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ORIGINAL ARTICLE

Remarkably higher efficacy and a wider safety window for nonfrontline over first-line drug combinations in the adenocarcinoma Colo 320DM cell line

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Summary

Purpose: *To determine in vitro, the efficacy and safety window of not-front-line and first-line anti-colorectal (CRC) drug combinations.*

Methods: *The adenocarcinoma cell line Colo 320DM and normal human mesenchymal stem cells derived from adipose tissue were used respectively to determine the anti-CRC efficacy (% of Colo 320DM cell death [CD]) and safety window [SW] - % Colo 320DM percent cancer death (PCD)/% of mesenchymal stem cell's death) of drug combinations, using the adenosine triphosphate-based chemotherapy response assay (ATP-CRA).*

Results: *First-line anti-CRC drug combinations (5-fluorouracil [5FU]/oxaliplatin [oxa] and 5-FU/Oxa /leucovorin [Leuco]) produced 57.7% and 52.4% CD, and 1.38 and 2.44 SW, respectively. Combinations of 5-FU/Oxa and 1 to 3 non-front line drugs led to 56.3–99.8% CD and to 0.96–2.2 SW. The highest safety window corresponded to 5FU/Oxa/carboplatin [Carbo] (93% CD and 1.4 SW) and to 5-FU/Oxa/cisplatin [Cispl] (93.5% CD and 1.4 SW). In contrast, non-front line drugs led to 89.8–97.4% CD and to 1.1–78.2 SW. Outstandingly, those combinations containing Carbo/Cispl/3,3'-diindolylmethane (DIM), aspirin (Asp), or 3,3'-DIM/ Asp showed a very high CD (91.9-96.9% [39.2–39.5 times higher than first-line-combined drugs]) and very wide SW (57.8–81.56 [66.6–40 times higher than the first-line drug combinations]).*

Conclusions: *Human mesenchymal stem cells could be an excellent alternative to laboratory animals, when testing the safety profiles of drugs. The most promising combinations of non-frontline drugs to treat CRC are Carbo/Cispl/Asp and Carbo/Cispl/DIM.*

Key words: *Alternative anti-cancer drugs, ATP-CRA, colorectal cancer, first-line anti-colorectal drugs, human mesenchymal stem cells, non-frontline anticancer drugs*

Introduction

CRC is the third-leading cause of cancer death worldwide [1]. FOLFOX, which includes 5-FU, Oxa and Leuco, is one of the most widely used combinations as first-line treatment in patients with CRC [2]. Standardized chemotherapeutic regimens to treat CRC have improved patients' quality of life and life expectancy [3]. However, most patients develop resistance to these treatments and die within 1–10 years after treatment initiation [4]. On the other hand, non-frontline chemotherapeutic agents have shown better results when compared to standardized first-line chemotherapeutic drugs regimens,

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mainly when used as adjuvant therapies. Carbo, doxorubicin (Doxo), Cispl, Asp, and DIM are of special interest because none of these compounds have yet been used as anti-CRC agents; they rather have been used to successfully treat other types of cancer [5-11]. Therefore, it appears to be advisable to test non-frontline anticancer drugs as adjuvants of conventional chemotherapeutic schemes to investigate if these alternative treatments increase both the survival and quality of life of patients with cancer. An essential prerequisite to finding promising alternative combinations of anticancer drugs consists of testing them *in vitro* on a CRC line. Furthermore, a very specific and sensitive method is needed to evaluate the efficacy of anticancer drugs, such as ATP-CRA (an adenosine triphosphate-based chemotherapeutic response assay) [5-7]. Another important issue in anticancer drug evaluation is to determine *in vitro* the SW of the drug combination of interest. The SW is an efficacy/safety ratio that estimates the difference in the therapeutic effect of interest on malignant and normal cell cultures [12]. In seeking new anticancer compounds, the cells used to evaluate their safety should ideally be of human origin; these cells should be normal, healthy, and easy to culture and expand *in vitro*. While considering these aforementioned factors, we chose adult human mesenchymal stem cells derived from adipose tissue (hMSC-AT), which meet all the aforementioned characteristics [13].

The aim of this study was to evaluate and compare the *in vitro* efficacy and safety of first- and nonfront line drug combinations.

Methods

Adenocarcinoma cell line

Colo 320DM cells (ATCC CCL-220; American Type Culture Collection, Manassas, VA, USA) were used to determine the anti-CRC efficacy of separate drugs or drug combinations. The cells were maintained in RPMI culture medium with 10% fetal bovine serum (FBS), 100 µg gentamycin/mL, 100 µg streptomycin/mL, 100 µg penicillin/mL, and 2.5 µg amphotericin B/mL (enriched RPMI).

Human mesenchymal stem cells

hMSC-AT cells were isolated from surplus adipose tissue, which was obtained by liposuction from the abdominal adipose panniculus of two men and two women, who underwent plastic surgery, providing informed consent at the Plastic Surgery Service of the University Hospital "José Eleuterio González", Autonomous University of Nuevo León (UANL). The samples were divided into two 25 mL aliquots, put into 100 mm diameter Petri dishes (NalgeneNunc International, Penfield, NY, USA), and treated with collagenase type I (0.04g/mL). Cells were subsequently incubated for 1 hr at 37°C in 5% CO_2 atmosphere. The digested adipose tissue was centrifuged four times at 500×g for 15 min, and the supernatant was discarded. The pellet was re-suspended with 40 mL of phosphate-buffered saline (PBS:Gibco; Thermo Fisher Scientific, Waltham, MA, USA) in α-Minimum Essential Medium (MEM) culture medium (Gibco; Thermo Fisher Scientific) with the addition of 30 mg bovine serum albumin/mL, 50 µg gentamycin/mL, and 2.5 µg amphotericin B/mL. Onwards, this medium is called enriched α-MEM. The number and percentage of viable cells was determined using the Trypan blue technique and 3.5×10^4 cells were inoculated in 25 cm^2 canted neck cell culture flasks with vent caps (Corning) containing 5 mL of enriched α-MEM. The flasks were incubated at 37°C in a 5% CO_2 atmosphere for 3 days. The supernatant was incubated in new flasks for 4 days, and the former flask, containing the remaining cells, was added with 5 mL of fresh enriched αMEM, as before. This procedure was repeated two more times.The cells from all cultures were mixed together and incubated (3.5×10^4cells), as before, until the cell cultures reached 80% confluence. Then, the cells were detached with 0.25% trypsin, washed with PBS, and suspended in 10% dimethylsulfoxide and 90% FBS. Then, their concentration was adjusted at 1×10^6 cells/mL and placed into 2 mL cryovials (Corning), in 1.0 mL aliquots and preserved in liquid nitrogen until used.

Immunocytochemistry characterization of hMSC-AT

Cells in one of the above cryovials were thawed, washed thrice with enriched a-MEM, their density was adjusted to 4×10^4 viable cells/mL and 500 µL were poured into each of the four culture microchambers from a Chamber Slide System (LabTEK, Naperville, IL, USA).

These were incubated at 37°C in a 5% CO_2 atmosphere until reaching 80% confluence. The cell monolayers were gently washed with PBS, fixed with ethanol/acetone (1:1 v/v) at 4°C for 20 min and flooded for 2 min in PBS at 4°C and, then, in Tris buffer plus Tween 20 (TBST) at 37°C for 5 min. The endogenous peroxidase was blocked by incubating these preparations with 150 µL of 3% hydrogen peroxide at 37°C for 10 min. These preparations were washed with TBST and added with 150 µL of anti-CD105 monoclonal antibodies (Abcam, Cambridge Science Park, Cambridge, UK), diluted with DakoCytomation antibody diluent, added with reducing components 1:10 (v/v) (Agilent Technologies, Dako Denmark A/S, Glostrup, Denmark), and incubated in a closed chamber and 4°C for 12 hrs. The preparation was washed with TBST and added with 150 µL of Dako'sEnVision system. These preparations were incubated at 37°C for 45 min, washed with TBST, added with 150 µL of 3-3´-diaminobenzidine (DAB) (Abcam), incubated at 37°C for 10 min, and washed thrice with distilled water. Cell nuclei were contrasted with 150 µL of Meyer's hematoxylin at 37°C for 1 min. The preparations were washed with distilled water at 37°C for 5 min, and dehydrated by successive immersions in 70, 95, and 100% ethanol dilutions and 100% xylol. The preparations were then mounted with Entellan montage medium (Mallinckrodt Baker, Inc., México City, Mexico); a coverslip was put over the

preparation and was observed under a Nikon Eclipse 50i microscope equipped with a Digital Camera Sight DS-2MV XCite120 EXFO and analyzed with Nikon's image analyzing system (Nikon, Konan, Minato-ku, Tokyo, Japan) with the aid of the ENIS-elements BR 2.30 software (ENIS-Elements B12 Model 2.30; Nikon). In this immunocytochemical characterization of hMSC-AT cells, human jejunum and liver histological cuts were included as negative (according to our own experience) and positive [14] controls, respectively. To perform the present study, liver and jejunum paraffin preparations were heated at 37°C for 30 min, immersed into 100% xylol for 5 min, rehydrated with 3-min successive immersions in 100, 96 and 70% ethanol, distilled water and TBST, respectively, with target retrieval solution (TRS) (Dako) at 90°C for 1.0 hr and labeled following the same procedure used for the hMSC-AT cells. The mean ± standard deviation (SD) of CD-105-positive and CD105-negative cells was determined by counting 5 fields on 3 different preparations of hMSC-AT, as well as negative and positive controls.

Cytotoxicity was evaluated with ATP-CRA [7], using the CellTiter-Glo Luminescent cell viability assay kit (Cat. G7572; Promega Corporation, Madison, WI, USA), following the manufacturer's instructions, with the next particularities: each of the 96 wells of a White Cliniplatemicroplate (Thermo Fisher Scientific) was added with 2×10^4 Colo320DM cells suspended in 50 µL RPMI cell culture medium (Gibco®; Thermo Fisher Scientific) and 50 µL of an individual drug (Table 1) or drug combination (Table 2). In all bioassays, the maximum concentration reached in the blood plasma by each drug [16] (named [1X]) was used as a relative concentration reference (Table 1). Carbo and Oxa were obtained from Asofarma de Mexico (México City, Mexico); Cispl, 5-FU, and Doxo were purchased from TEVA (Naucalpan de Juárez, Mexico). The cytotoxicity of individual drugs on hMSC-AT cells was evaluated using the same aforementioned method. All chemotherapy drugs used in this study were of reactive grade and were purchased from Sigma Aldrich Co. Saint Louis MO. USA.

Table 1. Cytotoxicity of anticancer first-line and nonfrontline individual drugs in Colo 320DM cell line

Drug	*PCD*[a]	*CD_{50}*[b] *(µg/mL)*	*r^2*[c]
5-FU	55.9±5.6	8.58	0.956
Oxa	97.1±0.8	0.40	0.904
Cispl	99.3±0.1	0.53	0.912
Carbo	96.5±0.6	5.47	0.929
Doxo	66.6±5.8	1.04	0.983
Asp	26.7±2.4	1,584	0.936
DIM	5.5±4.0	ND[d]	0.208[d]

[a]PCD: percentage of cell death produced by the highest concentration reached in blood plasma [1X]: 5-fluorouracil (5-FU) 10 µg/mL, oxaliplatin (Oxa) 2.9 µg/mL, carboplatin (Carbo) 12 µg/mL, doxorubicin (Doxo) 1.5 µg/mL, cisplatin (Cispl) 2.5 µg/mL, diindolylmethane (DIM) 0.6 µg/mL, and aspirin (Asp) 396.5 µg/mL; [b]CD_{50},concentration that caused 50% cell death; [c]r^2 correlation coefficient with the straight line; dDIM toxicity did not reach CD_{50}

PCD and CD_{50} estimation and statistics

These assays were performed using colo320DM as a target. The PCD and CD_{50} of each drug were calculated by interpolation of the correspondent dose/response curve. Each curve was constructed by including a 1x concentration of each drug (see the toxicity assay method detailed above), as well as two of its multiples (4x and 2x) and four of its submultiples (0.5x, 0.25x, 0.125x, and 0.065x). The results were expressed as PCD as a function of the $\log_{10}$ ng/ml of each of the aforementioned first-line and non-front-line anti-CRC drugs. The correlation with the straight line (r^2) of each dose–response curve was estimated with the aid of the Excel 2007 statistical package (Microsoft Co., Redmond, WA, USA).

All determinations were made in triplicate and three different experiments (n=9). The statistical comparisons of means presented in this study were performed by applying a two-tailed paired samples Student's t-test using the Excel 2007 statistical package (Microsoft Company).

Ethical aspects

This project was authorized by the Scientific, Biosafety and Bioethical Committee of the Medicine School, UANL (register BI114-004).

Results

Cytotoxicity of individual drugs on Colo320DM

The CD produced by each drug as a function of its concentration (expressed as Log_{10}) was linear (r^2>0.90), except DIM. The potency of [1X] evaluated agents, was as follows: Cispl 1.8 times >5-FU; 1.02 times >Oxa;1.49 times >Doxo;1.02 times >Carbo;3.72 times >Asp; and 22 times >DIM (Table 1).

Characterization of hMSC-AT

Figure 1A shows that the majority of hMSC-AT (55.68%±8.83) was positively labeled with anti-CD-105 antibodies. Positive and negative labeling controls for anti-CD105 are shown in Figures 1B and 1C.

Cytotoxicity of individual drugs on hMSC-AT and safety window

PCD tested on hMSC-AT was 5-FU, 30.07; Oxa, 18.02; Cispl, 89.78; Carbo, 65.55; Doxo, 89.34; Asp, 0.0; and DIM, 0.0. Their SW was 5-FU, 1.82; Oxa, 5.39; Cispl, 1.11; and Carbo, 1.47. The Asp and DIM SW were not toxic on hMSC-AT cells.

Cytotoxicity of drug combinations on Colo320 cells and safety window

Table 2 shows that the highest CD in group 1 was observed in C1, which featured a slightly lower CD (52.4%) than in C2. With the exception of C6 (Asp/DIM), the CD produced by all combinations

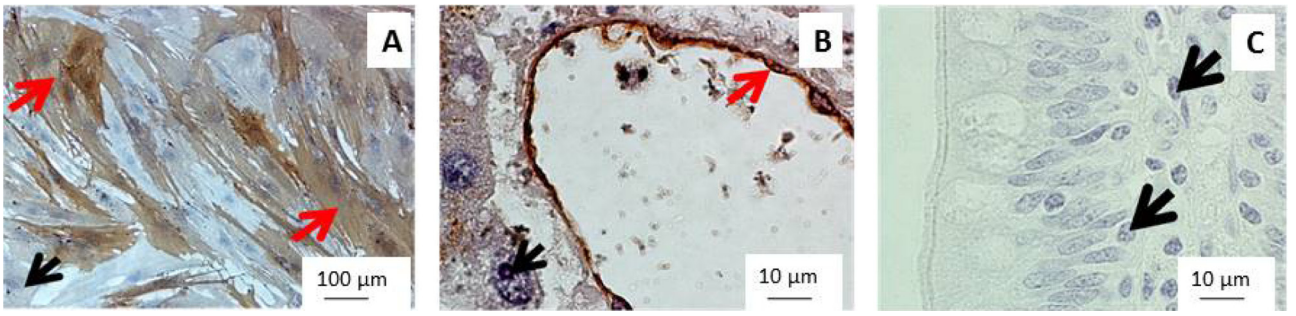

Figure 1. Immunocytochemistry characterization of hMSC-AT.
(A) shows the appearance of human mesenchymal stem cells derived from adipose tissue (hMSC-AT). These cells show a typical fibroblast shape and a heavy CD-105 label (red arrows). **(B)** as a positive control, this image shows the central vein of a human liver, where the surface of the endothelial cells was labeled with antiCD105 antibodies (red arrow). **(C)** as a negative control, this image shows human jejunum, which was treated following the same procedure employed with the hMSCs and the liver vein. The three images show the cell nuclei, which were counterstained with Meyer's hematoxylin (black arrows).

Table 2. Anti-ACRC and safety window of first-line and non-front line anti-ACRC drug combinations[a]

Number	*Drug combination*[b]	*PCD*[c]		
		Colo320DM	*hMSC-AT*[d]	*SW*[e]
Group 1. Drugs used in the FOLFOX schemes				
C1	5-FU/Oxa	57.7±4.94	40.1±10.6	1.38*
C2	5-FU/Oxa/Leuco	52.4±1.49	21.6±2.06	2.44**
Group 2. Non-front-line anticancer drugs				
C3	Carbo/Doxo	95.4±0.86	85.9±0.72	1.1**
C4	**Carbo/Cispl**	**96.9±0.34**	**2.5±1.77**	**57.79* 5****
C5	Carbo/Cispl/Doxo	99.3±0.03	91.7±0.61	1.1*,**
C6	Asp/DIM	2.7±3.72	0.0±18.91	0.07*,**
C7	Carbo/Doxo/DIM	90.9±0.24	71.5±1.02	1.3**
C8	Carbo/Doxo/Asp	97.5±0.92	79.5±1.83	1.2**
C9	Carbo/Doxo/Asp/DIM	89.8±0.67	80.4±0.41	1.1**
C10	**Carbo/Cispl/DIM**	**93.8±0.35**	**1.2±0.21**	**78.2*,****
C11	**Carbo/Cispl/Asp**	**94.6±0.58**	**1.6±0.55**	**59.1*,****
C12	**Carbo/Cispl/DIM/Asp**	**91.9±0.23**	**1.4±0.33**	**65.4*,****
C13	Carbo/Cispl/Doxo/DIM	97.4±0.30	86.3±2.7	1.1**
C14	Carbo/Cispl/Doxo/Asp	99.8±0.02	87.9±1.5	1.1**
Group 3. Drugs used in the FOLFOX scheme combined with non-frontline anticancer drugs				
C15	5-FU/Oxa/Asp	60.1±8.83	50.6±11.6	1.2**
C16	5-FU/Oxa/DIM	57.7±3.4	58.7±11.1	1.0*.**
C17	5-FU/Oxa/Asp/DIM	56.3±11.0	53.3±9.59	1.1*,**
C18	5-FU/Oxa/Carbo/Doxo/Cispl	99.8±0.11	97.5±0.83	1.0*,**
C19	5-FU/Oxa/Carbo/Cispl	98.8±0.32	83.3±5.73	1.2**
C20	5-FU/Oxa/Carbo/Doxo	97.8±1.1	97.0±1.26	1.0*,**
C21	**5-FU/Oxa/Cispl**	**92.75±3.46**	**45.4±12.0**	**2.2*5**
C22	5-FU/Oxa/Doxo	90.1±4.5	94.0±2.23	0.96*, **
C23	**5-FU/Oxa/Carbo**	**93.55±1.46**	**68.5±6.1**	**1.4**5**

[a]Anti-ACRC refers to anti-adenocarcinoma colorectal cancer acting against the Colo320DM cell line. [b]All drug combinations contained a concentration of each particular drug equivalent to that produced at 50% of CD (CD50), with the exception of DIM, which had a concentration equivalent to its highest concentration reached in plasma. Table 1 shows these values and the meaning of the drug abbreviations. Drug combinations, CDs, ratios, and P-values shown in bold typeface indicate the values belonging to the highest CD found in each of the three groups of drug combinations evaluated herein. [c]Percentage of cell death. dHuman mesenchymal stem cells derived from adipose tissue. [e]SW, means safety window. It is the ratio of PCD on Colo 320DM/PCD hMSC-TA cells. The bold typeface indicates a noticeably wide SW (Group 2) or a higher ACCR efficacy and comparable SW with respect to C1 (group 3). One asterisk indicates a significant difference ($p<0.05$) with respect to C1, and two asterisks indicate a significant difference ($p<0.05$) with respect to the C2 ratio (SW). Drug combinations, PCDs, ratios, and p values shown in bold typeface indicate the values belonging to the highest PCD found in each of the three groups of drug combinations evaluated herein.

of non-frontline anti-CRC drugs was greater than 89.8% and higher than C1–CD or C2–CD. The CD values were as follows: 1.6 times (C9–CD/C1–CD ratio) to 1.7 times (C14–CD/C1–CD ratio); and 1.9 times (C14–CD/C2–CD ratio) to 1.7 times (C9–CD/C2 times – expressed as ratios – of drug blends in group 3CDs with respect to C1–CD and C2–CD). This blend contained Cispl in addition to Carbo/Doxo. Nevertheless, when Carbo and Doxo were added with DIM or ASP (C7–C9), the cytotoxic potency diminished significantly (15.4 to 5.5%), and when Carbo/Cispl/Doxo was added with DIM or Asp (C13–C14) the CD diminished to 3.79–5.36%. CD produced by blends containing 5-FU/Oxa (C1) or 5-FU/Oxa/Leuco (C2) was lower than the drug combinations containing 5-FU/Oxa plus non-frontline drugs. The ranges were as follows: regarding CD–C1, the lowest difference was observed for CD–C15 (10.5% higher) and the highest difference was that of C18–CD at 57.4%. Regarding C2–CD, the lowest difference on hMSC-TA toxicity was C15–CD (29.0% higher), and the highest was for C18–CD (75.9% higher). All the aforementioned differences were statistically significant except for CD-C15 compared to CD-C1 or C2 (Table 2).

Safety window

In groups 2 and 3, most drug combinations showed safety windows of 1.1–1.3. Nevertheless, in group 2, we observed four noticeable exceptions corresponding to C4, C10, C11, and C12. All these blends contained Carbo/Cispl and showed SW of 41.9–56.7, which were wider than the C1 SW, and which was also 23.7–32 times wider than the C2 SW. Among these four exceptions, the combination that showed the largest safety window was the one featuring DIM, followed by the combination that contained both DIM and Asp, and then by the combination that contained Asp. Regarding the drug combinations from group 3, we noted that combinations containing Cispl or Carbo (besides 5-FU/Oxa) showed a SW similar to those of C1 (C21) and C2 (C23). Nevertheless, the CD of C21 and C23 was 1.59 and 1.62 times higher than that for C1–CD, and it was 1.75 and 1.78 times higher than that for C2–CD, respectively. All of these differences were statistically significant.

Discussion

The results of this study suggest that the ATP-CRA is useful for determining the sensitivity or resistance of cells derived from a human adenocarcinoma cell line, that adequate combinations of front-line anti-ACCR (adenocarcinoma of colon and rectum) drugs can be more efficacious and safer than first-line drug combinations (such as FOLFOX), and that even combinations of first-line and some non-front-line drugs can offer a similar SW, but a higher efficacy against CRC cells than those of first-line drugs. In addition, the results showed that hMSC-AT is a very good model for determining the SW of anticancer drugs.

The hMSC-AT model

In discovering and developing anticancer drugs, a preliminary screening in cell culture models is typically carried out to identify the extent and specificity of their antitumor activity. These experiments are followed by the evaluation of efficacy and toxicity in animal models. Animal screening models predominantly focus on tumor regression, animal survival, and safety [16]. As an alternative, in the present study, we propose to use hMSCs, which are normal and healthy. This approach could allow specific differences between cancer cells and normal human cells to be identified in a manner that is more practical, easier, more economical, and ethically more acceptable than using laboratory animals, especially when drug discovery or new uses of old drugs (as in the present case) are in their early phases. To this purpose, we currently used hMSCs without differentiating them in a preliminary screening to define the SW 23 anticancer drug combinations.

Cytotoxicity of individual drugs on Colo 320DM

The CD_{50} of Doxo that we determined in Colo 320DM was similar to that reported for other ACCR cell lines [23], with some quantitative differences [18-22], which could be due to the genetic heterogeneity among different cell lines [23] or to intrinsic characteristics of the bioassays used to evaluate these anticancer drugs.

Cytotoxicity of individual drugs on hMSC-AT

These experiments allowed us to determine the individual CD_{50}, and to be sure that this was not higher than the IX concentration and thus, neither any drug was too toxic for our cell models, nor it exceeded the physiological availability.

Drug combination cytotoxicity and SW

The main purpose of the present study was to determine if non-front-line anti-CRC drug combinations or combinations of non-front-line plus front-line anti-CRC drugs could be useful as new chemotherapeutic schemes to treat CRC. To achieve this, we made several interesting observations: 1) the first-line anti-CRC combination

containing Leuco(C2) was less potent against Colo 320DM and much less toxic against hMSC-TA than 5-FU/Oxa (C1);C2 also featured a SW that was 1.8 times greater than C1. This finding confirms the protective role of Leuco for normal cells. Nevertheless, at least in experiments performed *in vitro*, our results also showed that Leuco does not always enhance the anticancer effect of chemotherapy agents, as is generally accepted [24]. All non-frontline anticancer combinations showed a very high activity against Colo320DM, except for Asp/DIM, which showed almost null toxicity on normal cells (hMSC-TA). Furthermore, combinations containing Carbo/Cispl, Asp or DIM, and Asp plus DIM did not contribute considerably to enhance the anti-Colo320DM cytotoxicity of Carbo/Cispl, but they remarkably protected the normal cells, thus markedly opening the SW. Therefore, the combinations of Carbo/Cispl/Asp, Carbo/Cispl/DIM, or Carbo/Cispl/Asp/DIM could serve as excellent alternatives in chemotherapy schemes against CRC. Conversely, Carbo/Doxo plus DIM or Asp blends showed a very narrow safety window – very near to 1.0 – meaning that these combinations are almost toxic to both cancer cells and normal cells. The same unfavorable situation was observed in group 3 (first-line agents combined with nonfront-line drugs, which showed SW approaching 1.0). Exception to this finding were C21 and C23, which showed SW similar to those for C1 and C2; however, these agents also exhibited cytotoxicity toward Colo 320DM cells that was almost twice as high compared with 5FU/Oxa or 5-FU/Oxa/Leuco. Thus, 5-FU/Oxa/Cispl and 5-FU/Oxa/Carb could be more efficacious against CRC than 5-FU/Oxa or 5-FU/Oxa/Leuco, although these agents exhibit comparable toxicity against normal cells.

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Conflict of interests

The authors declare no confict of interests.

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