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Research review paper

Naturally occurring compounds in differentiation based therapy of cancer

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ABSTRACT

Differentiation of cancer cells entails the reversion of phenotype from malignant to the original. The conversion to cell type characteristic for another tissue is named transdifferentiation. Differentiation/transdifferentiation of malignant cells in high grade tumor mass could serve as a nonaggressive approach that potentially limits tumor progression and augments chemosensitivity. While this therapeutic strategy is already being used for treatment of hematological cancers, its feasibility for solid malignancies is still debated. We will presently discuss the natural compounds that show these properties, with focus on anthraquinones from *Aloe vera*, Senna, Rheum sp. and hop derived prenylflavonoids.

1. Introduction

Numerous literature data has correlated the application of chemo or radiotherapy with progression of advanced cancers. The repopulation of surviving tumor cells upon radiation and chemotherapy is thought to represent the cause of treatment failure (Huang et al., 2011). Recently, Karragianis et al. showed that neoadjuvant chemotherapy elevated the number of breast cancer metastasis (Karagiannis et al., 2017). Regardless of whether anti-cancer treatments are specific or non-selective, the main mechanism of their action is shrinkage of tumor mass through cell death induction. If so, why then standard care treatments are not capable of permanently limiting advanced cancer progression by simply killing the malignant cells? One possibility could be that the fact that the role of apoptotic cell death in cancer treatment is more complex than a simple dying process. In order to keep the tissue homeostasis, apoptotic cells deliver numerous signals to organize their removal, prevent further tissue damage but also for their own replacement (Chekeni et al., 2010; Lauber et al., 2003; Gude et al., 2008; Ravichandran, 2011). These signals influence behaviour of other cells in surrounding tissues and organs. Pattern of signals include exchange of membrane potential partly by externalization of phosphatydylserine (PS), release of exocytic vesicles, production of reactive oxygen species, anti-inflammatory peptides and numerous molecules which act as mediators of different processes. As suggested by Ravichandran (2011) and Ryoo and Bergmann (2012), these signals can be scattered into "find me", "eat me", "keep out inflammation" (Ravichandran, 2011; Ryoo and Bergmann, 2012). The list can be extended with signal named as "be loyal" to tissue in order to compensate the lost and retain tissue homeostasis. While "find me", "eat me", "keep out inflammation" represents connected cascades of events leading to apoptotic cell clearance, the "be loyal" signal is evoked in initial phase of apoptosis prior to or even independently of completion of the apoptotic process (Fan and Bergmann, 2008). Several examples exist indicating that compensatory proliferation might play a role in the conventional therapeutic failures in advanced cancers (reviewed in Zimmerman et al., 2013). The better understanding of the background of the tumor expansion as the consequence of aggressive treatment requires the proper comprehension of the relation occurring between cell death and proliferation in tumor tissue. Communication between apoptotic and neighbouring cells is defined by their phenotype and differentiation level. The term malignant phenotype is often identified as genetic abnormal cell with sustained proliferative capacity. However, several data prove that

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malignant transformation can follow the opposed route to embryogenesis. In fact, transformed cells undergo the process of dedifferentiation passing through different stages that at the end point are finalized by the establishment of the pluripotent phenotype (Gabbert et al., 1985; Volinia et al., 2014). For example, in advanced tumor mass, blastomera like polynuclear giant cells can be observed (Niu et al., 2017). In parallel, apoptotic cells precede the mitogen signal to stem cells, but not cells already functionally committed (Kurtova et al., 2015). Stem cells are unspecified cells able to renew themselves and develop into more specialized cell types with defined function. Cancer stem cells are a small population of tumor cells that share the same features of stem cells and represent the source of all other phenotypes in heterogeneous tumor mass. Cancer stem cells are less responsive or fully unresponsive to conventional therapies due to their ability to be in a state of dormancy allowing them to escape drugs' mediated toxicity that targets primarily proliferative neoplastic cells. Accordingly, it has been proposed that better tailored chemotherapeutic protocols should first aim at the killing of proliferating cells and subsequently differentiate or eliminate the cancer stem cell (Massard et al., 2006). However, this hypothesis takes into little account cell-cell communication specifically occurring inside the tumor mass. Since the presence of low differentiated cells is one of the main features of advanced cancers, induction of apoptosis under these circumstances may turn into powerful signal for proliferation. Trigger for this might be the death not only of bulk tumor cells but also vascular endothelial or stromal cells (Shekhani et al., 2013). In any case, through the activation of effector caspases, apoptotic cell can deliver the mitogen signal to the neighbour cells (Fan and Bergmann, 2008; Jäger and Fearnhead, 2012). Caspase 3 is crucial for the delivery of proliferative signal and the degree of its expression in several cancer types represents an unfavourable prognostic marker (Huang et al., 2011; Hu et al., 2014). These data are in apparent conflict with the notion that caspase-3 deficiency leads to apoptosis resistance. Nonetheless, inhibition of caspases sensitized lung and breast cancers to radiotherapy *in vivo* (Moretti et al., 2009). It is known that caspase activation is primarily mediated by binding of prostaglandin E2 (PGE2) to their receptors on the responder cells (Huang et al., 2011; Kuhrer et al., 1986) and it is also known that low or non-differentiated cells described as “stem” express those receptors on their membrane. Ligation of PGE2 to its receptor results in delivering of proliferative signal and subsequent division in the neighbouring cells (Ichim and Tait, 2016) (Fig. 1A). The consequential repopulation may stimulate the process of tissue regeneration in different physiological, but also pathological conditions, such as chronic inflammation. As already stated by Virchow in 1860, the tumor appears like “a wound that never heals”. This loop can be further amplified by other mediators produced by the apoptotic cells that in addition to direct caspase 3 dependent proliferative signal delivered to neighbouring pluripotent cell, can convey proangiogenic factors and recruit immune cells that may favour tumor progression through release of multiple mitogenic molecules (Scheme 1B) (Fogarty and Bergmann, 2017). As a consequence, dying cells can promote further dedifferentiation, which in some circumstances may lead to the presence of giant cells as a terminal process of phenotype regression (Niu et al., 2017). Moreover, in response to apoptotic mediators, epithelial-mesenchymal transition (EMT) can be triggered (Chaudhry et al., 2014). Subsequently in parallel with transition of epithelial to mesenchymal morphology acquirement of stem like properties has been observed (Singh and Settleman, 2010). For example, EMT promotes cancer stem phenotype in thyroid cancer leading to tumor progression (Lan et al., 2013).

If the above physio-pharmacological hypothesis is correct how can one improve efficiency of killing based treatments? One attractive alternative or complementary approach in cancer treatment that has emerged during the last decades is the induction of differentiation of tumor cells. Differentiation based therapies are strategies that lead to maturation of low differentiated cancer cells rendering them less aggressive and more sensitive to conventional treatments (Dela Cruz and Matushansky, 2012). It has been shown that despite genetic alterations, malignant cells forced by microenvironmental factors can regain normal phenotype upon transplantation in blastocysts and become a part of regular embryonic development (Illmensee, 1978). “Differentiation based therapy” represents a mainstream direction in regenerative medicine. Protocols on reprogramming or generation of induced pluripotent cells as a source of progenitors for further reprogramming are primarily focused on genetic manipulations. However, that has not eliminated the most important point of concern that is teratomagenesis (Cieślar-Pobuda et al., 2017). Transdifferentiation as a process of gaining of different phenotype escaping pluripotency may represent a harmless alternative. However, unpredictable microenvironmental influence on stability of transdifferentiated phenotype may persist. The concept of differentiation/transdifferentiation based therapies in regenerative medicine differs from that used for cancer treatment since the goal for this latter is the acquirement of a phenotype characterized by the loss of proliferative potential. In contrast to the approach in regenerative medicine, it is not necessary that in cancer tissue these “differentiated” cells re-establish their function. Most often the fate of such cells is the state of senescence and sponta theirneous involution. Even with recently described controversial role of cellular senescence in response to cancer therapy, in the context of proliferative response as a net effect of the interplay between apoptotic and non/low differentiated subpopulation, induction of quiescent phenotype can be promising (Schosserer et al., 2017).

Here we reviewed the potential of plant-derived compounds as differentiation inducers, with the focus on anthraquinones from *Aloe vera*, *Senna*, *Rheum* sp. and hop derived prenylflavonoids. Previously, we stated that several plant-derived and synthetic compounds promote the differentiation and/or transdifferentiation of advanced cancers like glioblastoma or melanoma.

2. Induction of differentiation as approach for cancer treatment

Differentiation therapy for cancer can be defined as a pharmacological intervention directed toward the maturation of tumor cells and loss of the cancer phenotype. On the contrary, traditional chemotherapy aims at the induction of necrosis or apoptosis in cancer cells. As a general note, differentiation agents should be less toxic than chemotherapy. As pointed out by Cruz and Matushansky, cancer differentiation can occur as: cancer-directed differentiation, in which the initial oncogenic pathway is not corrected; cancer-reverted differentiation, aimed at correcting the oncogenic defects in order to restore the normal cellular differentiation; or cancer-diverted differentiation (transdifferentiation), in which tumor cells are redirected to alternative differentiation pathways (2010). By doing so, tumor cells can differentiate toward a different lineage in which the differentiation pathway is not blocked (Cruz and Matushansky, 2012). The prototypical example of differentiation-based therapy is usage of retinoic acid in the treatment of acute promyelocytic leukemia (APML). All trans-retinoic acid dampens the PML/RARα (promyelocytic leukemia/retinoic acid receptor α) fusion protein, responsible for the blockade of myeloid cells maturation at the promyelocytic stage. It has been reported that 95.8% of APML patients who received both all trans-retinoic acid (ATRA) and chemotherapy, underwent remission without showing bone marrow hypoplasia (Huang et al., 1988). Although most of the studies on differentiation therapy focus on hematological tumors, more attention is recently being paid to the potential role of differentiation therapy in solid tumors. Up to now, no compound has proved to have a differentiation-inducing effect equivalent to that observed for ATRA in the setting of APL. This is probably due to the fact that the pathogenesis of APL relies on a single karyotype defect that may be easier to correct as compared to multiple defects (Cruz and Matushansky, 2012). On the contrary, most solid tumors are heterogeneous aberrant tissues and consequently, a single-targeted pharmacological intervention is likely to be inadequate to promote their differentiation. In addition, the

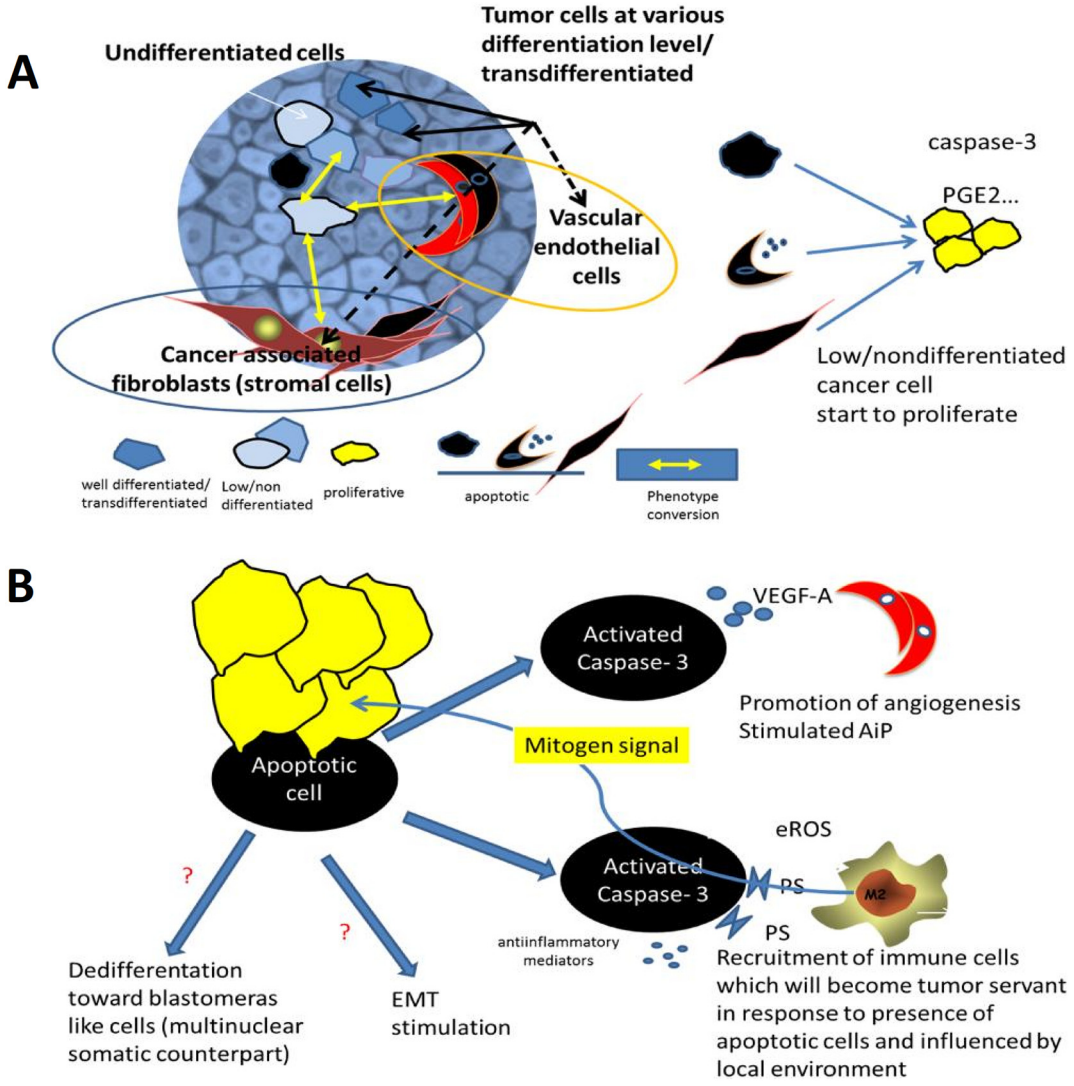

Fig. 1. Induction of apoptosis stimulates tumor repopulation. A). Tumor is a mass of heterogeneous cellular populations at different stages of differentiation and phenotypes. All actors might have the same ancestor and possess phenotypical plasticity. Induction of cell death in tumor mass will trigger the proliferation of cancer stem cells regarding to caspase 3 mediated synthesis of PGE2. B) Apoptotic cells stimulated tumor repopulation in long-lasting manner through: production of vascular endothelial growth factor (VEGF) promoting angiogenesis and stimulating AIP; production of extracellular ROS and inversion of PS leading to the recruitment of protumor immune cells which became main source of tumor mitogens; promoting further dedifferentiation; promoting EMT.

outcome of differentiation based therapy depends on intercellular communication between cells at different maturation stages, as well as other mediators like cytokines, chemokines, and reactive oxygen/nitrogen species (ROS/RNS) in tumor microenvironment. Accordingly, some agents may only promote differentiation in certain type of the cells and under certain biological conditions. Therefore, depending on the stage of the cell cycle the same agent can induce apoptosis, differentiation/transdifferentiation or senescence (Bulatović et al., 2014; Mijatovic et al., 2005a). The net effect of the drug treatment can be defined by the dosage, modality of delivering or regimen. Recently, it has been shown that a slow releasing formulation of etoposide conjugated with dextran, that mimicks metronomic therapy, changes the mode of action of the drug from apoptotic toward differentiation-related compound (De Nicola et al., 2017). Drugs or treatment protocols whose mechanism of action is not solely based on cell killing but rather includes change of cell phenotype toward more mature one, may prevent unwanted side-effects of proliferative responses. Increasing evidence indicates that induction of differentiation of solid tumor- derived cell lines is possible *in vitro*. However, clinical data are still sporadic. One of the first examples has been seen in in few patients with high grade lyposarcoma treated with the peroxisome proliferator-activated receptor-γ (PPARγ) ligand, troglitazone. This treatment decreased the proliferative potential of malignant cells leading to intensive accumulation of lipids inside cytoplasm, thus indicating that the treatment had induced a process of cellular maturation (Demetri et al., 1999). Also, the antitumor drug from Caribbean tunicate *Ecteinascidia turbinata*, trabectedin, changed the structure of tumor tissue in patients with myxoid liposarcomas, reducing their potential to divide and promoting subsequent shrinkage of the tumor mass (Grosso et al., 2007). This produced a long lasting effect in 17 out of 23 patients. In one additional case, the administration of histone deacetilase inhibitor, depsipeptide, to patients with recurrent non-medullary thyroid cancer, has reconstructed radiotherapy sensitivity (Sherman et al., 2009).

3. Plant-derived compounds as chemotherapeutics

Several plant-derived compounds are currently used as first and second line chemotherapeutics in cancer, *e.g.* paclitaxel, vincristine, etoposide and irinotecan. These drugs are characterized by different mechanisms of action, that include interaction with microtubules

(Vinca alkaloids and taxanes), inhibition of topoisomerases (camptothecins and epipodophyllotoxins), and cell cycle interference (reviewed in Nobili et al., 2009).

Taxanes, that include paclitaxel and docetaxel, are a class of diterpens that exerts anticancer activity by promoting the stabilization of microtubules and the prevention from depolymerisation. Paclitaxel, originally extracted from the plant *Taxus brevifolia*, is now obtained by a semisynthetic process, as docetaxel, starting from the 10-deacetylbaccatin III of the tree, *Taxus baccata*. Paclitaxel and docetaxel are poorly water-soluble and, therefore, they need to be administered in formulations that include polyoxyethylated nonionic surfactants, that may cause several adverse effects. Taxanes bind to the beta subunits of tubulin within the microtubular lumen, differently from colchicine and vinblastine, that instead interact with the microtubules at the interface between the α- and β-tubulin dimers and surrounding the GTP binding site, respectively. Paclitaxel is more effective at the G2-M phase, by targeting the mitotic spindle, while docetaxel exert its maximum cytotoxic effect during the S phase of the cell cycle, affecting centrosome organization. Paclitaxel and docetaxel are highly effective against a wide variety of tumors, including solid (breast, ovarian and non-small cell lung cancer) and hematological cancers. In order to overcome the problems in drug delivery and resistance to taxans, novel molecules and formulations have been developed. Among them, cabazitaxel has been approved in 2010 by FDA for the treatment of hormone-refractory prostate cancer, while Paclitaxel poliglumex and EndoTAG + paclitaxel represent formulations that do not involve cremophor, and that are being currently tested in various types of cancer (Muggia and Kudlowitz, 2014; Stanton et al., 2011).

Differently from taxans, vinca alkaloids, that are isolated from the plant *Catharanthus roseus* (a.k.a. *Vinca rosea*), bind to alpha-tubulin, blocking its polymerization with beta-tubulin, and disrupting the assembly of the mitotic spindle. At the ends of each microtubule, there are 16 to 17 high-affinity binding sites for vinca alkaloids. Vinca alkaloids share a dimeric chemical structure composed of two multi-ringed units, a terpene indole alkaloid (catharanthine), and the monoterpenoid indole alkaloid, vindoline. Vincristine, vinblastine and vindesine represent the first vinca alkaloids with proved anti-cancer action, while Vinorelbine represent the first second-generation vinca alkaloid. Vincristine is used in lymphomas, pediatric tumors, neuroblastoma, and rhabdomyosarcoma, as well as in CLL, sarcomas, and small-cell lung carcinoma, as combination therapy. Vinblastine is used in combination with cisplatin, and bleomycin (PVB regimen) in testicular carcinoma or in combination with doxorubicin, bleomycin, and dacarbazine (ABVD regimen) for the treatment of lymphomas. Vinorelbine has been approved for the treatment of unresectable non-small-cell lung cancer, and can be used in metastatic breast cancer. Vindesine has been used in ALL, melanoma, solid tumors of the childhood and metastatic renal, breast, esophageal and colorectal cancer. Vinflunine, the first fluorinated vinca alkaloid derivative, has been approved as second-line therapy for cell carcinoma of the urothelium (reviewed by Moudi et al., 2013).

Among plant-derived compounds, camptothecins and epipodophyllotoxins, have been found to inhibit a specialized class of nuclear enzymes, known as topoisomerases. Catalytic functions of topoisomerases include transient cleavage of DNA strands, DNA revolving and ligation of cut strands. Topoisomerases are classified as type I and II. Type I topoisomerases cleave single DNA strand, while type II cleave both strands. The semisynthetic camptothecins, irinotecan and topotecan, analogues of the camptothecins extracted from *Camptotheca acuminate*, act as topoisomerase I inhibitors, therefore preventing supercoiled DNA to relax and to allow gene expression or repair, while the plant-derived epipodophyllotoxins (etoposide and teniposide) act as inhibitors of topoisomerase II, a nuclear enzyme that promotes chromosome disentanglement. Camptothecins share a pentacyclic ring structure, with a pyrrolo[3,4-β]-quinoline moiety, conjugated pyridone moiety and one chiral center at position 20 within the lactone ring. In association to 5-fluorouracil, irinotecan is currently used for the treatment of metastatic colorectal tumors. Topotecan is indicated in second-line therapy against advanced ovarian carcinoma. Besides irinotecan and topotecan, other synthetic camptothechin are currently under development, such as lurtotecan, karenitecin and gimatecan (Nobili et al., 2009). The epipodophyllotoxins, etoposide and teniposide, derivatives of podophyllotoxins extracted from the root of the plant, *Podophyllum peltatum*, form a ternary complex with topoisomerase II and DNA, thus preventing the resealing of the DNA break. Hence, the topoisomerase II-DNA intermediate cannot be reversed, leading to cell death. Teniposide and etoposide are both 4-dimethyl-epipodophyllotoxins that differ only for the substitution of a methyl group with a thenylidene ring. The clinical indications for etoposide are lung cancer, choriocarcinoma, ovarian and testis cancers, lymphoma and acute myeloid leukemia. Teniposide is approved for brain tumors, lymphomas and bladder cancer (Nobili et al., 2009).

Other plant-derived molecules have been approved for use as anticancer chemotherapeutics or are under investigation, on the basis of their ability to affect cell cycle progression. Notably, homoharringtonine, an alkaloid isolated from the tree *Cephalotaxus harringtonia*, inhibits protein synthesis by acting on the ribosomes of cancer cells, thus blocking the progression of cells from G1 to S phase and from G2 to M phase. Homoharringtonine is now approved for the treatment for patients with chronic myeloid leukemia, resistant to tyrosine kinase inhibitors (Zhou et al., 1995). Flavopiridol, a semisynthetic flavone derived from rohitukine, an alkaloid isolated from *Dysoxylum binectariferum*, is the first potent inhibitor of cyclin-dependent kinases to enter clinical trial and is currently being tested for the treatment of acute myeloid leukemia (Shapiro, 2004), and for advanced solid tumors, in association to docetaxel (ClinicalTrials.gov identifier: NCT00331682), cisplatin (ClinicalTrials.gov identifier: NCT00083122) and vorinostat (ClinicalTrials.gov identifier: NCT00324480).

4. Naturally occurring compounds as differentiation inducing agents for hematological tumors

A number of molecules that promote the differentiation of leukemia cell have been described (Table 1). Gupta et al. showed that securine promotes cell differentiation *in vitro* in several AML cell lines and primary cancer cells. Securine treatment was found to induce myelomonocytic differentiation and monocytic differentiation in HL60 cells, as determined by the upregulation of CD11b and CD14, respectively. Microarray analysis showed that securine modulates the expression of several genes, such as c-myc and CEBP/β, underlying AML pathogenesis. Along the same lines, the authors showed that securine treatment increases by ~7-fold the expression of CD11b in THP1 cells (Gupta et al., 2011).

The isosteroidal alkaloid verticinone from the bulbs of *Fritillaria usuriensis Maxim*, used traditionally for its anti-inflammatory properties (Wu et al., 2010), was shown to induce CD11b upregulation in myeloblastic leukemia HL60 cells, indicating a differentiation toward the granulocyte phenotype.

The isoquinoline alkaloid berberine, extracted from the plant *Berberis vulgaris* L., administered to mice bearing the WEHI-3 leukemia cells, was able to significantly reduce the percentage of cells expressing the CD11b and Mac-3 surface markers and to promote B-cell differentiation, as determined by the induction of CD19 expression (Yu et al., 2007).

The flavonoid wogonine from *Scutellaria baicalensis Georgi* has been reported to significantly increase the expression of CD11b, but not CD14, in U937 cells, suggesting its ability to promote myeloid differentiation (Baumann et al., 2008; Zhang et al., 2008). Also, wogonine promoted erythroid differentiation in CML cells K562 cells, as determined by the increased expression of glycophorin A and CD71 and by the induction GATA-1, a transcription factor involved in megakariocytes and erythrocytes development (Yang et al., 2014a, 2014b).

Table 1
Pro-differentiative properties of plant-derived compounds.

Cell line(s)	Compound(s)	Effect(s)	References
Hematological cancer			
Human HL-60, THP-1 and OCI-AML3 leukemic cell lines	Securinine 15 μM	Securinine induced potent nitroblue tetrazolium (NBT) activity, increased CD11b and CD14 expression In HL60, securine treatment regulated the expression of genes involved in AML differentiation, such as c-myc and c-myb which are downregulated and CEBP/β, CEBP/δ, egr-1, mafB, fos, and jun that are upregulated	Gupta et al., 2011
Mouse WEHI-3 leukemia cells injected i.v. in BALB/c mice	Berberine 200 mg/kg	Berberine reduced the proportion of cells expression Mac-3 and CD11b and increased the number of CD19+ cells	Yu et al., 2007
Human NB4 promyelocytic leukemia cells	Wogonin 50 μM	Wogonine increased nitroblue tetrazolium (NBT) activity, increased CD11b and CD14 expression	Zhang et al., 2008
Human erythroleukemic K562 cells	Wogonin 20–80 μM	Wogonin dose-dependently increased glycophorin A, CD71 and γ-globin expression, as well as the expression of the transcription factors, GATA-1 and FOG-1	Yang et al., 2014a,b
Human chronic leukemia cell line K562	Apigetrin 75 μM	Apigetrin induced glycophorin A expression and fetal hemoglobin synthesis	Tsolmon et al., 2011
Human erythroleukemic cell line K562	Fagaronine	Fagaronine increased the expression such as γ- and α-globin, PBGD, and the expression of the transcription factors, GATA-1 and NF-E2	Dupont et al., 2005
Solid cancer			
Rat glioma cell line C6	Aloe emodin 20 μM	Aloe emodin promoted the maturation process toward the astrocytic lineage by increasing the expression of GFAP. Aloe emodin reduced the reactivity of C6 cells to the oligodendrocyte-specific O1 marker	Mijatovic et al., 2005a, 2005b
Mouse B16–F10 melanoma cells	Aloe emodin 10 μM	Aloe emodin induced transglutaminase 2 activity and increases the intracellular protoporphyrin IX content. Also, Aloe emodin induced melanogenesis and dendrite formation	Tabolacci et al., 2010
Mouse B16 melanoma cells	Aloe emodin 40 μM	Aloe emodin increased the expression of melanin and tyrosinase activity	Radovic et al., 2012
Human colorectal cancer cells, SW480 and SW620	Emodin 50 μM	Emodin induced alkaline phosphatase activity in SW620 cells and increased E-cadherin expression in both cell lines	Pooja and Karunagaran, 2014
Human breast adenocarcinoma, MDA-MB453 cells	Emodin 40 μM	Emodin induced the production of lipid droplets	Zhang et al., 1995
Mouse melanoma B16 cells	Isoxanthohumol 23 μM	Isoxanthohumol increased tyrosinase activity	Krajnović et al., 2016
Primary and metastatic human melanoma cells	Mezerein	Mezerein increasedn mda-6 expression	Jiang et al., 1995
Rat C6 glioma cell line	Taxol 100 nM	Taxol increased the percentages of nestin, βIII-tubulin, GFAP, and CNPase-positive cells	Chao et al., 2015
Rat C6 glioma cell line	Saikosaponins 0.1 to 100 μg/mL	Saikosaponin a increased enzymatic activities of glutamine synthetase (GS) and 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP). Saikosaponin d only increased of GS activity	Tsai et al., 2002
Mouse B16 melanoma cells	Saikosaponin b2 5 μM	Saikosaponin b2 increased expression tyrosinase	Zong et al., 1998

Erythroid differentiation of K562 cells has been also shown to be induced by aclacinomycin A from *Streptomyces galilaeus* and doxorubicin from *Streptomyces peucetius* (Morceau et al., 1996; Trentesaux et al., 1993). Among plant-derived compounds that have been found to induce erithroid differentiation *in vitro* in K562 cells, upon upregulation of GATA-1, the benzophenanthridine alkaloid, fagaronine, from the plant *Zanthoxylum zanthoxyloide*, and apigetrin, extracted from dandelion coffee, should also be mentioned (Tsolmon et al., 2011; Dupont et al., 2005).

Iriyama and collaborators have investigated the effects of ATRA and valproic acid (VPA) on the human acute promyelocytic leukemia cell line, NB4, bearing the t(15;17) translocation. VPA is a derivative of the valeric acid extracted from *Valeriana officinalis* L., which has been described to exert multiple biological effects, including inhibition of histone deacetylase, protein kinase C and WNT activity, as well as the activation the peroxisome proliferator-activated receptors and of the ERK-AP1 pathway (Iriyama et al., 2014). Both ATRA and VPA were able to induce differentiation of the NB4 cells and their effect was improved when administered concomitantly. The restoration of the granulocytic phenotype was associated to an increase in the levels of the transcription factors, C/EBP(β, ε) and PU1.

5. Natural compounds as differentiation inducing agents for solid tumors

Cell lines where a phenotypic transformation can be triggered include melanoma, pancreatic cancer, glioma, lung cancer, retinoblastoma, breast cancer, colon cancer, pheochromocytoma, liposarcoma and neuroblastoma (Guzhova et al., 2001; Ebert and Salcman, 1994; Vaudry et al., 2002; Fassina et al., 1997; Hartmann et al., 1997; El-Metwally and Adrian, 1999; Levy et al., 2003; Gaschott and Stein, 2003; Bartolini et al., 2004; Athanasiadis et al., 1995; Chang and Szabo, 2000; Demetri et al., 1999) (Table 1).

It has been demonstrated that numerous synthetic and naturally occurring compounds are able to prompt the differentiation of various malignant cells (Fig. 2) (Bulatović et al., 2014; Maksimovic-Ivanic et al., 2017; Maksimovic-Ivanic et al., 2009; Mijatovic et al., 2005a,b; Radovic et al., 2012). Diterpene extracted from *Coleus forskohlii*, known as forskolin, differentiate somatic cell hybrid NG108-15 neuroblastoma/glioma cells through upregulation of cAMP (Ammer and Schulz, 1997; Brodsky et al., 1998; Kim et al., 2004; Takanaga et al., 2004). In rat astrocytoma cells, C6 cells, forskolin enhanced the presence of GFAP regarding to maintaining of cyclin D1 at low expression level. The treatment of C6 cells with plant-derived compounds isolated from *Bupleurum* species, triterpenoid compounds, saicosaponins, or anthraquinone, aloe emodin, induced their commitment to astrocytic lineage (Mijatovic et al., 2005a,b; Tsai et al., 2002). As regards the impressive plasticity of gliomas, in addition to their possible astrocyte commitment, it should be mentioned that in certain conditions C6 cells can be conducted toward oligodendrocytes or neurons. It is possible to promote neural differentiation of C6 cells using commercial cytostatic drug taxol, isolated from *Taxus brevifolia*, which resulted in mixed phenotype appearance where some of the cells bearing oligodendrocytic, neuronal and astrocytic markers (Chao et al., 2015). Since glioma and melanoma cells are derived from the same neuroectodermal origin, the agents found to be efficient in the differentiation of gliomas were also found to be effective inducers of melanoma maturation. Treatment with saicosaponins differentiates B16 melanoma cells

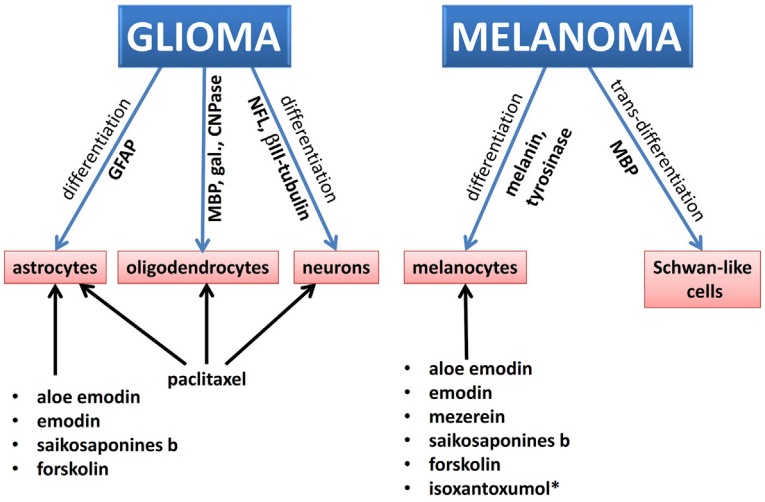

Fig. 2. Differentiation of cancer cells using experimental therapeutics of plant-derived origin *incomplete differentiation.

toward melanocytes *in vitro* (Zong et al., 1998). This effect can be prevented by PKC activator phorbol ester, indicating the essential role of abrogated PKC function in observed effect. Forskolin induced differentiation of UISO-Mel-6 cells through increased expression of microphtalmia transcription factor, since it is known that this plant-derived compound is an alpha-melanocyte stimulating hormone agonist (Lekmine and Salti, 2008). Similarly, the antileukemic drug, mezerein, a diterpene ester present in the juice of *Daphne mezereum*, caused the maturation of melanoma phenotype, characterized by altered morphology, and upregulation of melanogenesis (Jiang et al., 1995). Unfortunately, cell differentiation triggered by many experimental agents results in transitory changes in phenotype, dependent on the presence of the therapeutic in the culture. *In vitro* and *in vivo* data have also shown that VPA is able to promote differentiation in the neuroblastoma cell lines, UKF-NB-2 and UKF-NB-3, as determined by upregulation of the neural cell adhesion molecule, NCAM, and elongation of neuronal processes (Blaheta and Cinatl Jr, 2002). In addition to highly invasive forms of tumors such as glioma, glioblastoma and melanoma, there is a long list of different malignant pathologies prone to differentiation since they are characterized as anaplastic. Thus, it has been reported that the histone deacetylase inhibitor, trichostatin A, induces significant changes in the morphology of the small cell lung carcinoma cell line, DMS53, which acquires a flattened round shape with neuritis-like processes that suggests a neural differentiation (Platta et al., 2007). Also, treatment with another histone deacetylase inhibitor, vorinostat, determined major morphological changes in MCF7 breast cancer cells, indicative for epithelial mammary differentiation (Munster et al., 2001). Other examples of differentiation–inducing agents come from Rephaeli and collaborators, who have observed that administration of AN-7, a prodrug of butyric acid, to mice bearing the 22Rv1 prostate tumor, increases the expression of PSA by 15-fold in the cancer cells as compared to the control, suggesting cellular differentiation (Rephaeli et al., 2005). Finally, in an *in vivo* model of chondrosarcoma, depsipeptide (FK228) treatment was associated to an increase in alkaline phosphatase expression and a decrease in glycosaminoglycans production, suggesting an on-going differentiation process toward a chondrocytic-like phenotype (Sakimura et al., 2007). One more example comes from the observation that the differentiation of nasopharyngeal carcinoma cells is disrupted due to a downregulated expression of IkB kinase α (IKKα). IKKα is an ubiquitous helix-loop-helix kinase involved in the activation of the nuclear factor-kB (NF-kB) transcription factor, but it also controls the differentiation of keratinocytes by an indirect regulation of Myc. Indeed, IKKα - KO mice are characterized by undifferentiated and thickened epidermis, with keratinocytes lacking the physiological differentiation markers (Liu et al., 2008). Further investigations revealed that the methylation of the H3K27 histone at the IKKα promoter is responsible for the downregulation of IKKα and that retinoic acid is able to restore the normal H3K27 methylation pattern, thus increasing the differentiation of nasopharyngeal carcinoma cells (Yan et al., 2014).

6. Hydroxianthraquinones from *Aloe vera* as differentiation inducing agent for solid tumors

Aloe emodin and emodin are antraquinones extracted from leaves, roots or barks of numerous plants used in Chinese traditional medicine (Fig. 3) (Dong et al., 2016; Srinivas et al., 2007). Aloe emodin (AE) and emodin (EO), own pleiotropic biological activities like antimicrobial, laxative, diuretic, vasodilator, immunosuppressive, anti-inflammatory and antitumor (Shrimali et al., 2013). The effectiveness of these compounds in such a variety of different pathologies is suggestive of their influence on several signaling pathways involved in proliferation, differentiation or death (Shrimali et al., 2013; Liu et al., 2015). It was shown that both EO and AE induced apoptosis, autophagic cell death or cell cycle arrest in numerous tumor cell lines *in vitro* (Lin et al., 2016; Liu et al., 2015; Chang et al., 2011; Chun-Guang et al., 2010; Radovic et al., 2012; Harhaji et al., 2007; Mijatovic et al., 2005a; Mijatovic et al., 2004; Mijatovic et al., 2005b). However, *in vivo* data are modest, probably due to poor solubility, stability and rapid inactivation by CytP450 (Chen et al., 2015; Teng et al., 2012; Mueller et al., 1998). Also, the capability to interact with DNA resulted in genotoxicity *in vitro* (Srinivas et al., 2007). On the other hand, genotoxicity was not observed in healthy animals exposed to high dose of AE (Heidemann et al., 1996). Pecere et al. published in 2000 that Aloe emodin induced regression of neuroectodermal tumors in xenograft model without acute and chronic toxicity (Pecere et al., 2000). Further research revealed that AE strongly changed the morphology of cancer cells. Morphological transformation due to the treatment was accompanied with elevated GFAP expression. This effect coincides with the ERK1/2

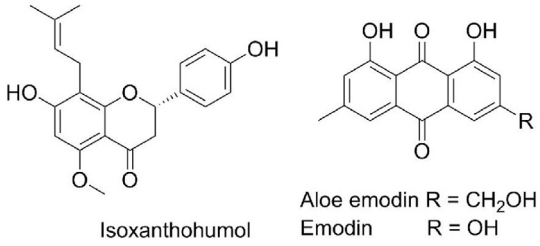

Fig. 3. Chemical structure.

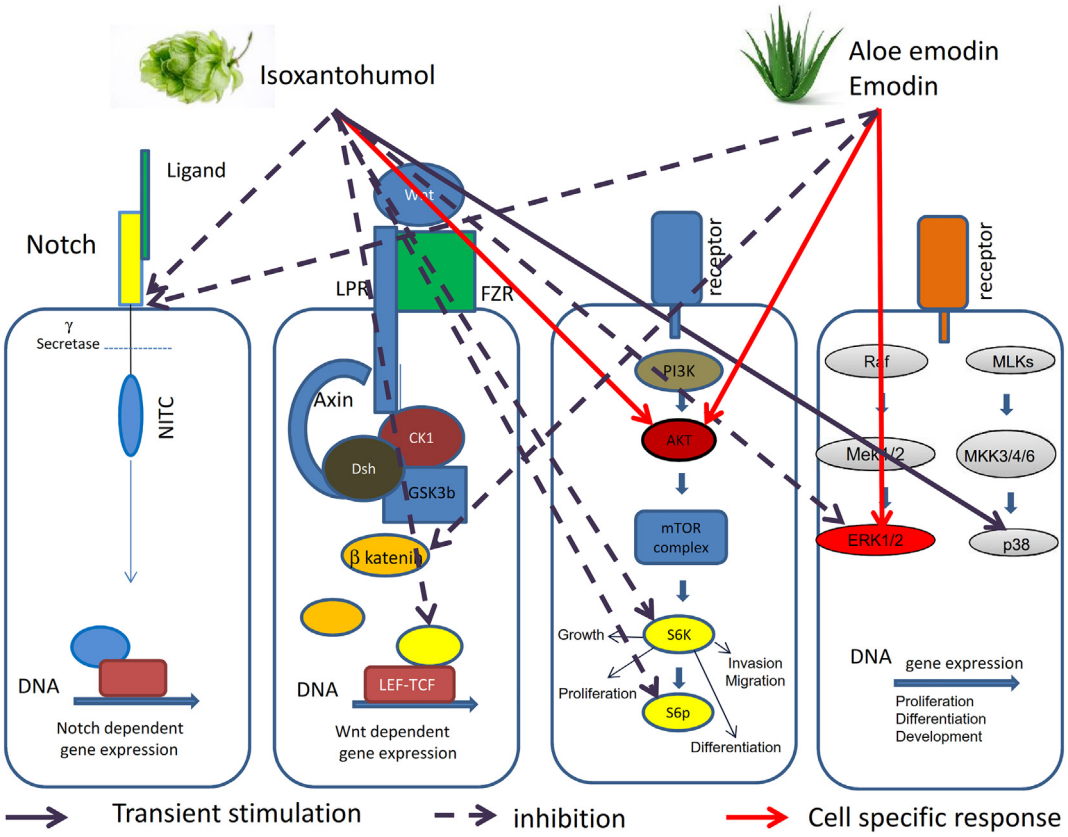

Fig. 4. Signaling pathways in the route of differentiation: potential targets of herbal compounds.

inhibition. The importance of down regulated expression of ERK1/2 in enhanced GFAP quantity was proved by simulating it with specific inhibitor PD98059 (Mijatovic et al., 2005a). However, the abrogation of ERK1/2 is not the general consequence of AE treatment, since in other cell types the same compound up-regulated its activity (Mijatovic et al., 2005b; Radovic et al., 2012). Cultivation of B16 melanoma cells in the presence of AE led to proliferative potential waste while the morphology of treated cells was characterized by appearances of dendritic-like prolongation and flattening of the cell body. The morphological changes were simultaneous with strong potentiation of tyrosinase activity, as well as melanin production, indicating that cells acquired the phenotype resembling mature melanocytes. Upregulation of p53, cyclin D and ERK1/2 expression reflected AE influence at intracellular level. The most important observation is that differentiation of B16 cells under AE is persistent as the so-treated cells lost tumorigenic potential in syngeneic mouse model (Radovic et al., 2012). Tabalocci et al. showed that AE was efficient in similar manner in metastatic clone of B16-F10 cells changing their metastatic properties such as cell adhesion and aggregation (Tabolacci et al., 2010). Apart from mentioned examples of glioma and melanoma differentiation it is found that these anthraquinones are able to differentiate other types of tumors, like breast, colon and cervical cancer (Pooja and Karunagaran, 2014; Luo et al., 2014; Guo et al., 2007; Zhang et al., 1995).

7. Hop derived penylflavonoids as differentiation inducing agents for solid tumors

Prenylflavonoids belongs to a class of compounds, known as flavonoids. These molecules are broadly distributed in the world of herbs. Prenylflavonoides are phytoestrogens and antioxidants, but in last few decades their antitumor potential has been frequently explored (Rossi et al., 2014; Gerhäuser, 2005). Since they are used as beer ingredients, their biological activity is deeply studied. A hop, the basic constituent for beer preparation, is a rich source of prenylflavonoids: xanthohumol (XN), isoxanthohumol (IXN) and 8-prenylnaringenin (8-PN) (Gerhäuser, 2005). Besides, the plant *Sophora flavescens*, used in traditional Chinese medicine, is also abundant in IXN (Jin et al., 2010). All hop-derived prenylflavonoids are proved phytoestrogens, antioxidants, antiinfective, antiinflammatory, antiangiogenic and anticancer agents (Rossi et al., 2014; Gerhäuser, 2005; Żołnierczyk et al., 2015). The list of the cell lines sensitive to those compounds is long and includes different tumor types like breast, melanoma, ovarian, colon, and prostate (Rossi et al., 2014; Gerhäuser, 2005; Żołnierczyk et al., 2015; Krajnović et al., 2016; Negrão et al., 2013; Tronina et al., 2013; Allsopp et al., 2013; Serwe et al., 2012; Yang et al., 2007; Delmulle et al., 2008). So far, few groups showed the favourable effect of XN *in vivo* in different models in prostate, leukemia, breast, and T cell lymphoma models (Monteiro et al., 2008; Venè et al., 2012; Benelli et al., 2012). As far as we know, IXN (Fig. 3) has been shown to suppress the melanoma growth in subcutaneous model in C57Bl/6 mice concomitantly to subtoxic dose of paclitaxel (Krajnović et al., 2016). In some experimental settings, those compounds induced blockade in cell cycle progression while in other circumstances apoptotic cell death appears as the consequence of the treatment. This effect is probably secondary to the reduced expression of antiapoptotic proteins like BCL2, BCLXL, survivin, xIAP, cIAP which can further influence sensitivity of malignant cells to chemotherapy (Gerhäuser, 2005; Żołnierczyk et al., 2015; Kunnimalaiyaan et al., 2015a; Kunnimalaiyaan et al., 2015b). Also, caspase independent cell death resembling autophagic cell death can be behind their activity (Delmulle et al., 2008; Krajnović et al., 2016). Down regulation of inflammatory and angiogenic mediators makes these compounds a powerful tool against tumor progression and metastasis (Negrão et al., 2013; Serwe et al., 2012). XN and IXN are inhibitors of efflux transporters like MDR proteins and also CYP450s, influencing the efficacy of applied therapy. Potential of XN and IXN to differentiate colon cancer cells was showed in HT29 and SW620

(Żołnierczyk et al., 2015; Allsopp et al., 2013). Concordantly, we reported that IXN decreased proliferative potential of B16 mouse melanoma and A375 human melanoma that belongs to the resistant iNOS positive melanoma (Krajnović et al., 2016). Both type of melanoma cells exposed to IXN acquired different morphology as compared to their precursor cells, in term of size and shape. However, the enhanced tyrosinase activity, enzyme crucial to the synthesis of melanin, was not accompanied with elevated melanin content, indicating an aberrant differentiation into melanocyte lineage in B16 cells. This was attributed to ROS scavenging potential of the drug. Accordingly, numerous data confirmed the importance of ROS in completing melanin synthesis, which can be prevented by endogenous or exogenous antioxidants. Altogether, these properties qualify this group of compounds as potential therapeutics for aggressive malignancies. It has also been observed that IXN treatment is able to suppress the phosphorylation of p70 S6 kinase and its target, S6 protein, were suppressed. Given the role of p70 S6 kinase in protein synthesis, rearrangement of cytoskeleton, cell survival and proliferation, this is probably connected with the differentiation inducing property of the IXN (Pearce et al., 2010) (Fig. 4).

8. Naturally occurring compounds in STEM cell differentiation

The tumor has the features of multicellular unit composed of diverse cells, endowed with functional autonomy. Concerning continuous dynamic in phenotype changes, cancer can be described as a disease of reprogramming and differentiation (Huang et al., 2015). Since differentiated cells can dedifferentiate into cancer stem cells or cancer stem-like cells, the *vice versa* is also possible. That establishes the platform for cancer treatment, considering that tumor is not a group of random non-orchestrated cells but rather multicellular entity which functions at highly organised level even with disrupted morphology. It is well described that therapeutic failure in advanced malignancies is connected with the presence of stem cells that, owing to dormant state, don't respond in similar way as proliferating bulk cells (Massard et al., 2006). However, as we have previously mentioned, the death of cells induced by chemo- or radiotherapy in the neighbouring or bystander cells triggers their proliferation, making the frame for tumor progression (Fogarty and Bergmann, 2017). To prevent the undesirable effect of killing based therapy, induction of differentiation can be alternatively or complementarily considered. Synergistic effects of differentiating agents and chemotherapeutic agents have been described through this review. In recent years, a lot of examples in literature describing the potential of naturally occurring compounds to conduct cancer stem cells toward more differentiated phenotype appeared (Murakami and Tashiro, 2015). We will mention just few of them. Studies on glioblastoma multiforme (GBM) and breast cancer have demonstrated the ability of ATRA to induce the differentiation of cancer stem cells. In particular, treatment with 10 μM ATRA of GBM neurospheres reduced the percentage of cell expressing the neuronal stem cell marker, nestin, and increased the number of cells expressing the astrocytic marker, GFAP, and the neural marker, TUJ1 (Karsy et al., 2010). Also, Yan et al. (2016) found that treatment of MCF7/C6 breast cancer cells with 10 μM ATRA for 72 h was associated to a significant reduction in CD44+/CD24−/low and NANOG-positive cells, while concomitantly increasing the expression of the maturation markers, syndecan 3 and involucrin. It has been found that emodin inhibited self-renewal of glioma stem cells *in vitro* as well as maintenance of stemness through suppression of one of the main signals for stem-persistence, Notch. Additionally, nonphosphorylated β-catenin, as well as phosphorylated STAT-3, was also affected. Anti-stem feature of emodin is probably connected with its ability to promote proteasome degradation of EGF receptor (Kim et al., 2015). A similar effect was observed in gallbladder carcinoma stem like population where emodin suppressed ATP- binding cassette protein, ABCBG2 and eliminated sphere formation through ROS related mechanism (Li et al., 2013). Through the same mechanism, emodin downregulated Wnt signals in colon cancer cells, resulting in downstream matrix metalloproteinases 2 and 9 expressions (Pooja and Karunagaran, 2014). Aloe emodin also exerted antimetastatic properties by downregulating cancer stem phenotype and inhibiting EMT, *e.g.* in HER-2-overexpressed breast cancer xenograft model (Ma et al., 2016). Another biological active substance isolated from hop, xanthohumol, can influence stemness in chemotherapy resistant MCF-7/ADR cells. Reduced stemness was manifested through decreased colony formation, migration, sphere formation, as well as abrogated expression of stemness related biomarkers (Liu et al., 2016). In hepatocellular carcinoma treatment with this compound resulted in Notch signaling pathway inhibition, evidenced by declined expression of Notch 1 and HES1 proteins (Kunnimalaiyaan et al., 2015a). Similarly, IXN was found to block the transdifferentiation of cancer stem cells toward vascular conduits *in vitro* and *in vivo* (Shekhani et al., 2013). On the other hand, highly aggressive, amelanotic A375 cells showed deprivation of stem markers upon the same treatment (Krajnović et al., 2016). Downregulation of Wnt/β catenin and Notch 1 signaling pathway, critical regulators of melanocyte lineage development, as well of the differentiation marker Oct-3/4, indicated that morphological changes triggered by IXN, correlate with their lower invasive potential. The same pattern was noticed for hepatocellular, pancreatic and ovarian cancers, where XN inhibited Notch 1 signaling (Kunnimalaiyaan et al., 2015a; Kunnimalaiyaan et al., 2015b; Drenzek et al., 2011).

9. Signaling pathways in differentiation process triggered by naturally occurring compounds

Taking all the above into account, it emerges that naturally occurring compounds can trigger differentiation process. Conversely to molecular targeted therapies, where the exact target is single and well defined, it is usually hard to predict complexity of intracellular response to naturally occurring compounds. Namely, the activity of different signaling pathways is simultaneously changed, and it is not clear what the direct result of the treatment is and what is the consequence. However, complexity of signals affected by the treatment with naturally occurring compounds surmounts the limits of molecular targeted therapies. Several data confirms that plant-derived compounds downregulate signals involved in stem phenotype maintenance, but can also promote maturation through modulation of signals triggered by microenvironmental factors upon their ligation to membrane receptors (Murakami and Tashiro, 2015; Jin et al., 2017). The appearance of signaling pathways involved in stem phenotype maintenance coincides with the development and establishment of multicellularity. Wnt/β catenin, Notch and Hedgehog/Gli pathways are essential for tissue development and homeostasis but their activity is also tightly related with cancer stem phenotype (Jin et al., 2017). Moreover, members of mentioned signaling network are often overexpressed in cancers and responsible for therapy resistance. For example, Wnt/β catenin is important for EMT but is also a key mediator of proliferative response of pluripotent cells to apoptotic signal in the surroundings (Ryoo and Bergmann, 2012). Hedgehog signaling possesses a pivotal role in tumor initiation, metastasis and cancer stem cells renewal (Gupta et al., 2010). Notch signals are of great importance for cross talk between cancer stem cells and cells in the neighbourhood leading to tumor progression through CST renewal and tumor vascularisation (Wang et al., 2012). JAK/STAT pathway activated through growth factors and cytokines is responsible for keeping of adult tissue homeostasis and tumorigenesis (Chen, 2012). Mutations in this signaling pathway leading to constitutive activation in ligand independent manner have been reported in hepatocellular, prostate, hematological and other cancers (Jin et al., 2017). Abnormal or constitutive activation of NF-kB promoted cancer progression, dissemination and vascularisation. This transcriptional factor is also in charge for the development of tumor promoting M2 phenotype of macrophages (Hagemann et al., 2008). Finally, important members in cancer intracellular signaling network are the PI3K/Akt

and MAP kinase pathways (Lee et al., 2006). Single compound like IXN (Krajnović et al., 2016) can trigger multiple targets within the cell like p70 S6 kinase, S6 protein, Wnt, Notch 1 and Oct3/4. On the other hand, molecular signature in response to therapeutics is defined not just by the type of tumor but also vary inside of the same group. For example, we showed that AE oppositely regulated ERK1/2 in glioma and melanoma, but also its influence on ERK1/2 activity can be extremely dissimilar even between melanoma cell lines at different maturity stage (Mijatovic et al., 2005a; Radovic et al., 2012). The essential advantage of naturally occurring compounds in comparison to synthetic small molecules created is their plasticity and potential to accommodate to cell specificities. Despite diverse intracellular responses at the level of signaling molecules, the outcome of the treatment is almost always desirable one- establishment of nonproliferative and less aggressive phenotype. Abundance of biological active compounds in nature is fascinating, so scientific discipline such as nutrigenomic will offer the short-cut for selection of substances with differentiating potential. Nutrigenomic appointed nutrition-gene connections looking at the gene expression affected by phytochemicals as well as molecular mediators between nutrients and cellular response (Braicu et al., 2017; Lundstrom, 2013). According to this, it will be possible to predict and select naturally occurring compounds able to decrease stemness in cancer tissue and promote maturation of tumors cells toward non-proliferative/less aggressive form.

10. Conclusion and future direction

Natural compounds and plant extracts, in particular, have been for centuries used as therapeutic agents, and are still object of extensive research for their manifold properties and mechanisms of action, that make them promising molecules for novel cancer treatment strategies. To this regard, it is relevant the ability of a number of molecules to induce cancer cell differentiation. Although differentiation based strategies find their place in regenerative medicine apart from cancer, the concept of their application is basically different. While in regenerative medicine, the goal is to obtain functional and stable phenotype, in cancer both aspects are irrelevant. Differentiation therapy in cancer should aim at a nonproliferative cell preventing undesirable compensatory proliferation that follows chemotherapy treatment in advanced malignancy. The properties of several molecules to induce maturation of hematopoietic cells have been deeply investigated, but increasing data are available for solid tumors. These molecules hold promise as potential alternatives to traditional anti-tumoral chemotherapies. It is likely that favourable effects of therapy-induced differentiation will be observed for cancer types that have underlying dominant genetic drivers. Therefore, the characterization of the causes underlying the development of pluripotent phenotype in advanced cancers would represent the baseline for the design of specific molecules able to trigger differentiation toward less or nonproliferative functionally-committed lineage, without the necessity of active phenotype reestablishment. The reversion of the malignant phenotype to a more benign, or at least, lower grade, may significantly influence patients prognosis and turn a potential fatal disease into a chronic, and more amenable to management, one. The success of a differentiation therapy may not necessarily consist in the elimination of all tumor cells and in their complete differentiation to a mature phenotype, but in minor changes of their pathological status, *i.e.* from high to low grade.

Moreover, integrative approaches where differentiation inducing agents from plants are applied concomitantly with subtoxic doses of conventional chemotherapy or radiation will improve the efficacy of standard protocols together with a decrease in general toxicity. Exact identification of molecular targets of differentiation based agents might trace novel strategies in healing of cancer as well as to predict combined treatments that could be promising.

It should also be pointed out that, although cancer cells may undergo differentiation, it is not clear whether terminal cell cycle arrest and stable differentiation can be achieved. The possibility of reversion and de-differentiation, along with the selection of differentiation-resistant cells poses a major problem and should be addressed by the identification of the mechanisms through which cancer cells escape differentiation.

Plant-derived molecules can be used as lead compounds and their therapeutic effects improved by chemical modifications of their molecular structures. As for many other drugs currently in clinical use, semisynthetic processes of novel drugs, from lead compounds, can produce chemical analogues with improved pharmacological activity and lower side effects. Genome-wide Association Studies (GWAS), computational chemistry and bioinformatics, coupled to high-throughput screening methodologies, may allow the identification of molecules with both cell-targeted and favourable toxicity profiles.

Finally, the properties of some molecules to promote cellular differentiation, can be exploited for cancer chemoprevention, in subjects at a high risk of developing malignancies, before cancer is clinically detectable or when precursor lesions of cancer are identified.

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