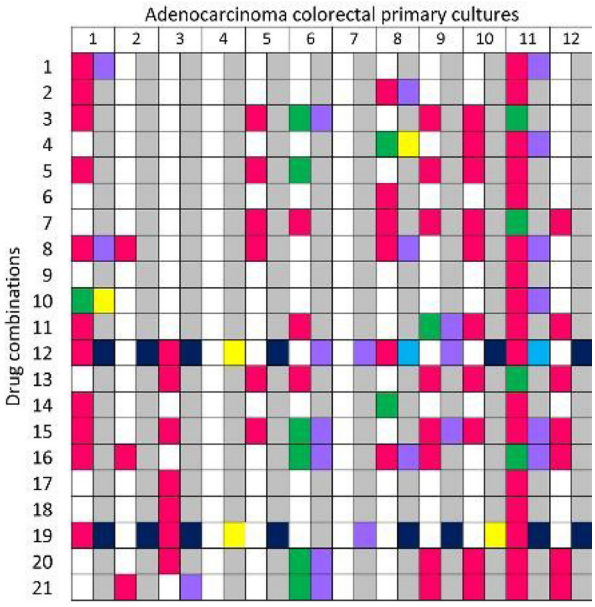

Figure 3. Individualized resistance or sensitivity and safety windows (SWs). At the top of this figure, a set of numbers are observed, which correspond to each of the 12 CRAC-PCs used in the present study. The left column, under each number, corresponds to the sensitivity or resistance of each CRAC-PC to every DC, whose number is placed on the left of this figure, and its composition is depicted in Table 2. Color codes of the resistance/sensitivity of each CRAC-PC are as follows: White, resistant; red, sensitive; green, very sensitive. The color codes of the SWs are as follows: Gray, closed; purple, narrow; yellow, open; blue, broad; and sky blue, very broad. Only the pairs formed by a sensitive or very sensitive CRAC-PC and a DC showing a not-closed SW were considered as acceptable. DC, drug combination; CRAC-PC, colorectal adenocarcinoma primary culture.

Individualized analysis of the efficacy and the SWs of the DCs

Figure 3 is a color-coded chart illustrating the results of sensitivity or resistance of each of the 12 CRAC-PCs, currently assayed with every one of the 21 DCs; the SW classification is also represented in this Figure. Those cell pairs that are colored differently from white or gray represent the results collating sensitive CRAC-PCs and not-closed SWs. On the other hand, white cells correspond to resistant CRAC-PCs, and gray cells are representative of the closed SWs. Results codified by the gray cells are comparable with SWs determined with 5FU/Oxa. In total, 10/12 (83.3%) sensitive or very sensitive CRAC-PCDs, which were coupled with 27/252 (10.7%) assays with a not-closed SW, were identified. The CRAC-PCs that formed pairs with two or more DCs were as follows: With two DCs, CRAC-PC3 with DC-12 and -19, and CRAC-PC9 with DC-11 and -15; with four DCs, CRAC-PC-1 with DC-1, -8, -12, and -19; with five DCs, CRAC-PC8 with DC-2, -4, -8, -12, and -16, and with eight DCs, CRAC-PC-11 with DC-1, -4, -8, -10, -12, -15,-16, and -19. In contrast, 6/12 (50%) CRAC-PCs (2, 4, 5, 7, 10 and 12) did not match with susceptibility or non-closed SWs with any DC.

Discussion

The present study was performed to offer preliminary information that may be useful for the development of new chemotherapeutic regimens to treat patients with CRC in a personalized way. Personalized chemotherapeutic regimens thereby obtained will hopefully improve on the current survival rates of patients with CRC treated with conventional procedures. The findings of the present study have demonstrated that combinations of the first-line drugs (5FU/Oxa) plus NFLDs, or of only NFLDs, may be more efficacious against CRAC-PCs, and offer wider SWs than using only 5FU/Oxa.

A total of 21 DCs were assayed against 12 CRAC-PCs. These CRAC-PCs were obtained by modifying the original method [25]. This modification con-

sisted of preventing the centrifugation step of the tumor cell suspension in a density gradient. This modification was made since an unknown number of malignant and tumor-associated cells may be lost during their separation and in the recovering procedures, which has a significant impact when the tumor sample is rather small. In this scenario, and as would be the case in the present study, it would be challenging to recover the cells of interest in a sufficient quantity via a cell-enrichment step in a density gradient to permit the necessary series of drug-sensitivity assays to be performed. Furthermore, the tumor-associated cells, such as fibroblasts, vascular endothelial cells, pericytes, adipocytes, and bone marrow mesenchymal stem cells, can help malignant cells to proliferate, and thereby resist the effect of the anti-cancer drugs [27]. Therefore, assays that were to be performed without the original number of malignant and supporting cells from a tumor may result in an overestimation of the efficacy of an anti-cancer drug.

The 21 DCs assayed in the present study belonged to two categories: The first (10 DCs) included only 5FU/Oxa, or 5FU/Oxa and between one and three NFLDs. The other group, featuring the other 11 DCs, comprised NFLDs (2-4) exclusively. In all these assays, the efficacy of the DCs determined as the PCDs and SWs, measured in comparison with the 5FU/Oxa treatment, were taken as the reference parameters. The concentration of all drugs in the 21 DCs was equivalent to the CD_{50} of each of these drugs, previously determined with the COLO-320DM cell line [6]. The highest concentrations that each drug may attain in blood plasma were not used, taking into consideration that, by using submaximal doses of each drug, a sufficient margin was available for the combined effects of all DCs to be evaluated.

A significant proportion of the DCs was identified to have as little efficacy as the first-line treatment, i.e., 5FU/Oxa was little effective against the majority of CRAC-PCs. These findings suggested that patients from whom the CRAC-PCs were assayed, and treated with FOLFOX-6, would not able to respond satisfactorily to this regimen. Notwithstanding this fact, all DCs, except one, were active, or very active, against CRAC-PCs in 92 of the determinations. The number of assays where the CRAC-PCs were identified to be either sensitive or very sensitive to the DCs was 30% higher in those cases where the DCs included only NFLDs, compared with those where the DCs contained 5FU/Oxa plus NFLDs. It was interesting that the DCs that were associated with the highest number of sensitive, or very sensitive CRAC-PCs contained Doxo and Carbo, or Doxo/Carbo and Cis. This phenomenon was observed whether or not the DCs contained 5FU/Oxa. The beneficial effect of DIM or Asp appeared to depend on the other components of DCs (i.e., 50% of CRAC-PCs were sensitive to 5FU/Oxa plus Asp, and eight CRAC-PCs were sensitive (seven) and very sensitive (one) to Doxo/Carbo and DIM, and seven CRAC-PCs were sensitive (five) or very sensitive (two) to Doxo/Carbo and DIM. In opposite terms, only one CRAC-PC was sensitive to 5FU/Oxa plus DIM or 5FU/Oxa and DIM/Asp; and none CRAC-PC was very sensitive to 5FU/Oxa/DIM or 5FU/Oxa/DIM/Asp.

The SWs were determined to estimate the balance between the efficacy (in terms of PCD) of DCs on CRAC-PCs, and their cytotoxicity (also measured by the PCD) on healthy hMSCs cells. In other words, the larger the quotient of the PCD of CRAC-PC/PCD of healthy cells, the wider was the SW, and therefore the lower would be the toxicity for healthy cells, and *vice versa*. In this regard, a previous publication from our laboratory proposed the use of hMSC-TA cells, considering all the advantages that these cells offer [6]. The SWs were classified according to the ranges of the quotients above. Closed SWs corresponded to all the results [12] obtained with 5FU/Oxa, as well as the highest number of assays performed with the other 20 DCs. To obtain the result of a closed SW suggests that the DC in question could be as toxic for certain patients as 5FU/Oxa treatment would be. Notwithstanding this, 43 non-closed SWs, suggesting that some of DCs evaluated in the present study, or new alternative DCs could be safer than conventional anti-CRC drugs for healthy human cells, and, by analogy, for patients. In a future clinical trial, the source of hMSC-TA cells must be autologous, i.e., the adipose tissue is required to be derived from the same patient from which the CRAC-PC was obtained.

Despite the valuable information produced by the above analyses, wherein the efficacy of DCs or their SWs were assessed, these parameters still have limited usefulness, if the information concerning to the DCs efficacy or their SWs are considered separately. It does not matter if, in a group of patients, each of these parameters is analyzed as a whole, or broken down in sets, or individually. This situation is due to neither the DCs´ efficacy nor their SWs allow, by itself, knowing to the two essential qualities of a DC: to be efficacious and safe enough for a specific CRAC-PC. Therefore, a color-coded chart has been included in the present study, showing the sensitivity (PCDs) and SW for each of the 12 CRAC-PCs, determined with each of the 21 DCs here evaluated. In this way, the following observations may be made: i) The efficacy with PCDs (0-0.6), their SWs (closed), or both parameters of 8 out of 21 of the DCs on 12/12 CRAC-PCs was

unsatisfactory. Therefore, in a clinical trial, these eight DCs could be as little efficacious and iatrogenic as 5FU/Oxa. Therefore, it could be worth to discard these DCs and not used them to treat any of those patients who donated the 12 tumor samples. In clinical cases like these, alternative DCs could be advisable to employ upon specific CRAC-PCs derived from each patient: ii) Thirteen DCs showed acceptable efficacy and SW on between one and four of the CRAC-PCs. In these cases, the oncologist could choose the most efficacious and safe DC: iii) It was noticeable that, of the 252 assays, only 26 (10.3%) of them were shown to be efficacious for 7 of the 12 CRAC-PCs, and their SWs were also acceptable. Of these CRAC-PCs, four were susceptible to one DC, and this DC had an acceptable SW (in the majority of the cases, exhibiting a narrow SW). In those cases that showed resistance to the available DCs or a closed SWs, the health team should design and test novel DCs with the corresponding CRAC-PCs; iv) The high variability in drug susceptibility and SW observed in the CRAC-PCs suggests that each tumor from which the CRAC-PC was derived had a particular pattern of gene expression, which is congruent with the conclusions of previously published studies [28,29]. The individual susceptibility to different DCs, and the resistance presented by all 12 CRAC-PCs that were analyzed in the present study, reinforce the hypothesis that the drug susceptibility should be tested in order to select the appropriate treatment in a personalized manner, as has been previously suggested [29,30], rather than applying, *a priori* a conventional treatment; v) It is noteworthy that the DC that exhibited suitable efficacy and an acceptable SW on most CRAC-PCs (4) was DC12, which was composed by Carbo and Cis. The efficacy of DC12 was followed by that of DC8, DC15 and DC16, which showed their usefulness on three CRAC-PCs. These latter three DCs were composed by 5FU/Oxa/Asp, Doxo/Carbo/DIM and Doxo/Carbo/Asp. Our group demonstrated previously that DIM and Asp made an important contribution to increasing the SW for the CRAC cell line, COLO-320DM [7]. Furthermore, Asp has been found to be efficacious against prostate cancer [31], and to control the incidence of breast cancer [32]. Therefore, it may be worth including DC-8, -12, -15 and 16 and novel drug combinations including DIM or Asp in future evaluations of CRAC drug susceptibility and DC SWs to analyze in more detail the protective roles of Asp and DIM, as well as to investigate their ability to enhance the anti-cancer efficacy of 5FU/Oxa and other anti-cancer drugs. Regarding this approach, Kim et al [8] pointed out that DIM may potentiate the anti-cancer effects of other anti-cancer drugs, a finding that was corroborated by the findings of the present study; vi) As mentioned above in the Introduction, all the NFLDs included in this study are used intensively in daily medical practice as anti-cancer medications. Therefore, the pharmacological, toxicological, and therapeutic characteristics of all the five NFLDs used in the present study are widely known. Thus, the 12 DCs that were identified as efficacious and safe for hMSCs could readily be tested in a clinical assay, supported by a previous *ex vivo* evaluation.

The individualized evaluation of those DCs and personalized treatment using the approach described in the present study could be applied for other types of cancer, and bring to physicians the opportunity to repeat the *ex vivo* assays periodically, using primary cultures of primary tumors or metastases. Furthermore, considering that malignant cells frequently change their gene expression [32], the *ex vivo* assays should be performed periodically in each patient to opportunistically evaluate whether the previous chemotherapy is no longer efficacious and to identify alternative DCs for a change in the chemotherapy regimen. In this way, the quality of life and survival time of patients may be improved.

In conclusion, the present study has identified the following features: i) It has been demonstrated that sATP-CRA, CRAC-PCs, and hMSCs constitute a useful system to determine the susceptibility of CRAC tumors to alternative DCs, and to evaluate their SWs; ii) All the CRAC-PC samples were resistant to 5FU/Oxa, and the associated SWs were revealed not to be satisfactory for any CRAC-PC; iii) NFLDs were shown to increase the efficacy and safety of 5FU/Oxa; iv) Each CRAC-PC reacted in a particular way to DCs containing conventional first-line drugs and NFLDs, indicating that it is mandatory to treat the patient concerned with CRC in a personalized manner, taking into account the specific DC to which his/her cancer is susceptible. Using alternative DCs and personalized treatments that are based on *ex vivo* results demonstrating efficacy and safety can improve the life expectancy of patients and diminish the iatrogenic effects of conventional therapeutic schemes.

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Conflict of interests

The authors declare no conflict of interests.

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