The polyphenolic-rich Aronia melanocarpa juice kills teratocarcinomal cancer stem-like cells, but not their differentiated counterparts

Tanveer Sharif, Mouni Stambouli, Benjamin Burrus, Fathi Emhemmed, Israa Dandache, Cyril Auger, Nelly Etienne-Selloum, Valérie B. Schini-Kerth, Guy Fuhrmann*  
UMR 7213 CNRS, Laboratoire de Biophotonique et Pharmacologie, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

ABSTRACT
A diet rich in plant-derived products is expected to have anticancer chemopreventive effects by acting on the appearance and growth of cancer stem cells (CSCs). Thus the effects of Aronia melanocarpa juice (AMJ) on the mouse embryonal carcinoma (EC) stem cell line P19 were investigated. AMJ inhibited cell proliferation, induced cell cycle arrest in S phase and triggered apoptosis. A pronounced upregulation of tumour suppressors p53 and p73 was observed in association with caspase-3 activation and a downregulation of the anti-apoptotic protein UHRF1 and the stemness factor Oct-4. Overall the results strongly suggest that AMJ is functionally able to counteract the carcinogenesis process by targeting CSCs. Interestingly AMJ selectively kills undifferentiated EC cells, without significant effects on normal restricted pluripotent cells (i.e. NIH/3T3 fibroblasts) or even differentiated EC cells. This argues that a differentiation therapy might normalize the pathological phenotype of a CSC which becomes insensitive to further plant-derived pharmacological treatment.

1. Introduction
On the basis of epidemiological data, it has recurrently been reported that a diet rich in herbs, spices, fruits and vegetables has cancer-protective properties (Surh, 2003). Indeed, increased consumption of fruits and vegetables has been shown to be associated with a reduction of risk of cancer death by 6% (Pauwels, 2011; Sofi, Cesari, Abbate, Gensini, & Casini, 2008). Similarly, prevention of cancer has been linked with intake of tea or grape-derived products (Chung, Schwartz, Herzog, & Yang, 2003; Walter et al., 2010). The presence in these plant-derived products of both micronutrients (for instance vitamins and minerals) and non-nutritive components (known as phytochemicals), especially polyphenols, could explain such anticancer effects. High intake of such functional food is therefore essential in the prevention, management and treatment of cancer (Manchali, Kotamballi, Murthy, & Patil, 2012; Tsuda et al., 2004). Accordingly numerous bioactive compounds isolated from plants of terrestrial or marine origins have shown strong chemotherapeutic potential in vitro (Isha et al., 2012; Kang et al., 2012; Lee & Fan, 2012). Although their effects on cancer cells are well documented (Shu, Cheung, Khor, Chen, & Kong, 2010), their impact on cancer stem cells (CSCs) remains poorly understood, to a large extent because of the absence of well characterized experimental models.
It is now believed that CSCs are responsible for tumour initiation and development, metastatic spreading, and resistance to radiotherapy and, at first sight, to numerous current chemotherapeutic compounds (Sagar, Chaib, Sales, Winslet, & Seifalian, 2007; Sell, 2004). Since John Dick’s laboratory pioneering work in 1994 (Lapidot et al., 1994), growing realizations have shown that CSCs arise from embryonic, fetal or adult stem cells (SCs) exposed to repetitive mutation-inducing stress injuries; it is also expected that CSCs can arise from closely related dedifferentiated descendants. Such a hierarchical and dynamical relationship between these two cell type populations thus indicates that future anticancer treatments should not only target cancer cells, but also CSCs.

It is now accepted that serial xenotransplantation and tissue cultivation allows to recapture the malignant phenotype and to isolate CSCs from any tumor tissue (Sell, 2004). For these reasons, different CSC lines are now available and the chemopreventive and chemotherapeutic effects of bioactive compounds present in various food products can therefore be systematically analyzed in vitro. Actually, we have recently suggested that the malignant counterparts of the embryonic stem cell lines, namely the embryonal carcinoma (EC) stem cell lines, could be suitable models of CSCs. These cell lines are poorly differentiated pluripotent germinal stem cells with a highly aggressive malignant phenotype and could be used as surrogated investigational tools for the evaluation of potential anticancer agents (Sharif et al., 2011).

Several reports have highlighted the anticancer chemopreventive and chemotherapeutic properties of polyphenols. In a strict sense, phytochemicals with chemopreventive properties hinder the (re)appearance of a cancer by targeting CSCs, whereas phytochemicals with chemotherapeutic properties destroy a preexisting cancer by targeting cancer cells. However these latter can conceptually be considered, at least in part, as potential CSCs with very limited cell competencies. For that reason, chemotherapy and chemoprevention become hard to distinguish from each other, since they can theoretically target the cancer cell as well as the CSC. It is therefore not surprising that numerous plant-derived compounds might act on both cell types and have therapeutic and preventive effects (Aggarwal, Takada, & Oommen, 2004). In this point of view, we have recently described the pro-apoptotic properties of red wine polyphenols (RWP)s on both cancer cells and cancer stem-like cells (Sharif et al., 2010, 2011). Interestingly, RWPs kill malignant cells, without having any toxic effects, in a reasonable range of concentrations, on their normal counterparts. Actually RWPs, by a redox sensitive mechanism, inhibit cell proliferation by modulating several signal transduction pathways which leads to cell cycle arrest and enhanced apoptosis (Sharif et al., 2010, 2011).

Aronia melanocarpa, also known as black chokeberry, is a plant native to eastern North America. A. melanocarpa juice (AMJ) is one of the richest sources of natural polyphenolic substances (Bermúdez-Soto & Tomás-Barberáns, 2004), and products derived from A. melanocarpa (juices, extracts) have been shown to have anti-proliferative effects on various solid tumor models (Bermudez-Soto et al., 2007; Malik et al., 2003; Sharif et al., 2012). However, until now, the effects of AMJ have only been extensively studied on cancer cells. The aim of the present study is therefore to determine whether AMJ can also exert a chemopreventive activity by targeting CSCs. Since AMJ is expected to target specifically CSCs without affecting normal stem-like cells, we have used the non-pathological embryonic fibroblast NIH/3T3 cell line as control. In order to get better insights into the cellular and molecular mechanisms underlying the selective activity of AMJ, we have developed a more appropriate comparative normal stem-like cell line. For that purpose, we used all-trans-retinoic acid (ATRA)-differentiated P19 cells. Actually, the aggressiveness of a CSC is proportional to its lineage-specific competencies (Al-Hajj, Becker, Wicha, Weissman, & Clarke, 2004). It is assumed that the differentiation therapy allows CSC to switch from a highly aggressive state to a harmless state. That is the case of the most exhaustively studied differentiation-inducing pharmacological compounds, namely retinoids including vitamin A and its derivatives. As an adjunct to clinical therapy, ATRA treatment allows complete remission of about 90% of patients with acute promyelocytic leukemia (Freemantle, Spinella, & Dmitrovsky, 2003). This example highlights the strong positive impact of the differentiation strategy which is able to block the tumor burden. Actually, an increasing number of in vitro and in vivo studies show that various plant-derived compounds are potential anticancer agents since they can specifically target the self-renewal properties of CSCs and induce their differentiation (Li, Schwartz, & Sun, 2011).

In this contribution, we show that AMJ selectively induces apoptosis in the undifferentiated P19 cancer stem-like cells, likely via the activation of tumour suppressors-dependent cell cycle checkpoint signaling pathways. Moreover, AMJ has no deleterious activity on both embryonic fibroblast NIH/3T3 and ATRA-differentiated P19 cell lines. Since AMJ has no effects on these normal/normalized pluripotent cells, it is concluded that AMJ has a strong chemopreventive activity, additionally to its chemotherapeutic activity. Our findings also show that the differentiated cancer stem-like cell is a suitable model for the evaluation of the side effects of pharmacological agents expected to target CSCs and not normal SCs.

2. Material and methods

2.1. Preparation of AMJ

A. melanocarpa juice concentrate (66° Bx) was provided by Eckes-Granini (Nieder-Olm, Germany). AMJ was reconstituted by dilution to 15° Bx in distilled water. One liter of AMJ yielded 7.15 g of phenolic compounds, expressed as gallic acid equivalents measured by Folin–Ciocalteu method.

2.2. Cell lines and culture conditions

P19 and NIH/3T3 cell lines (ATCC, Rockville, MD, USA) at low passages were maintained in humidified incubator with 5% CO2 at 37°C. Both cell types were cultured in DMEM (Sigma–Aldrich, Saint-Quentin-Fallavier, France), supplemented with 10% (v/v) of fetal bovine serum (BioWhittaker, Verviers, Belgium), 2 mM glutamine, 50 μM non essential amino acids, 50 U/ml penicillin and 50 μg/ml streptomycin (Sigma–Aldrich,
Saint-Quentin-Fallavier, France). P19 cells were also induced to differentiate, by treating them with ATRA (Sigma–Aldrich, Saint-Quentin-Fallavier, France) at 5 μM for 6 days. Several cell lines have been derived from these differentiated P19 cell cultures and are expressing GFAP (Gial fibrillary Acidic Protein) concomitantly with an absence of Oct-4 expression (Guy Fuhrmann, personal data). These cell lines were then maintained in exponential growth prior to treatment. For that purpose, undifferentiated and differentiated P19 cell lines, as well as NIH/3T3 cell line were treated with the appropriate working concentrations of AMJ mixed with the cell culture medium.

2.3. MTS assay

Cells were seeded in triplicate on 96-multiwell plates at a density of 5 × 10^3 cells/well, grown for 24 h and exposed to AMJ at different concentrations for an additional 24 h. Cell proliferation rate was then assessed by colorimetric assay using the CellTiter 96 AQueous One® Solution Cell Proliferation Assay (MTS), following the manufacturer's recommendations (Promega, Charbonnières-les-Bains, France). Absorbance was measured at 490 nm on a multiwell ELISA plate reader. The percentage of living cells was calculated as a ratio of the OD value of each AMJ-treated cell sample to the OD value of the corresponding control AMJ vehicle.

2.4. Trypan blue exclusion assay

Cells were seeded on 6-multiwell plates at a density of 4 × 10^5 cells/well, grown for 24 h, exposed to AMJ at different concentrations for an additional 24 h and then collected after trypsinization. Cell viability rate was determined by haemocytometric cell counting using the trypan blue exclusion method, according to the manufacturer's instructions (Sigma–Aldrich, Saint-Quentin-Fallavier, France). The viability rate was obtained by dividing the number of Trypan blue-negative cells by the total number of cells.

2.5. Cell cycle phase distribution analysis

Cells were cultured on 80 cm² culture flasks at a density of 2 × 10^5 cells/ml, grown for 24 h and exposed to AMJ at different concentrations for an additional 24 h. Cells were then washed with phosphate-buffered saline (PBS), resuspended in ethanol 70% and incubated for 10 min at 4 °C. The cell suspensions were then sonicated and the supernatants were collected after centrifugation at 10,000g for 15 min. The protein concentration was determined by a modified Lowry method (DC Protein Assay, Bio-Rad, Marnes la Coquette, France). Immunoblotting was then performed by using either a mouse monoclonal anti-p53 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), a mouse monoclonal anti-p73 antibody (BD Biosciences Pharmingen, San Diego, CA, USA), a rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA, USA), a rabbit monoclonal anti-cyclin B1 antibody (BD Biosciences Pharmingen, San Diego, CA, USA), a mouse monoclonal anti-β-tubulin antibody (Abcam, Paris, France), and a rabbit polyclonal anti-beta tubulin antibody (Abcam, Paris, France), according to the manufacturer's instructions.

2.6. Apoptosis rate analysis

Cells were cultured, treated with AMJ and collected as described above. Apoptosis rate was then assessed by flow cytometry (FACScan) using the “In situ cell death detection kit, Fluorescein” (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's recommendations. At least 10,000 events were recorded to assess the percentage of apoptotic cells.

2.7. Western blot analysis

Exponentially growing cells were treated either with AMJ at different concentrations or with the vehicle and incubated for 24 h. Cells were then harvested and centrifuged at 200g for 10 min at room temperature; the pellets were resuspended in RIPA buffer (25 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Sigma–Aldrich, Saint-Quentin-Fallavier, France) and incubated on ice. The cell suspensions were then sonicated and the supernatants were collected after centrifugation at 10,000g for 15 min. The protein concentration was determined by a modified Lowry method (DC Protein Assay, Bio-Rad, Marnes la Coquette, France). For Western blot analysis, proteins were resolved on 10% SDS-polyacrylamide gels and transferred onto PVDF membranes (Millipore, Saint-Quentin-en-Yvelines, France). Immunoblotting was then performed by using either a mouse monoclonal anti-p53 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), a mouse monoclonal anti-p73 antibody (BD Biosciences Pharmingen, San Diego, CA, USA), a rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA, USA), a mouse monoclonal anti-β-tubulin antibody (Abcam, Paris, France), and a rabbit polyclonal anti-beta tubulin antibody (Abcam, Paris, France), according to the manufacturer's instructions.

2.8. Statistical analysis

Data were presented in a bar graph form and expressed as means ± S.E.M. of three independent experiments. Statistical evaluation was performed using one-way ANOVA test followed by Bonferroni post hoc test using GraphPad Prism (Palm version 5.04 for Windows, GraphPad Software Inc., San Diego, CA, USA); a P value less than 0.05 was considered as significant.

3. Results

3.1. AMJ selectively inhibited the proliferation of P19 cells and induced a S phase cell cycle arrest

The effects of AMJ on the growth parameters of P19 EC and NIH/3T3 fibroblastic cells were assessed by the MTS and the trypan blue exclusion assays. As shown in Fig. 1A, AMJ decreased the percentage of living P19 cells in a
concentration-dependent manner with a significant effect observed already at 0.3% (v/v). AMJ had no significant effect on the cell proliferation of NIH/3T3 fibroblasts. Moreover cell viability was significantly decreased in AMJ-treated P19 cells (Fig. 1B). On the opposite, no significant changes were detected in AMJ-treated NIH/3T3 fibroblasts. These results show that AMJ selectively inhibited the growth of P19 cells, while preserving that of NIH/3T3 fibroblasts. Since cell growth is associated with the ability of a cell to progress through the different phases of mitosis, we have investigated the effects of AMJ exposition on the cell cycle phase distribution. As shown in Fig. 2A and B, AMJ, at a concentration from 0.3% to 0.5% (v/v), significantly increased the accumulation of cells in S phase in P19 cells. Thus, AMJ inhibited the growth of P19 cells by promoting their cell cycle arrest in the S phase.

3.2. AMJ selectively induced apoptosis of P19 cells without affecting NIH/3T3 fibroblasts

Cells induced to arrest at one of the cell cycle checkpoints may undergo programmed cell death if the DNA damage cannot be properly repaired. We therefore investigated whether AMJ induced apoptosis in P19 cells. As indicated in Fig. 3A, AMJ increased the apoptosis rate in the teratocarcinoma cells, with a statistically significant effect already observed at the concentration of 0.3% (v/v). In contrast, no significant change in the apoptosis rate was observed in AMJ-treated NIH/3T3 fibroblasts (Fig. 3A). These data are in line with the results obtained from the cell proliferation assays and cell cycle studies and show that AMJ treatment selectively induced apoptosis in P19 cells.

3.3. Effect of AMJ on various molecular targets related to cell cycle and apoptosis in P19 cells

To better characterize the AMJ-induced cell cycle signaling pathway involved in cell cycle arrest, we analyzed the expression level of the tumour suppressor protein p53 (a well-characterized inducer of cell cycle arrest and apoptosis) and p73 (known to function as a p53 homolog). As shown in Fig. 3B, p53 was undetectable in control cells, whereas after AMJ treatment there was an increase in the expression level of p53. This effect was accompanied by a significant increase in the expression level of p73 and a strong increase in the expression level of the cleaved caspase-3 (one of the main executors of apoptosis). Cyclins play a critical role in the transition of cell cycle through different phases. Therefore, we studied the expression level of cyclin B1 in AMJ-treated P19

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Fig. 1 – Concentration-dependent effects of RWPs on the proliferation and viability rates of P19 and NIH/3T3 cells. Cells were exposed to AMJ at the indicated concentrations and incubated for 24 h. The proliferation and viability rates of P19 cells or NIH/3T3 cells were respectively assessed by colorimetry using the MTS assay (A) or assessed by cell counting using trypan blue dye exclusion assay (B). The absolute value obtained for each AMJ-treated sample was expressed in a second step as percent relative to the corresponding absolute value obtained for the untreated sample and set at 100. (n = 3); *P < 0.05.
cells. AMJ caused a concentration-dependent down-regulation of the expression of cyclin B1 (Fig. 3C), which most likely contributes to the cell cycle arrest.

Spontaneous or induced deregulation of the expression of UHRF1 (Ubiquitin-like, containing PHD and RING Finger domains, 1), an epigenetic integrator, has been correlated with the proliferation and survival capacity of numerous types of cancer cells (Abusnina et al., 2011; Achour et al., 2009; Alhosin et al., 2011). Accordingly, AMJ caused a marked down-regulation of UHRF1 (Fig. 3D) in P19 cells, providing further evidence for the pro-apoptotic properties of AMJ.

Unrestricted pluripotency and self-renewal are the characteristic features of embryonic stem cells, wherein Oct-4 plays a critical role. We therefore studied the effect of AMJ treatment on the expression level of Oct-4 in P19 treated cells. The expression of this stemness factor decreased with increasing concentrations of AMJ, while at higher doses (≥0.3% (v/v)), Oct-4 became barely detectable (Fig. 3D). These results indicate that AMJ selectively induced apoptosis in P19 EC cells by affecting the expression of different proteins implicated in the regulation of self-renewal, cell cycle and apoptosis.

Fig. 2 – Concentration-dependent effects of AMJ on the cell cycle phase distribution of P19. Cells were exposed to AMJ at the indicated concentrations and incubated for 24 h. Cell cycle phase distribution was assessed by a cytometric detection assay. (A) Shows representative DNA content histograms for treated (left panel) or untreated (right panel) P19 cells. (B) Recapitulates in a bar graph form the distribution of P19 cells in G1, S or G2/M phase; the number of cells in each mitosis phase was determined and expressed as percent relative to the total cell number. (n = 3); *P < 0.05.
3.4. AMJ has no effect on the proliferation of ATRA-differentiated P19 cells

There are several lines of evidence showing that cancer stem cells lose their cancer potential after differentiation (Al-Hajj et al., 2004; Freemantle, et al., 2003) and, therefore, become insensitive to putative anticancer plant-derived treatment. Since a selective pro-apoptotic effect of AMJ on teratocarcinoma P19 cells has been observed, it was expected that their differentiated counterparts would not be affected by such a treatment. As shown in Fig. 4A, ATRA-differentiated P19 cells did not express Oct-4 while a significant expression of GFAP was detectable. Moreover, MTS assay revealed that AMJ had no significant effect on the proliferation of these cells, providing further evidence for the selective growth inhibiting effect of AMJ (Fig. 4B). This result also suggests that AMJ only kills cells with a cancer signature and has no significant toxic effect on normal cells.

4. Discussion

It has generally been accepted that prevention of any disease is preferable over treatment as illustrated by Benjamin Franklin in the famous “An ounce of prevention is better than a pound of cure”. Chemoprevention is a strategy aimed at preventing tumour initiation and progression by targeting the cells which have the oncogenic potential; this is a relatively promising approach for controlling cancer that uses specific natural or synthetic compounds to suppress or prevent premalignancy before transformation into irreversible cancer (Colic & Pavelic, 2002; Rafter, 2002). Bioactive compounds isolated from various plant-derived products have been shown to exert chemopreventive effects (Colic & Pavelic, 2002; Lee & Pan, 2012; Manchali, et al., 2012). As phytochemicals, the polyphenols also have received a great deal of interest because of their potential ability to act as effective chemopreventive and chemotherapeutic agents (Bode & Dong, 2004; Ramos, 2008; Thomasset et al., 2007). Indeed, it has been shown that products rich in polyphenols have healthy effects since they are able to affect the overall process...
of carcinogenesis by inhibiting various target genes involved in cell proliferation, apoptosis and angiogenesis (Bode & Dong, 2004; Ramos, 2008; Thomasset et al., 2007). In addition, the low toxicity and the very few side effects linked to the consumption of polyphenols give them a potential advantage with respect to the commonly used chemotherapeutic agents (Thomasset et al., 2007; Yang, Wang, Lu, & Piccinich, 2009).

CSCs have been recently proposed to be the cancer initiating cells responsible for tumorigenesis; they can contribute to the ability to resist to chemo- and radiotherapy, and give rise to cancer metastasis and recurrence (Houthuijzen, Daenen, Roodhart, & Voest, 2012). Therefore, eradication of CSCs may lead to complete cancer cure. In the process of developing CSC-targeted therapy, it is necessary to identify the products having the ability to selectively kill the CSCs while preserving their normal counterpart. The goal of this work was to study the chemopreventive ability of AMJ on the P19 EC, NIH/3T3 and ATRA-differentiated P19 EC cell lines. The present findings indicate that AMJ effectively down-regulated the proliferation of P19 EC. Indeed, 0.3% (v/v) treatment with the AMJ caused an inhibition of proliferation together with an S phase cell cycle arrest of P19 EC. Further experiments revealed that AMJ-induced cell cycle arrest leads to an apoptotic cell death in P19 EC cells. A TUNEL assay showed that starting from 0.3% (v/v) treatment with AMJ, a significant increase in P19 EC cells number undergoing apoptosis was observed. To better understand the molecular mechanisms underlying the inhibition of proliferation, cell cycle arrest and apoptosis, the expression levels of various proteins related with these cell processes were studied in AMJ-treated P19 EC cells. Different types of cyclin/cyclin-dependent kinase complexes are involved in the cell cycle progression, while on the other side the cell cycle progression is blocked by several tumour suppressors such as p53 and p73 (Collavin, Lunardi, & Del Sal, 2010; Lapenna & Giordano, 2009). It is important to mention that p33 is a structural and functional analogue of p53, which has the ability to interact with various genes responsible for cell cycle arrest and, hence, apoptosis (Zawacka-Pankau, Kostecka, Sznarkowska, Hedstrom, & Kawiak, 2010). Therefore as a first line of evidence, the effect of AMJ on the expression levels of cyclins and tumour suppressors was studied. The results show that AMJ treatment downregulated the expression levels of cyclin B1 and up-regulated those of p53 and p73. Furthermore, the AMJ treatment of P19 EC led to a dramatic increase in the expression levels of cleaved caspase-3, the main executor of apoptosis. These findings show that AMJ induced an activation of a p53/p73-dependent proapoptotic pathway in P19 EC cells.

Fluropotency and self-renewal are the main properties of normal SCs, as well as of CSCs. These characteristics are, at least in part, controlled by specific proteins also involved in cell proliferation and survival of CSCs (Chen, Du, & Lu, 2012). Therefore, it was of interest to explore the effect of AMJ treatment on the expression levels of such proteins in P19 EC cells. Oct-4, as a marker of the stemness and unrestricted pluripotency, plays a central role in the survival of poorly differentiated and highly aggressive CSCs (Chen et al., 2012; Hu et al., 2008). Our results show that AMJ treatment at 0.4% and 0.5% (v/v) in P19 EC cells markedly decreased the expression levels of Oct-4, suggesting that Oct-4 downregulation could be a triggering event in AMJ-induced apoptosis. On the other hand, an interesting finding of this study is the marked downregulation of UHRF1 by the polyphenolic-rich AMJ in P19 EC cells. UHRF1, as an oncogenic protein, plays a role in the proliferation of numerous cancer cells by promoting cell cycle progression. The effect of natural products on the expression of UHRF1 in several cancer cell lines has already been reported in vitro and in vivo (Alhosin et al., 2011; Walter et al., 2010), while this report provides for the first time an insight about the effects of a nutritional source of polyphenols on the expression levels of UHRF1 in teratocarcinomal cancer stem-like cells. Finally, our findings show that AMJ has the potential to affect the expression of proteins related with cell proliferation and survival in CSCs, such as cyclin B1, tumor suppressors p53 and p73, and the active form of caspase-3, similarly to previous studies investigating the cytotoxic properties of polyphenols on CSCs (Shankar et al., 2011).

Selective cytotoxicity of a chemopreventive product against CSCs, and not normal SCs, provides an edge for the chemotherapy by limiting the side effects. As AMJ has a pronounced cytotoxic effect on CSCs, the selectivity of AMJ activity remains questionable. To address this question, we studied the effects of AMJ on NIH/3T3 cell line, a model of normal SC; surprisingly the results showed that high doses of AMJ, which were able to strongly decrease the viability of P19 EC cells, had no obvious toxic effect on NIH/3T3 cells. Indeed MTS data revealed that AMJ treatment has no significant effect on the proliferation of NIH/3T3 cells. The TUNEL assay confirmed the absence of survival downregulation as AMJ treatment was without any significant apoptotic effect on NIH/3T3 cells. These results clearly provide evidence about the selective pro-apoptotic effect of AMJ on CSCs, but not on normal SCs. In order to better evaluate the selectivity of the AMJ-induced cytotoxic effect, we investigated the consequence of AMJ treatment on ATRA-differentiated P19 cells; this ATRA-model provides the opportunity to study the effect of AMJ on differentiated cells that are more closely related to P19 EC cells. The disappearance of Oct-4 and the appearance of GFAP after treatment with ATRA confirmed the differentiation state of the P19 cells. The MTS assay indicated that AMJ had no significant effect on the proliferation of ATRA-differentiated P19 cells; these results indicate therefore that the anti-proliferative effect of the AMJ is selective for cells having the ability to give rise to cancer, without having major toxic effect on the normal SCs and differentiated CSCs.

Overall, the findings point out that the polyphenolic-rich AMJ has selective chemopreventive properties against CSCs. This work also indicates that differentiated CSCs which have lost their cancer properties become resistant to the antiproliferative and cytotoxic effects of natural polyphenolic mixtures, like AMJ. Indirectly, this highlights the benefits of the differentiation therapy on CSCs. Indeed, differentiated counterparts of CSC seem to have a normalized behavior and, therefore, remain unaffected after anticancer pharmacological agents or mixtures. However, the chemopreventive properties of AMJ on CSCs need to be confirmed by further studies, especially by using models of self-renewal. Interestingly, other polyphenol compounds, such as curcumin, blueberry phenolic acids or resveratrol, have been shown to
be able to reduce self-renewal by using models of mammospheres and spheroids formation in breast and pancreas CSCs, respectively (Kakarala et al., 2010; Montales et al., 2012; Shankar et al., 2011). All together, the present studies, and other recent investigations about the potential of polyphenolic products to reduce CSC viability and to inhibit their self-renewal in vitro and in vivo, suggest that nutritional sources of polyphenols might represent an effective approach in cancer prevention.

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