Biochemical and Biophysical Research Communications 424 (2012) 45–51

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Diindolilmethane (DIM) selectively inhibits cancer stem cells

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ARTICLE INFO

Article history:
Received 12 June 2012
Available online 19 June 2012

Keywords:
Diindolylmethane
Cancer stem cells
Chemoresistance

ABSTRACT

Epidemiologic studies repeatedly have shown chemopreventive effects of cruciferous vegetables. Indole-3-carbinol (I3C) and its metabolite diindolylmethane (DIM) were identified in these plants as active ingredients and theirs anti-tumor activities were confirmed in multiple *in vitro* and *in vivo* experiments. Here, we demonstrate that DIM is a selective and potent inhibitor of cancer stem cells (CSCs). In several cancer cell lines, DIM inhibited tumor sphere formation at the concentrations 30–300 times lower than concentrations required for growth inhibition of parental cells cultured as adherent culture. We also found that treatment with DIM overcomes chemoresistance of CSCs to cytotoxics, such as paclitaxel, doxorubicin, and SN-38. Pre-treatment of tumor spheres with DIM before implantation to mice significantly retarded the growth of primary tumors compared to tumors formed by untreated tumor spheres. The concentrations of DIM required to suppress CSCs formation are in the close range to those achievable in human plasma after oral dosing of the compound. Therefore, DIM can potentially be used in cancer patients, either alone, or in combinations with existing drugs.

1. Introduction

In spite of the progress in the development of new anti-cancer drugs, acquired chemoresistance of tumors, their recurrence and metastases are still the main obstacles in anti-cancer therapy. Cancer stem cell hypothesis has been recently introduced to explain this phenomenon. According to this theory, tumors contain a small number of tumor-forming, self-renewing, cancer stem cells (CSCs) within a majority population of non-tumor-forming cancer cells [1]. Unlike most cells within the tumor, CSCs are resistant to chemotherapy and radiotherapy, and may contribute to tumor progression, formation of metastases and disease recurrence [2]. New agents that selectively target CSC could offer a great promise for cancer therapy and prevention. So far, only very few drugs have been shown to be specific for CSCs. One such drug, salinomycin, a potassium ionophore used as an agricultural antibiotic, reduced the proportion of CSCs by more than 100-fold relative to paclitaxel [3]. Another drug, metformin, a standard agent for diabetes, selectively killed CSCs in several breast cancer cell lines *in vitro* and *in vivo* [4]. Unfortunately, salinomycin is very toxic to humans [5] that prevents its clinical use. Metformin, on the other hand, is not very potent; against CSCs it works only at high concentrations of 100–300 μM, which is beyond physiologically achievable limit. Thus, more potent and less toxic inhibitors of CSCs are in great demand.

Due to their intrinsic tumor-forming ability, CSCs could be a target for agents preventing cancer formation. 3,3′-Diindolylmethane (DIM), emerged from epidemiology studies demonstrating that dietary consumption of cruciferous vegetables including broccoli, cauliflower and Brussel's sprouts may provide protection from several types of cancer [6]. Phytochemicals identified in cruciferous vegetables include indole-3-carbinol (I3C) and DIM, its major metabolite. Because I3C in cell culture medium or in stomach is readily converted into DIM, most of the activities observed for I3C *in vitro* and *in vivo* have been related to DIM action [6,7]. DIM targets multiple components of cancer cell cycle regulation and survival including Akt-NFkB signaling, caspase activation, cyclin-dependent kinase activities, estrogen receptor signaling, and endoplasmic reticulum stress [7,8]. DIM also activates aryl hydrocarbon receptor (AhR) [9], which plays an important role in its chemopreventive effects [7]. Recently it was shown that DIM can repress sphere-forming capacity of prostate cancer cells [10].

In this work, we have investigated the specificity of DIM against CSCs. We also compared the effects of DIM to those of Tranilast, an anti-allergic drug with established anticancer activities [11] that can inhibit the growth of breast cancer stem cells [12]. The latter strongly depended on activation of AhR because its knockdown entirely abrogated the anti-proliferative and anti-mammosphere

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http://dx.doi.org/10.1016/j.bbrc.2012.06.062

activity of Tranilast [12]. We found that DIM is selective and potent inhibitor of CSCs in several carcinoma and melanoma cell lines. We also found that treatment with DIM overcomes chemoresistance of CSCs to conventional cytotoxics and that pre-treatment of CSCs with DIM before implantation inhibits the growth of primary tumors in mice.

2. Materials and methods

2.1. Cell lines

H460 (human non-small cell lung carcinoma) cell line was maintained in RPMI-1640 medium. MCF-7 (human breast carcinoma), KB (head and neck carcinoma), B16 and B16/F10 (mouse melanoma) cell lines were maintained in DMEM. B16/F10 is *in vivo* selected more metastatic and multidrug resistant variant of B16 cells. MESSA (human uterus carcinoma) and Lovo (human colon carcinoma) cell lines were cultured in McCoy5A and F12 medium, respectively. All cell lines were cultured in a 5% CO_2 atmosphere at 37 °C. All media were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cell culture reagents were from Invitrogen.

2.2. Reagents

DIM was from Alexis Corporations, Tranilast was from Sigma, SN-38 was from Haorui Pharma-Chem Inc., doxorubicin was from Zhejiang Hisun Pharmaceutical Co. Ltd., and paclitaxel was from Bioxel Pharma. For the growth of tumor spheres progenitor cell targeted mammary epithelium medium CnT-27 and growth supplements (CELLnTEC Advanced Cell Systems) were used according to manufacturer's recommendations.

2.3. Culture of tumor spheres

Suspension growth of tumor cells was assessed as described [12]. Briefly, cells were suspended in serum-free CnT-27 medium containing growth factors and plated on 6-, 24- or 96-well ultra-low-attachment plates (Corning). The medium was supplemented with fresh growth factors twice a week. Self-renewal capacity of the CSCs was assessed by producing next generations of tumor spheres. First-generation spheres were collected by gentle centrifugation, dissociated into single cell suspension, counted and re-plated under the conditions described above.

2.4. Cytotoxicity studies

Cells (4000/well) were plated in triplicates in 125 μl of corresponding medium supplemented with 10% FBS on 96-well plates. Twenty four hours after seeding, seven 2-fold dilutions of DIM or Tranilast were added to the culture medium. Cell viability was measured after 72 h with Cell Proliferation Reagent WST-1 (Clontech) according to the manufacturer's protocol. In separate plate absorbance of control cells was measured at time zero, i.e. before addition of drugs.

Optical densities from WST-1 assays were converted to the percentage cell growth, according to [13]. The percentage growth is calculated at each drug concentration level as:

$$[(T_i - T_0)/(C - T_0)] \times 100\% \text{ for concentrations for which } T_i \geqslant T_0$$

$$[(T_i - T_0/(T_0 - M)] \times 100\% \text{ for concentrations for which } T_i < T_0,$$

where T_0 is absorbance at time zero, C is absorbance in control not treated cells, M is absorbance of medium, and T_i is absorbance in test wells with specific drug concentration. IC_{50} parameters were calculated by nonlinear fitting of percentage cell growth curves to four-parameter logistic equation using GraphPad Prism software (GraphPad Software Inc.).

To assess the resistance of CSCs to cytotoxic agents, tumor spheres were dissociated into single-cell suspensions and 3000 cells/well were plated in triplicates in corresponding medium supplemented with 10% FBS on 96-well plates. Twenty four hours after seeding, seven 2-fold dilutions of doxorubicin, paclitaxel or SN-38 were added to the culture medium. For comparison, parental adherent cells were also treated in the same conditions. Cell growth was measured after 72 h with Cell Proliferation Reagent WST-1 (Clontech) as above.

2.5. Animal studies

C57BL/6 mice were purchased from Charles River Inc. All mouse procedures were approved by the Animal Care and Use Committees and performed in accordance with institutional policies. B16 mouse melanoma cells were cultured as tumor spheres in the absence (B16/CSC) or presence of 2 μM DIM (B16/CSC + DIM). Before implantation, tumor spheres were collected by gentle centrifugation, washed and tripsinized, trypan blue staining was used to assess cell viability. One thousand cells were implanted subcutaneously in 200 μl of medium containing 30% Matrigel in both flanks of mice. Five mice were in B16/CSC group, and six mice were in B16/CSC + DIM group. Tumor volume was measured every 3 days according to the formula: $TV = L \times W^2/2$, where TV – tumor volume, L – length, and W – width. Animals were sacrificed when tumor size reached the limit or animals demonstrated signs of morbidity.

2.6. Statistical analysis

Statistical analyses were performed with GraphPad Prism 3.0 program (GraphPad Software Inc.). Comparisons between values were performed using two-tailed Student's t test. Survival curves were analyzed by log-rank test. In all experiments $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Cytotoxicity of DIM and Tranilast in cancer cell line

We first evaluated cytotoxic activity of DIM in adherent tumor cell lines of various origins. For comparison, cytotoxicity of Tranilast was also tested in the same cell lines. DIM or Tranilast in various concentrations were added to cell cultures and cells were grown for 72 h, after which the numbers of viable cells were measured by WST-1 assay. The IC_{50} values were calculated and presented in Table 1. These results demonstrate that compared to Tranilast, DIM does not possess strong cytotoxic activity. Its IC_{50} in carcinoma cell lines are in a narrow range, 140–200 μM, which is comparable to the cytotoxic activity of quercetin, another

Table 1
Cytotoxicity of DIM and Tranilast in cancer cell lines.

Cell line	DIM IC_{50} (μM)	Tranilast IC_{50} (μM)
H460	154	38
MCF-7	156	46
B16	46	96
B16/F10	45	122
KB	205	54
MESSA	139	42

DIM or Tranilast in various concentrations were added to cell cultures and cells were grown for 72 h, number of alive cells was measured by WST-1 assay. Concentrations inhibiting the growth of cancer cells by 50% were calculated by nonlinear fitting of titration curves.

well-known phytochemical with established anticancer properties [14]. Effect of DIM does not depend on p53 status because its activity in MCF-7 and MESSA cells, possessing wild-type p53, did not differ significantly from the activity in other cell lines with mutant p53 that confirms the previous results in prostate cancer cells [15]. In melanoma cells, DIM was more active with IC_{50} being around 45 μM; however, Tranilast demonstrated an overall higher cytotoxic potential. Its IC_{50} concentrations in carcinoma cell lines are in the range of 40–55 μM.

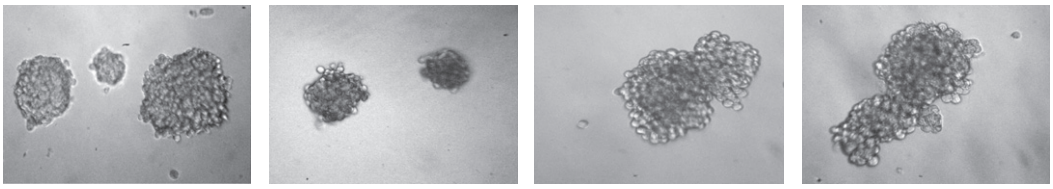
3.2. Tumor sphere formation

Recently, the concept of tumor-initiating or cancer stem cells was proposed to explain tumor initiation, relapse, metastases and acquired chemoresistance. To better understand the cancer preventive role of DIM and its anticancer activities, we have evaluated growth-inhibiting activity of DIM against CSCs. The ability to form spheres in serum-free medium in non-adhesive conditions is fundamental property of cancer stem cells [1,2]. Because of a clonal origin of tumor spheres, their number reflects the number of CSCs in the original heterogeneous cell population [16]. Sphere-forming assays have been adapted for use with a number of different tissues types for the quantification of stem cell activity and self-renewal [17], especially when the specific CSC markers have not been identified [18]. The results presented in Fig. 1 illustrate the ability of the tested cell lines to form tumor spheres. Percentage of CSCs in various cell lines varied between 0% and 3.5%. These numbers correspond well to the percentage of CSCs detected by

A Efficiency of tumor sphere formation.

Cell line	Number of tumor spheres per 1 000 cells	% of CSCs
H460	35	3.5
B16	16	1.6
KB	16	1.6
B16/F10	11.6	1.2
MCF-7	0.5	0.05
Lovo	0.3	0.03
MESSA	0	0

B



KB H460 B16 B16/F10

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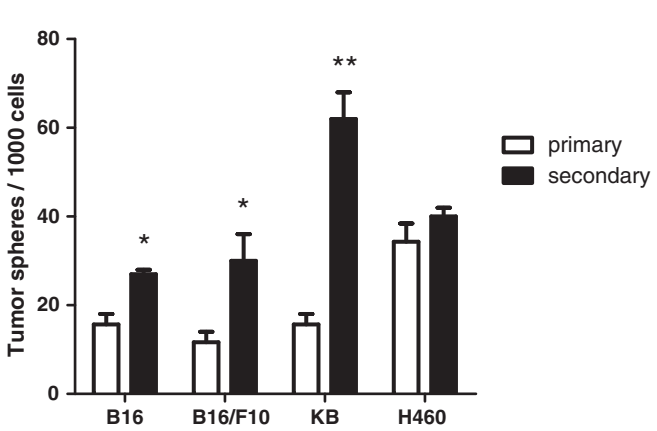
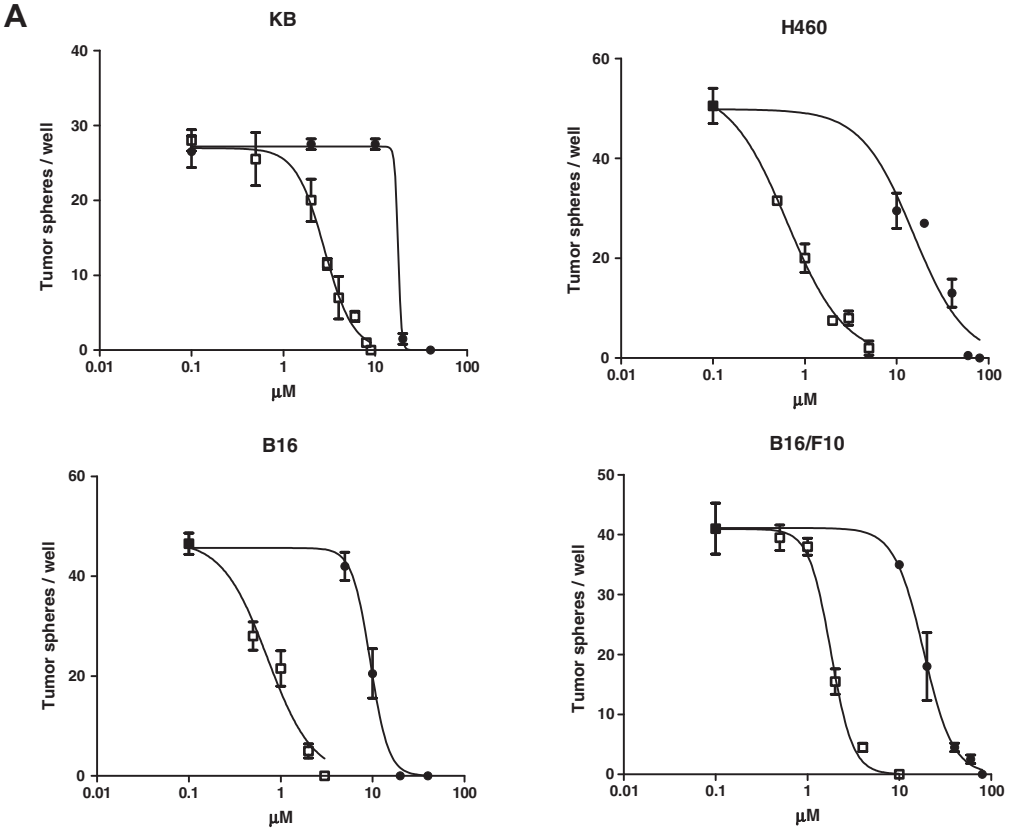


Fig. 1. Formation of tumor spheres in various cancer cell lines. (A) 4–6000 cells per well were plated on ultra-low attachment 6-well plates and grown in serum-free medium for 8–14 days. Number of tumor spheres was calculated under microscope (40×) and recalculated per 1000 cells. Percentage of cancer stem cells (CSCs) was calculated as number of tumor spheres per 100 cells. (B) representative images of tumor spheres formed in KB, H460, B16, and B16/F10 cells (magnification 100×). C, number of primary and secondary tumor spheres formed per 1000 cells in B16, B16/F10, KB, and H460 cells. To produce secondary tumor spheres, primary tumor spheres grown on 6-well plates were collected, tripsinised and 500 cells per well were plated on ultra-low attachment 96 well plates and grown in serum-free medium for 8–14 days. Number of secondary tumor spheres was counted under microscope (40×). $^{*}p < 0.05$; $^{**}p < 0.01$ by *t*-test.

side population method [19]. The highest percentage (3.5%) of CSCs was detected in H460 cells followed by KB, B16 and B16/F10 cells. Significantly lower percentage of CSC was found in MCF7 and Lovo cells, no CSCs were detected in MESSA cells (Fig. 1A). Recently, side population cells from H460 cell line were shown to harbor stem-like properties forming self-renewal tumor spheres, possessing higher tumorigenicity in mice and expressing stem cell markers ABCG2 and SMO [19].

The four cell lines (H460, KB, B16, and B16/F10) showing the highest tumor sphere formation capacities were selected for



B Inhibition of tumor sphere formation by DIM and Tranilast.

	DIM IC_{50}, μM	Decrease in DIM IC_{50}, relative to parental adherent cells, -fold	Tranilast IC_{50}, μM	Decrease in Tranilast IC_{50}, relative to parental adherent cells, -fold
H460	0.5	308	14	2.7
KB	2.8	73	18	3.0
B16	0.7	66	10	9.6
B16/F10	1.7	26	19	6.4

C

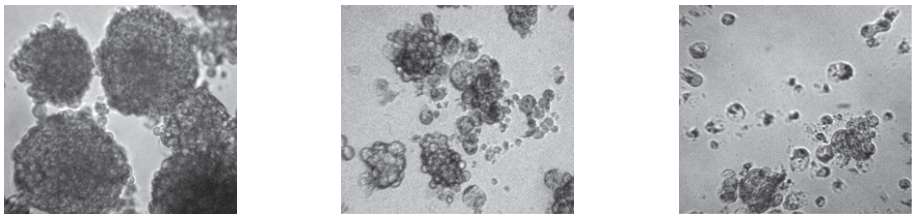


Fig. 2. Effect of DIM and Tranilast on tumor sphere formation. 500 cells per well were plated on ultra-low attachment 96 well plates and grown in serum-free medium for 8–14 days. (A) DIM (open squares) or Tranilast (closed circles) in various concentrations were present during all time of incubation. Number of tumor spheres was calculated under microscope (40×). (B) concentrations inhibiting the formation of tumor spheres by 50% were calculated by non-linear fitting of titration curves shown in (A). (C), representative images of control, DIM- and Tranilast-treated tumor spheres in KB cells (100×).

further experiments. Fig. 1B demonstrates representative images of tumor spheres in these four cell lines. In KB and H460 cells tumor spheres were compact and had regular, spherical shape. In B16 and B16/F10 cells, spheres were looser, had more irregular shape and frequently stick each other.

Besides the ability to produce tumor spheres, another specific property of CSCs is their capacity for self-renewal [1,2]. We have tested the self-renewal potential CSCs in selected cell lines. The primary tumor spheres were tripsinized and plated on ultra-low attachment plates in serum-free medium for the formation of secondary spheres. We found that secondary tumor spheres were formed in all four tested cell lines. Moreover, efficiency of tumor sphere formation was higher in second generation compared to the first one, especially in the case of KB cells (Fig. 1C).

3.3. Effect of DIM and Tranilast on tumor spheres

In the four cell lines, effect of DIM on tumor sphere formation was evaluated and compared to that of Tranilast. The ability of DIM to inhibit tumor sphere formation was substantially more pronounced compared to that of Tranilast. Fig. 2A demonstrates titration curves for these two compounds. The IC_{50} values for DIM and Tranilast are presented in Fig. 2B. IC_{50} values for inhibition of tumor sphere formation by DIM are in low micromolar range, 0.5–2.8 μM. It is about the same potency as those of salinomycin, the first selective inhibitor of cancer stem cells identified by high-throughput screening [3]. Another selective anticancer stem cells agent, metformin, inhibited tumor sphere formation at the concentrations 50–100 times higher [4] than those of DIM. One of possible mechanisms of DIM activity against CSCs can involve up-regulation of microRNA let-7, one of microRNAs frequently implicated in carcinogenesis (11).

Importantly, DIM inhibited growth of tumor spheres 30–300 times more efficiently then the growth of parental adherent cells (Table 1). Tranilast also inhibited formation of tumor spheres, but at 10-fold higher concentrations than DIM, ranging from 10 to 19 μM, which is only 3–10 times lower than the concentrations required to inhibit growth of the parental adherent cells. These results indicate that DIM is not only considerably more potent, but also more selective CSC inhibitor as compared to Tranilast. In addition to inhibition of the number of tumor spheres their size in the presence of DIM and Tranilast was also reduced. Representative images of tumor spheres after DIM or Tranilast treatment are shown in Fig. 2C.

3.4. DIM decreases the self-renewal potential of CSC

To measure the effect of DIM on self-renewal potential of CSC we tripsinized primary tumor spheres produced from four cell lines and continued culturing them in low adherent plates in serum-free medium to produce second generation tumor spheres. DIM produced negative effect on the self-renewal potential of CSCs as evidenced by decreased number of secondary tumor spheres formed from 1000 cells (Fig. 3). Efficiency of secondary tumor sphere formation decreased by 1.9- ($p < 0.05$) and 2.4-fold ($p < 0.01$) in KB and H460 cells, respectively. In the case of B16 and B16/F10 cells, the efficiency of secondary tumor sphere formation decreased 1.7- and 3.0-fold, respectively, although the effect did not reach significance.

3.5. DIM reverses the drug resistance of CSC

Recently, CSCs were shown to play a critical role in the development of multidrug resistance of tumors [20]. To analyze multidrug resistance of CSCs and the effect of DIM on this resistance, tumor spheres grown in the presence or in the absence of DIM were tripsinized, seeded into regular 96-well plates and cultured for 3 days in the presence of various concentrations of the following cytotoxic drugs: doxorubicin, paclitaxel, and SN-38. Parental adherent cells were seeded on the same plates for the comparison. The IC_{50} values for growth inhibition by these drugs were evaluated using WST-1 assay and the results are presented in Table 2. Three out of the four tested cell lines (except H460 cell line) cultured as tumor spheres demonstrated increased resistance to these drugs. Their resistance comprised 2.7- to 3.7-fold in the case of paclitaxel, 2.3- to 5.4-fold in the case of doxorubicin (except B16/F10 cells, which demonstrated extremely high intrinsic resistance to doxorubicin), and 2.0- to 12.0-fold in the case of SN-38. These data correspond well to previously published results [21]. Presence of DIM during the growth of primary spheres sensitized them to the cytotoxic effect of the drugs. IC_{50}s of all drugs in DIM-treated spheres were only 1.0- to 1.8-fold higher than corresponding IC_{50}s in parental adherent cells. In two cases, paclitaxel and SN-38 in B16/F10 cells, DIM-treated spheres were even more sensitive than adherent cells. Therefore, the sensitizing effect of DIM is evident in the case of all tested cytotoxics. These drugs were chosen because they have different molecular targets: microtubules, topoisomerase II, and topoisomerase I. Thus, DIM increases the sensitivity of CSC to cytotoxic drugs irrespective of their primary mechanism of action. These results suggest that DIM could be efficiently used together with existing cytotoxic drugs in patients with refractory or relapsing tumors.

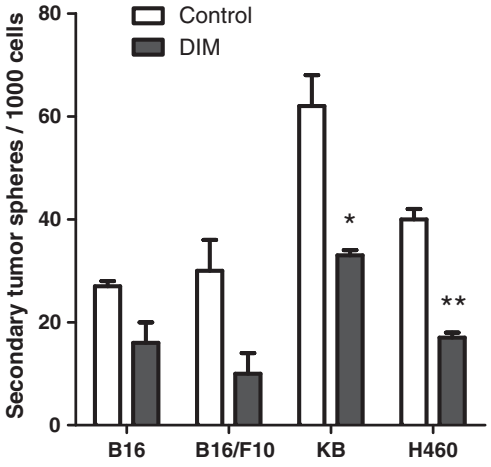


Fig. 3. DIM decreases self-renewal potential of CSCs. Primary tumor spheres were grown on ultra-low attachment 6 well plates in serum-free medium for 8 days. Cells were grown in the absence or presence of 2 μM DIM. To produce secondary tumor spheres, primary tumor spheres were collected, tripsinised and 500 cells per well were plated on ultra-low attachment 96 well plates and grown in serum-free medium for 8–14 days. Number of secondary tumor spheres was calculated under microscope (40×). $^{*}p < 0.05$; $^{**}p < 0.01$ by *t*-test.

3.6. DIM inhibits in vivo growth of CSC-derived tumors

To explore whether the inhibitory properties of DIM detected *in vitro* are translatable into *in vivo* settings, we have compared the growth of primary tumors in mice initiated by CSCs or CSCs pre-treated with DIM. To this end, tumor spheres produced from B16 cells and tumor spheres obtained in the presence of 2 μM DIM were injected s.c. into syngenic mice and the tumor growth was followed for 30 days (Fig. 4A). Despite the fact that only 1000 cells were implanted, tumors have developed in all mice underlying very high tumorogenic potential of these melanoma cells. The growth of tumors formed by CSCs pre-treated with DIM was significantly retarded ($p = 0.02$), relative to untreated CSCs. These results suggest that depletion of cancer stem cells

Table 2
DIM increases drug sensitivity of CSCs.

(*A*) *Paclitaxel*					
	PTX IC_{50} (μM)			Resistance, -fold	
	Adherent cells	Control spheres	DIM-treated spheres	CSCs relative to adherent cells	DIM-treated CSCs relative to adherent cells
B16	0.041	0.15	0.042	3.7	1.0
B16/F10	3.1	9.9	2.2	3.2	0.7
KB	0.0017	0.0046	0.0021	2.7	1.2
(*B*) *Doxorubicin*					
	Dox IC_{50} (μM)			Resistance, -fold	
	Adherent cells	Control spheres	DIM-treated spheres	CSCs relative to adherent cells	DIM-treated CSCs relative to adherent cells
B16	0.12	0.27	0.21	2.3	1.8
B16/F10	>10	>10	>10		
KB	0.08	0.43	0.12	5.4	1.5
(*C*) *SN-38*					
	SN-38 IC_{50} (μM)			Resistance, -fold	
	Adherent cells	Control spheres	DIM-treated spheres	CSCs relative to adherent cells	DIM-treated CSCs relative to adherent cells
B16	0.09	0.17	0.14	2.0	1.2
B16/F10	0.30	0.62	0.12	2.1	0.4
KB	0.07	0.84	0.10	12.0	1.4

100 000 cells per well were plated on ultra-low attachment 6 well plates and grown in serum-free medium containing growth factors for 8 days. Tumor spheres were grown in the absence or presence of DIM, afterwards tumor spheres were collected, tripsinised and 3000 cells per well were plated on regular 96-well plates; parental adherent cells were plated on the same plates as well. Cell were grown in FBS-containing medium as adherent cultures for 3 days in the presence of various concentrations of (A) paclitaxel (PTX), (B) doxorubicin (Dox) or (C) SN-38. Concentrations of drugs inhibiting the growth of cells by 50% were calculated by non-linear fitting of titration curves. Resistance of CSCs relative to parental adherent cells was calculated as IC_{50} of control spheres/IC_{50} of adherent cells, resistance of DIM-treated CSCs relative to adherent cells was calculated as IC_{50} of DIM-treated spheres/IC_{50} of adherent cells.

substantially decreases the growth rate of primary tumors. Moreover, pre-treatment of tumor spheres with DIM also significantly improved the survival of animals, $p = 0.03$ (Fig. 4B).

As it was mention above, DIM inhibits the growth of adherent cells with IC_{50} in the range of 45–200 μM, while it can inhibit formation of tumor spheres with IC_{50} in the range of 0.5–2.8 μM. It is important to compare these concentrations with DIM concentrations that can be achieved in human patients. In healthy subjects, BR-DIM (oral formulation of DIM with higher bioavailability compared to crystalline compound) was well tolerated at single dose of up to 200 mg and further dose increase to 300 mg did not change Cmax. The single 200 mg dose produced a mean Cmax of 104 ng/ml or 423 nM [22]. In phase I study in prostate cancer patients, after oral administration of BR-DIM at maximum tolerated dose of 300 mg, mean Cmax reached 236 ng/ml or 959 nM [23]. Therefore, concentrations of DIM achievable in cancer patients overlap DIM concentrations required for the inhibition of CSCs. Therefore, it is possible that DIM could be safely used in cancer patients at the doses that inhibit CSCs; however, a more efficient formulation with additionally increased oral bioavailability of the compound, if developed, could help fully explore potential of DIM as pharmaceutical agent. The most feasible way to use DIM in oncology is to combine it with existing chemotherapeutic drugs and/or radiation. In this case a dual therapeutic effect could be achieved. A bulk of cancer non-stem cells in tumor can be efficiently eliminated by conventional cytotoxics or by irradiation, while cancer stem cells could be eradicated by DIM, thus preventing the development of multidrug resistance, metastases and relapse.

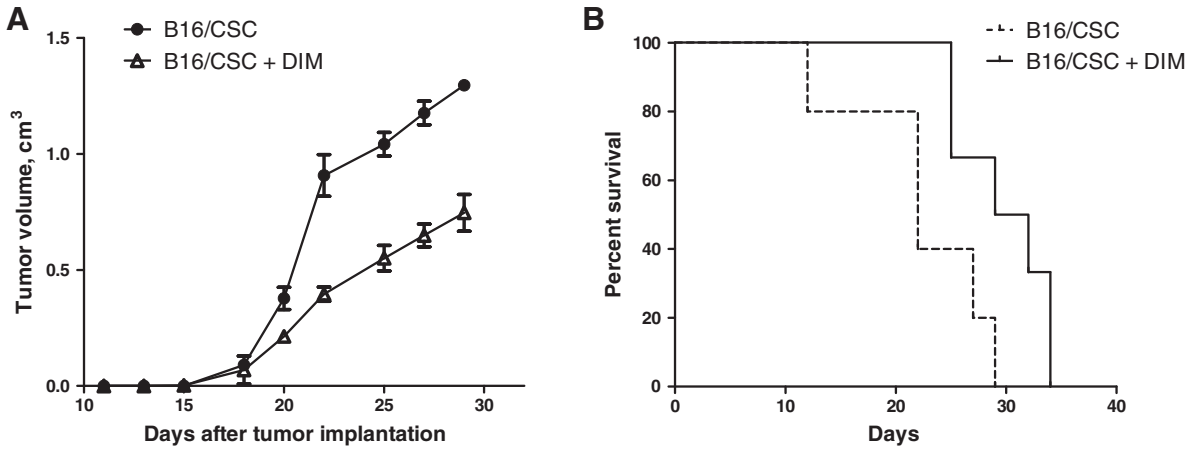


Fig. 4. Effects of DIM on tumor growth and survival of mice. (A) Inhibition of primary tumors growth. Before implantation, murine melanoma B16 cells were grown as tumor spheres (B16/CSC) or as tumor spheres in the presence of DIM (B16/CSC + DIM). 1000 cells produced from tumor spheres were implanted s.c. in C57BL/6 mice, tumors were examined three times a week and volumes were calculated. DIM treatment of tumor spheres significantly inhibited the growth of primary tumors, $p = 0.02$ by paired *t*-test. (B) Survival curves of mice shown in panel (A). DIM pre-treatment of tumor spheres significantly improved the survival of tumor-bearing mice, $p = 0.03$ by log-rank test.

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