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Review

Understanding genistein in cancer: The "good" and the "bad" effects: A review

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ABSTRACT

Nowadays, diet and specific dietary supplements are seen as potential adjuvants to prevent different chronic diseases, including cancer, or to ameliorate pharmacological therapies. Soybean is one of the most important food components in Asian diet. A plethora of evidence supports the *in vitro* and *in vivo* anticancer effects of genistein, a soybean isoflavone. Major tumors affected by genistein here reviewed are breast, prostate, colon, liver, ovarian, bladder, gastric, brain cancers, neuroblastoma and chronic lymphocytic leukemia. However, it is not always clear if and when genistein is beneficial against tumors (the "good" effects), or the opposite, when the same molecule exerts adverse effects (the "bad" effects), favouring cancer cell proliferation. This review will critically evaluate this concept in the light of the different molecular mechanisms of genistein which occur when the molecule is administered at low doses (chemopreventive effects), or at high doses (pharmacological effects).

Contents

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1. Introduction

"Cancer is a pleiotropic disease." This definition, by Nancy R. Gough, is reported in a recent editorial (Gough, 2014) and encompasses very well the complexity of the term "cancer" which is not only caused by the abnormal growth of cells with the potentiality to invade different organs, but also by an impaired differentiation. A classical example is acute promyelocytic leukemia, APL, or a block in cell death programme (chronic lympocytic leukemia). A second level of complexity regards the ability of cancer cells to change over time. DNA massive parallel sequencing of tumors now enables the rapid identification of a myriad of genetic mutations that alter signaling pathways, affecting drug efficacy or resistance. This explains why drug resistance represents a common and undesirable event, occurring randomly in patients affected by the same tumor and in the presence of "molecular targeted" drugs. For these reasons, cancer still remains an incurable disease.

The World Health Organization (WHO) reports that every year there are approximately 38 million new cases of non-communicable diseases (NCD) with cancer representing the second cause of NCD with 8.2 million deaths, corresponding to 22% of all NCD in 2012 (World Health Organization, 2015a). It has been well recognized that 90–95% of cancers are caused by epigenetic factors, while the remaining are related to genetic factors (Anand et al., 2008; Esteller, 2008; Taby & Issa, 2010). Among the epigenetic factors, the WHO reports that one third of cancer deaths are caused by the five leading behavioral and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco and alcohol use (World Health Organization, 2015a). Moreover, infections, radiation, and environmental pollutants are known as other common causes of cancers (Ames, Gold, & Willett, 1995; Boffetta, 2006). Currently, there is a strong urgency to find new therapeutic strategies for the treatment of cancers, especially for those that show drug-resistance, high risk of relapse, unavailability and/or poor therapeutic strategies. For this reason, much attention is paid to the therapeutic use of natural products, due to their high efficacy and low adverse effects (Cragg, Kingston, & Newman, 2011; Cragg & Newman, 2013; Demain & Vaishnav, 2011; Mehta, Murillo, Naithani, & Peng, 2010; Newman & Cragg, 2012).

Since ancient times, medicinal plants have been used for the treatment of different diseases due to their content of bioactive compounds (Balunas & Kinghorn, 2005; Nabavi, Daglia, Moghaddam, Habtemariam, & Nabavi, 2014; Nabavi, Nabavi, Mirzaei, & Moghaddam, 2012; Nabavi et al., 2013). It has also been reported that over than 60% of common anticancer drugs originate in nature (Cragg & Newman, 2005). In addition, the National Cancer Institute (NCI) in the USA examined the anticancer effects of different plant extracts, as well as other natural products (Snader & McCloud, 1994). Among them, flavonoids, widely found in different parts of plants, are known as the most important group of natural anticancer compounds (Bilotto et al., 2013; Clere, Faure, Carmen Martinez, & Andriantsitohaina, 2011; Genoux, Nicolle, & Boumendjel, 2011). The main chemical signature of flavonoids is the 15-carbon skeleton which contains two phenyl rings as well as one heterocyclic ring (Nabavi, Nabavi, Eslami, & Moghaddam, 2012; Nabavi, Nabavi, Mirzaei, et al., 2012).

Genistein, daidzein and glycitein (Fig. 1) are the most common and well known isoflavones in nature (Song, Barua, Buseman, & Murphy, 1998; Wang & Murphy, 1994). They contain a 3-phenylchromen-4-one skeleton without hydroxyl group substitution on position 2 (Coward, Barnes, Setchell, & Barnes, 1993). Genistein, present in soy foods at concentrations ranging from 1.9 to 229 mg/g, is reported to be the major anticancer constituent of soybean (Fukutake et al., 1996; Spagnuolo et al., 2015).

Although the literature of the past decade reports several excellent reviews on the biological activities of genistein, many of them are focussed on pathological conditions different than cancer and, even in the latter case, generally, the effects of genistein on a specific type of cancer have been reviewed. Therefore, the aim of the present work is to critically analyze the available data on the molecular targets of genistein in twelve different types of cancers, trying to identify common mechanisms of action of the molecule and its efficacy in enhancing chemotherapeutic protocols. In addition, depending on the data present in the literature on specific forms of cancers, e.g., breast cancer, we will try to highlight, not only the desired ("good") anticancer and chemopreventive effects of genistein, but also the unexpected and potentially dangerous consequences of its uses for treatment (Table 1).

2. Genistein

Many reports claim that consumption of soybean, because of the presence of genistein, reduces the risk of development of several types of cancer, including breast, prostate and colon cancer (Fournier, Erdman, & Gordon, 1998).

Search for the terms "genistein and cancer" in PubMed, reveals that the main molecular targets of genistein are estrogen receptors (ER), protein tyrosine kinases (PTK) and mammalian DNA topoisomerase II (Akiyama et al., 1987; Kuiper et al., 1998). Early reports have identified genistein as a potent inhibitor of PTK activity associated with epidermal growth factor receptor (EGFR), the designed target of Erlotinib, one of the first personalized drugs. A large part of these studies has focussed on the use of *in vitro* models and applied micromolar concentrations of genistein, revealing the "good" anticancer effects of the molecule. However, we are not only "what we eat", but essentially "what we absorb"; in other words, given the low plasmatic bioavailability of genistein (similar to other bioactive compounds present in the diet), it is necessary to distinguish between the potential chemopreventive effects of genistein (administered at low doses) and its pharmacological effect when administered at high doses. Essentially, the "bad" aspects of genistein, may derive from its *in vivo* effects which are strictly related to its circulating concentration.

We recently reviewed that the ability of genistein to inhibit cell growth (in both hormone-dependent and -independent cancer cells) is dose-dependent (Russo, Spagnuolo, Tedesco, & Russo, 2010; Spagnuolo et al., 2015). In fact, it has been reported that preferential activation of ERβ by genistein is lost when genistein is increased from low (6 nM) to higher concentrations. At hundreds nanomolar concentrations, genistein activated both ERs (α and β); therefore, the final effect on gene expression and cell fate depends on ligand dose and on the differential ability of ligand–ER complexes to recruit modulators at the ER binding sites of hormone-regulated genes (Chang et al., 2008). Reasonably, the antiproliferative activity of genistein at pharmacological doses (higher than 10 μM) is mediated by PTK inhibition, suggesting that genistein might exert *in vivo* anticancer effects.

In Fig. 2, several possible molecular targets of genistein are represented. The cartoon illustrates a key feature shared by several bioactive molecules, i.e., their "pleiotropic" activity, or the capacity

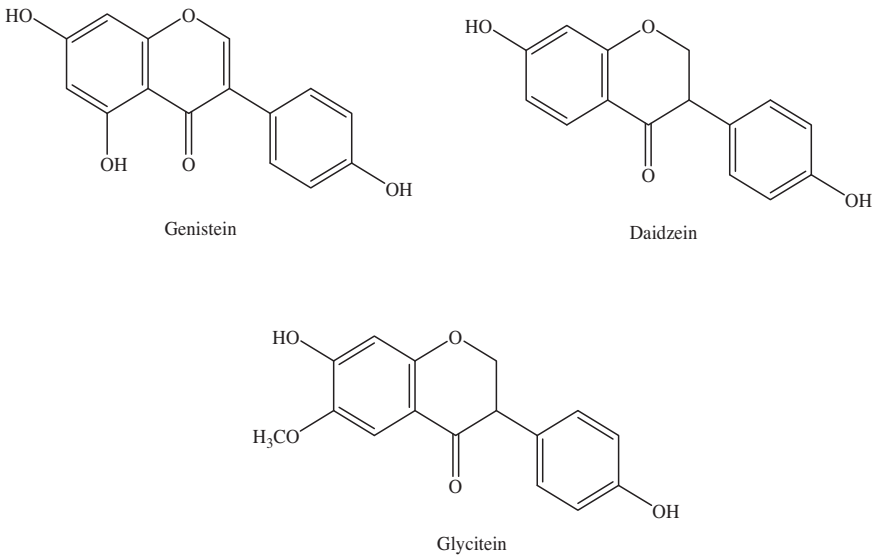

Fig. 1. Chemical structures of genistein, daidzein and glycitein.

to recognize and modulate independent cellular targets. Therefore, if cancer is a pleiotropic disease and genistein a pleiotropic compound, a molecular convergence may exist and will be explored in the current review.

3. Molecular targets of genistein in breast cancer

3.1. General

Breast cancer (BC) is the most common cancer type occurring in women worldwide. The WHO reported that BC is diagnosed in 1.4 million women with 521,000 deaths in 2012 (World Health Organization, 2015b). However, since 2008 (Siegel, Naishadham, & Jemal, 2013), a global increase in breast cancer incidence (>20%) and deaths (<500,000) has been observed. BC is characterized by considerable genetic heterogeneity, which corresponds to variable prognosis and treatments, depending on the mutational landscape that can affect essential cellular pathways regulating cellular proliferation (ERα-β) and/or the signaling pathway of the receptor tyrosine kinase family (HER-Heu2, EGFR/PI_3K) or DNA repair (BRCA1-2, Chk-2, p53, ATM) (Davis et al., 2014). In addition to genetic mutations, other important risk factors for the onset of BC are "epigenetic variables", including lifestyle and nutrition. Since genistein modulates the estrogen-regulated gene expression and exerts a range of biological effects (Prietsch et al., 2014), it has become one of the most studied natural compounds to be tested in view of potential clinical applications against BC.

Historically, BC has been divided into three large subtypes: Triple Negative BC (TNBC, which do not express the estrogen, progesterone and HER2 receptors); HER2 and ER expressing subtypes. We will review genistein action in this triple scenario.

3.2. Genistein as a modulator of ER in BC: in vitro and in vivo studies

The molecular effect of genistein on ER in BC-derived cell lines has been earlier described (Kuiper et al., 1998). Estrogens play an important role in the development and progression of BC, acting through two types of ERs: ERα and ERβ, encoded by different genes and with different tissue distributions and ligand specificities. They show 55% identity in their estrogen-binding domains and approximately 97% similarity in the DNA-binding domains (DBDs). Given their homology, both receptors interact with the same conserved estrogen response element (ERE) on DNA, as either homodimers or α/β heterodimers. However, ERα homodimer is more efficient than are ERβ or ERα/β heterodimers in promoting transcription of genes controlling growth and differentiation in uterus and breast (Thomas & Gustafsson, 2011). On the other hand, ERβ, when present together with ERα, counteracts its proliferative effects on breast cancer cells (Paruthiyil et al., 2004). However ERβ is frequently lost in BC, where its presence generally correlates with a better prognosis of the disease. ERα expression, on the contrary, promotes the development of resistance against anticancer drugs. About ~70% of BC cells express ERα; therefore, human cell lines derived from BC, like MCF-7, express mostly ERα. In a recent study, the interactions between different botanical-estrogens, besides genistein (daidzein, equol, and liquiritigenin), and ER or other key transcriptional co-regulators (steroid receptor coactivator, SRC3 and receptor interacting protein 140, RIP140) have been examined in different MCF-7 cell lines expressing ERα, ERβ, or both (Jiang et al., 2013) The authors compared the affinity of selected isoflavones versus ERα and/or ERβ and their co-regulators with their physiological counterpart, e.g., E2. In addition, the DNA binding sites of ERα, ERβ or both were identified by CHIP assay, together with the gene selectively expressed and their downstream effects on cell proliferation in these different genetic backgrounds. These cellular models mimic the different ratios between ERα and ERβ in human BC. The authors confirmed that, compared with other phytoestrogens, genistein possesses an affinity towards ERβ ligand (*K*d of 7.4 nM) much greater than that of daidzein (Fig. 1), probably due to the presence in the former of a phenolic hydroxyl group essential for the formation of an intra-molecular hydrogen bond, which stabilizes the binding within the ER pocket. Interestingly, the authors also observed that daidzein is metabolized by the gut microflora to S-equol and this conversion increases its affinity for both ERs by 50- to 70-fold, which is comparable to the affinity of genistein for ERβ. A novelty in the study is the quantification of the interaction between the co-activators and co-repressors with ERs in the presence of E2 compared to natural estrogens. As an example, SCR3, a co-activator, is found at a high level in BC and binds the ERα-E2 complex with an EC_{50} of 0.13 nM. Genistein has the highest affinity in the complex ERα–SCR3, with respect to other isoflavones. By contrast, the affinity of all tested isoflavones towards the ERβ-SCR3 complex was comparable to that of ERβ–E2. The authors concluded that transcriptional potency of genistein or other phyto-estrogens,

Table 1
The "good" and the "bad" effects of genistein associated with its main molecular targets in the different types of cancer reviewed.

Type of cancer	"Good" effects	Molecular targets	"Bad" effects	Molecular targets
Breast cancer (*in vitro*)	Decreases proliferation (low doses) Decreases proliferation (high doses)	Cells expressing ERβ Inhibits HER2 expression EGFR, PDGFR, IR, Abl, Fgr, Fyn and Src Inhibits NF-κB signaling	Increases proliferation (high doses)	Cells expressing ERα
Breast cancer (*in vivo*)			Increases markers of proliferation	Over expression of FGFR2, cell cycle genes (E2F5, cyclin B, CDK1)
Leukemia (*ex vivo*)	Induces apoptosis	Cells expressing ZAP-70 Co-treatment with miR-16		
Prostate (*in vitro*)	Induces apoptosis	Inhibits nuclear translocation of NF-κB and reduces NF-κB DNA binding Activates caspase-3		
Colon (*in vitro*)	Inhibits cell growth Promotes apoptosis Induces p53-dependent cell cycle arrest	Inhibits PI_3K/Akt pathway Stimulates FOXO3		
Liver (*in vitro*)	Decreases metastasis formation Induces cell cycle arrest	Inhibits TGF-β-induced EMT Inhibits NFAT1 Inhibits FAK expression		
Lung (*in vitro*)	Enhances growth inhibition Induces apoptosis	Enhance the activity of EGFR inhibitors Down-regulates NF-κB expression and prevents NF-κB-DNA binding		
Ovarian (*in vitro*)	Inhibits cell growth Induces cell cycle arrest	Inhibits the expression of VEGF and VEGF receptors Down-regulates the expression of miR-27a	At low concentrations (5–7.5 μM) abolishes cytotoxic or genotoxic effect	ROS scavenging
Bladder (*in vitro* and *in vivo*)	Inhibits cell growth Induces G2/M cell cycle arrest Induces apoptosis Inhibits angiogenesis	Down-regulates NF-κB		
Neuroblastoma (*in vitro*)	Induces mitochondria-mediated apoptosis Inhibits cell growth	Down-regulates Bcl-2 (mRNA and protein) Up-regulates the expression of death factors (TNFR-1, Fas, TNF-α, FasL, TRADD, FADD) Activatescaspase-8		
Brain (*in vitro*)	Induces cell cycle arrest	Increase expression of p21 Decreased expression of cyclin B1 and CDK1 Decreases the expression of TERT and TR		
Gastric (*in vitro*)	Enhances chemosensitivity	Down-regulates the activity transporter proteins involved in multi-drug resistance (ABCC1, ABCCD5, ABCG2, ERK 1/2)		

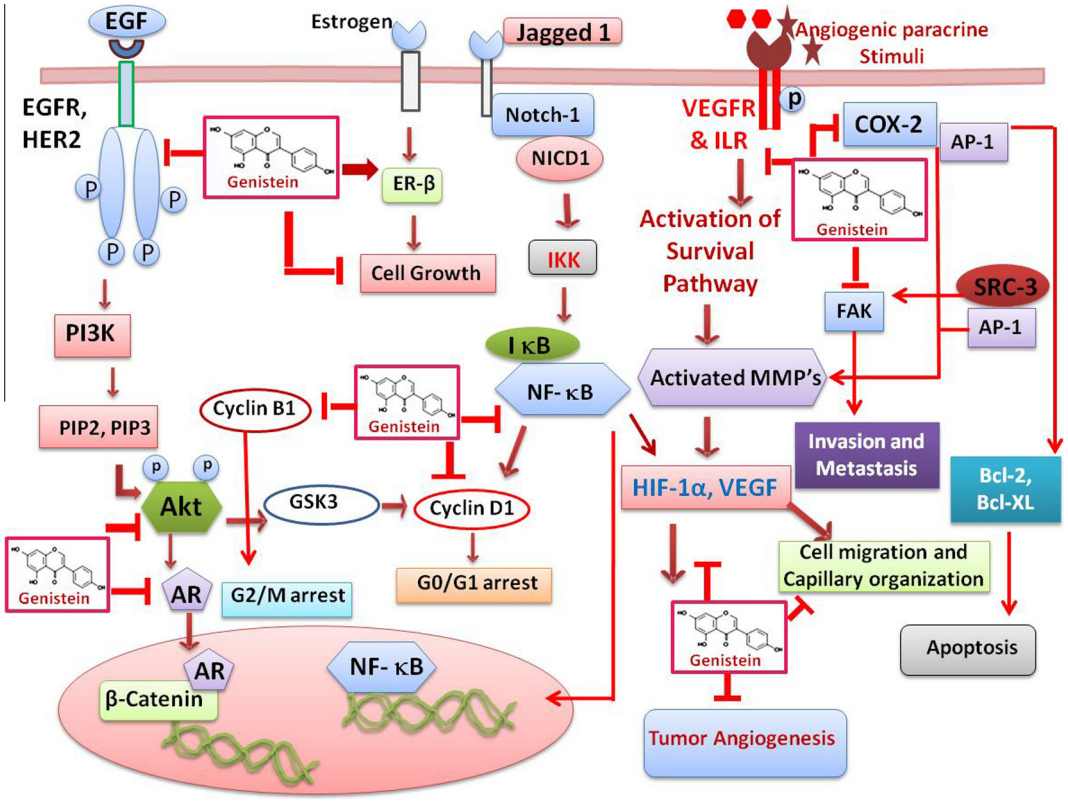

Fig. 2. Pleiotropic effect of genistein (see description in the text).

compared to E2, depends upon their ligand binding and co-activator affinities (Jiang et al., 2013). Comparing the expression of genes transcribed by E2, such as the progesterone receptor (PrR) in the different MCF-7 cell lines, the authors found that low concentrations of genistein (EC_{50} 4 nM) stimulated gene expression and reduced proliferation more effectively when ERβ was present. However, when ERα was the predominant form, all isoflavones elicited gene stimulation at high concentrations (24–300 nM) compared with E2.

Confirming the dose–response effects in the gene expression experiments, genistein stimulated proliferation in MCF-7/ERα cells only at concentrations of hundreds, nanomolar. Co-expression of the two ER isoforms α/β reduced the proliferative response of cells to genistein and other phyto-estrogens at all concentrations tested while, in MCF-7/ERβ, coherently, the proliferation rate was lower than in MCF-7/ERα. The overall conclusions indicate that the potential beneficial effects of genistein and other phyto-estrogens depend upon dose applied, tissues analyzed and relative levels of ER isoforms α/β. Therefore, when ERβ is present, estrogens and phytoestrogens could have generally beneficial ("good") effects, diminishing cell proliferation; by contrast, relatively high concentrations of the same compounds may have a proliferative effect ("bad") on BC cells expressing ERα, the isoform associated with worse prognoses. Considering that a meal rich in soy food increases blood levels of total isoflavones (aglycones and/or conjugates) in women to about 1–2 μM (Soukup, Al-Maharik, Botting, & Kulling, 2014) and, under these conditions, genistein aglycone alone is around 40 nM, we can hypothesize that the molecule may have a measurable ERβ selectivity *in vivo*.

The lesson learned from the previous *in vitro* study (Katzenellenbogen, Kendra, Norman, & Berthois, 1987) indicates that ER-positive BC cells possess higher hypersensitivity to exogenous estrogens, such as genistein and other soy isoflavones. In other words, BC cells from young or early post-menopausal (<5 years) women, use isoflavone as a "substitute fuel" to grow and survive in estrogen-austere conditions (Jordan, 2014). In a different cellular background, when BC cells grow independently of estrogens, as in older post-menopausal woman (>5 years), high levels of phyto-estrogens could induce massive apoptotic cell death (Obiorah, Fan, & Jordan, 2014).

This represents a key issue to be considered when clinical studies are designed and interpreted. In fact, in support of the mentioned *in vitro* study, a recently published clinical trial analyzed the effects of soy supplementation on gene expression in BC (Shike et al., 2014). In this randomized placebo-controlled study, 140 women with early stage BC (mainly HER2 negative and ER positive) were randomly divided into two groups and supplemented with soy protein (25.8 g) versus placebo for 30 days. The study evaluated plasma levels of genistein and BC biopsies before and after supplementation, changes in gene expression, proliferating and apoptotic markers (Ki67 and caspase-3, respectively). Surprisingly, the conclusion from this trial was that women with soy supplementation and high plasma genistein showed, after only 4 weeks, an over-expression of tyrosine kinase receptor FGFR2 and other genes that drive cell cycle and proliferation pathways (E2F5, cyclin B, CDK1). This negative scenario may result in "bad" effects of genistein in BC, although no changes in proliferation (Ki67) and apoptosis markers (caspase-3) between two groups were observed. Perhaps, the study period was too short. Another limit is represented by the observation that the majority of women enrolled in the trial were only early post-menopausal.

In summary, the data from recent *in vitro* and *in vivo* studies converge in one key concept: estrogen and phytoestrogen are "bad" when supplemented in "5 year gap" menopausal women but, if the plasmatic concentrations of these compounds reach

hundred nanomolar a decade following menopause onset (>5 years), then their chemopreventive effect could switch to "good" (Jordan, 2014).

3.3. Genistein as a selective protein tyrosine kinase inhibitor: in vitro studies

Another potential possible mechanism of action of genistein in BC cells is represented by its ability to inhibit PTK. This class of kinases has been considered among the main targets of new specific anti-cancer drugs. Two well-known examples are Gleevec (Imatinib) and Herceptin, which target BCR-ABL in leukemia and HER2/ErbB2 in BC, respectively (Davis et al., 2014; Ferrarelli, 2013). The first paper demonstrating that genistein inhibits PTK was published almost 30 years ago (Akiyama et al., 1987) (Fig. 2). In this work, genistein inhibited different PTKs (e.g., EGFR, IC_{50} 0.7 μg/ml). However, since genistein is not structurally similar to ATP, the authors concluded that inhibition of EGFR kinase might not be due to a competition for exactly the same ATP binding site. They formulated the hypothesis that genistein, like quercetin and other flavonoids, "binds in multiple places in the reaction pathway". In the same paper, genistein did not efficiently inhibit serine and threonine protein kinases, such cAMP-dependent protein kinase A (PKA) and Ca^{2+}/phospholipids-dependent enzyme protein kinase C (PKC).

This initial study was confirmed by several others where micromolar concentrations of genistein were tested. It is not easy to identify the "primary" target of genistein in the ocean of the cellular kinome. Genistein specificity towards PTK was recently demonstrated by an "omics" approach (Yan et al., 2010); in this study, the authors identified the alterations of tyrosine phosphorylation after genistein treatment, by a phospho-proteomic array, using 40 μM concentration for 48 h. The elevated concentration applied and the length of the treatment represented a limit of this and other studies. Briefly, they identified 183 phospho-proteins regulated by genistein. Among these, 8 cell surface receptors, 5 protein phosphatases and 7 transcriptional regulators were not previously associated with the anticancer effects of genistein. The integration of these data with functional analysis led the authors to suggest that genistein regulated cancer cell growth mainly by inhibiting the activity of core signaling PTK: EGFR, PDGFR, insulin receptor, Abl, Fgr, Fyn and Src. However, as previously discussed, the key issue is the identification, among these PTKs, of the "first hit" targeted by genistein (Fig. 2).

HER2 (human epidermal growth factor receptor 2)-positive BC is a type of more aggressive and fast growing BC. This oncogene encodes trans-membrane receptor tyrosine kinase. Overexpression of HER2 was observed in BC and in many other cancer types. HER2-positive BC patients develop resistance against chemotherapeutic drugs and are also less responsive to hormonal treatment (Singh, Jhaveri, & Esteva, 2014). In a study performed using a low micromolar (about 1 μM) concentration of genistein on BT-474 human BC cells (expressing only ERβ), the molecule inhibited HER2 expression, phosphorylation and promoter activity throughout an ER-independent mechanism. When these cells were genetically transformed to express ERα, genistein mimicked E2 and inhibited HER2 protein phosphorylation (Sakla, Shenouda, Ansell, Macdonald, & Lubahn, 2007). In a different study, the authors documented an anti-proliferative and apoptotic effect of genistein and quercetin, using a canonical MCF-7 cell line and MCF-7 overexpressing HER2. Differently from the previous study, in this system, both genistein and quercetin did not inhibit the expression of HER2, nor its tyrosine phosphorylation activity (Seo et al., 2011). However, the authors showed an involvement of the extrinsic apoptotic pathway in flavonoid-induced cell death. In particular, genistein and quercetin induced CD95/Fas/Apo1 receptor-dependent cell death in MCF-7/HER2 cells. In addition, a reduced level of phosphorylation of IκBα, that finally inhibited the nuclear translocation and phosphorylation of p65 within the nucleus, was observed (Seo et al., 2011). Perhaps, the explanation of this event resides in the interference of genistein with upper kinases (IKKα/β for example), as reported for its methoxy form, biochanin (Manna, 2012). We believe that inhibiting NF-κB signaling pathway in MCF-7/HER2 cells may represent a further indirect molecular target of genistein in this model system. We are also critical of the large concentration of genistein applied (100 μM) and the prolonged time of treatment (72 h) necessary to detect the apoptotic effects (Fig. 2).

3.4. Genistein action in TNBC: in vitro studies

As previously reported, triple negative breast cancer TNBC is an aggressive subtype of BC, characterized by negative expression of ERα, progesterone receptor (PR) and HER2. It is diagnosed in 15% of all BC, more frequently in younger and pre-menopausal women (Dent et al., 2007). Differently from the other subtypes, TNBC cannot be approached with novel therapeutic strategies, such as hormones. Resistance to chemotherapy is often present, calling for the identification of new therapeutic targets. A study investigating the effects of genistein on TNBC cells revealed that genistein had a dramatic effect on cell growth inhibition in a dose- and time-dependent manner (Pan et al., 2012). They used MDA-MB-231 cell line treated for 72 h with different concentrations of genistein (5–10–20 μM) and measured the induction of apoptosis and a G2/M cell cycle arrest. This effect was due to genistein inhibition of NF-κB, via the NOTCH-1 signaling that finally affected the expression of Bcl-2 and Bcl-xL as a consequence of NF-κB inhibition.

4. Molecular targets of genistein in leukemia

4.1. General

Leukemia is a cancer of early blood-forming cells. In most cases, leukemia is a cancer of the white blood cells, but some types of leukemia start in other blood cell types. There are different types of leukemia, such as acute leukemia (e.g., acute myeloid leukemia, acute lymphocytic leukemia in adults) and chronic leukemia (e.g., chronic lymphocytic leukemia, chronic myeloid leukemia and chronic myelomonocytic leukemia). The American Cancer Society states that, in the United States for 2015, about 54,270 new cases of leukemia will be diagnosed, with about 24,450 deaths from all kinds of leukemia (The American Cancer Society, 2015a).

4.2. Chronic lymphocytic leukemia: from in vitro to in vivo studies

In the United States, about 14,620 new cases of chronic lymphocytic leukemia (CLL) for 2015 are expected. CLL is the most frequent form of leukemia among adults in the western world, with an incidence of 3.5 and 6.15 per 100,000 per year in the United States and UK, respectively (The American Cancer Society, 2015a). Despite the improved efficacy of CLL treatments (Rituximab, an anti-CD20 monclonal antibody, or Alemtuzumab, an anti-CD52 monoclonal antibody, and their combination with fludarabine and/or cyclophsphammide), resistance to treatments and relapse are not rare events. In this context, there is a need to improve CLL therapy, mainly in old non responsive or resistance-developing patients. In previous works, the natural flavonoid quercetin, at not toxic concentrations, proved able to sensitize leukemia cell lines or B-cells isolated in CLL patients to fludarabine or experimental drugs, such as death ligands (TRAIL and CD95L) or BH3 mimetics (ABT-737), enhancing cell death response in a synergistic manner with respect to single treatments (Russo, Russo, & Spagnuolo, 2014; Russo et al., 2013). Following the

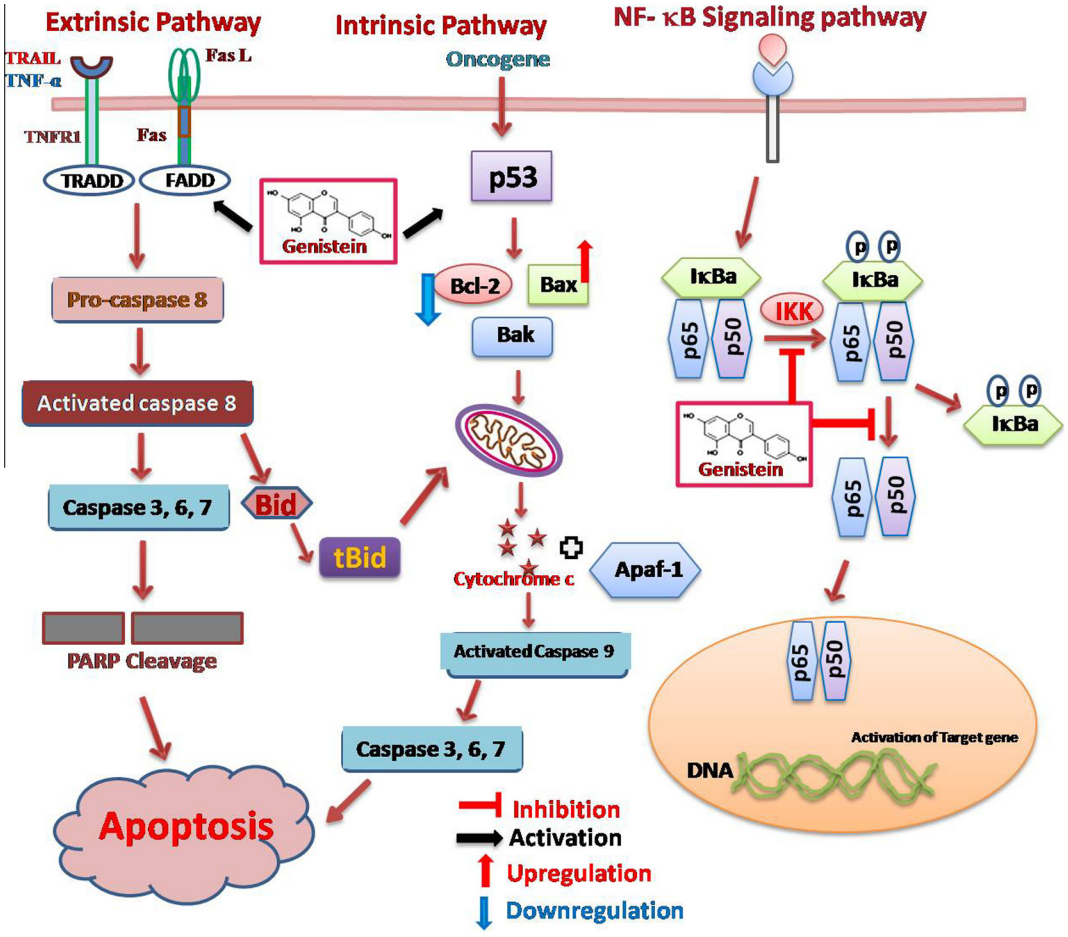

Fig. 3. Molecular targets of genistein in apoptotic and NF-κB signaling pathways (see description in the text).

same rationale, we have reviewed articles where genistein was applied in mono-treatment, or in combination with other drugs, against CLL or other forms of leukemia.

The immunoconjugate, genistein-CD19 (called "B43-Genistein"), was able to kill leukemic cells in a SCID mouse xenograft model of human B-lineage acute lymphoblastic leukemia (B-ALL) with a survival of 100%. Moreover, B43-Genistein was more effective than were classical chemotherapeutic drugs and conferred a long tumor-free survival with respect to control animals (Uckun et al., 1995). Starting from these results, the same authors moved towards a clinical trial involving 15 selected patients with B-ALL and one B-CLL patient. All subjects were resistant to previous chemotherapeutic treatments, or relapsed after bone marrow transplantation. They were treated (i.v.) with B43-genistein (0.1–0.32 mg/kg/day of immune-conjugate) and several parameters, such as safety, immunogenicity and therapeutic response, were evaluated. They concluded that B43-genistein was well tolerated, with no severe adverse effects and with a good pharmacokinetic profile. However, the therapeutic effects were less encouraging (Uckun et al., 1999).

In a different study, the expression of PTK ZAP-70 in B-CLL was related to the *ex vivo* response, using a combination of genistein (15–60 μM) plus fludarabine (3 μM) (Mansour, Chang, Srinivas, Harrison, & Raveche, 2007). ZAP-70 is expressed in a subset of CLL patients with worse prognosis when associated with the unmutated form of $IgGV_H$ gene (Trojani et al., 2010). Leukemic cells, isolated from patients belonging to the high ZAP-70 group, responded to genistein mono-treatment (>30% versus untreated cells) and genistein plus fludarabine combination (Mansour et al., 2007). This observation suggests that the un-mutated form of CLL is "addicted" to ZAP-70 to survive and resist cell death and genistein counteracts this process, making B-CLL more sensitive to treatment. This effect is confirmed by its low toxicity in normal peripheral lymphocytes where ZAP-70 is expressed at low levels (Mansour et al., 2007).

B-CLL represented the first example of the involvement of microRNA (miRNA) in cancer development. In fact, a deletion found in DLEU2 gene determined the loss of 15a and 16 miRNAs, resulting in tumor suppression activity in CLL (Liu et al., 1997). Using New Zeland Black (NZB) mice, characterized by a point mutation six bases downstream from pre miRNA 16-1 on mouse chromosome 14, resulting in a decreased expression of 15a and 16 miRNA, the addition of exogenous miR-15a and miR-16 led to a significantly greater accumulation of cells in G1 phase and a reduction in D1 cyclin, a direct molecular target of miR-15a and miR-16 regulation, in New Zealand Black-derived malignant B-1 cell lines (Salerno et al., 2009). These cells also showed an impressive apoptotic induction by relatively low genistein concentration (10 μM) or nutilin 1 (a p53-stabilizing drug). In summary, this study demonstrated that genistein and miR-16 synergize in terms of apoptosis response and may be potential therapeutic targets in human CLL (Salerno et al., 2009).

5. Molecular targets of genistein in prostate cancer: *in vitro* studies

The WHO reports that prostate cancer (PCa) is the second most common type of cancer diagnosed in 2012, after lung cancer

(World Health Organization, 2015b) and the most commonly diagnosed cancer type among men in 2012 (Chiyomaru et al., 2013). It is placed next to lung cancer since PCa causes severe cancer-related death in the male population. Death rate increases when PCa reaches the incurable metastatic stage. Hormonal deprivation therapy showed an excellent initial response, but PCa relapse was noted and death occurs within several years. Androgen-independent PCas are poorly responsive and there is no successful treatment available for this type of PCa (Chiyomaru et al., 2013). Davis, Kucuk, and Sarkar (1999) reported the mechanism of action of genistein using androgen-sensitive (LNCaP cells) and insensitive (PC3 cells) PCa model cell lines. The study confirmed that genistein blocks the nuclear translocation of NF-κB and reduces NF-κB DNA binding, which leads to the activation of the apoptotic pathway (Davis et al., 1999) (Fig. 3). The PCa model cells pre-treated with 50 μM genistein for 48 h inhibited NF-κB activation even after the addition of inducers (H_2O_2 and TNF-α). It has been observed that genistein prevents NF-κB-DNA binding by blocking the nuclear translocation of p50 and p65 subunits. Moreover, PCa model cells, pre-treated with genistein, reduced the amount of phosphorylated IκBα, which leads to the formation of unphosphorylated IκBα-NF-κB complex, thus facilitating the docking of NF-κB in the cytoplasm and preventing the nuclear translocation of NF-κB. Inhibition of NF-κB leads to the activation of pro-apoptotic response. Furthermore, genistein has been shown to activate caspase-3 and to induce apoptosis in LNCaP and PC3 prostate cancer cell lines (Fig. 3).

Li and Sarkar (2002) reported the gene expression profile of the PC3 cell line after 6, 36 and 72 h of genistein treatment at 50 μM. Genistein was observed to alter the expression of nearly 832 genes, in which 774 genes were down-regulated and 58 genes were up-regulated. All these genes are associated with the regulation of cell cycle, cell growth, cell signaling transduction, apoptosis, tumor cell invasion, metastasis and angiogenesis. Genistein down-regulated the expression of MMP-9, PAR-2, protease M, urokinase uPA, uPAR, VEGF, VEGFR, TGF-β, BPGF, LPA, and TSP, and up-regulated the expression of connective tissue growth factor and connective tissue activation peptide, which are fundamental genes involved in angiogenesis, tumor cell invasion and metastasis.

A recent report shows that genistein activates caspase-dependent apoptotic pathway in PC3 cells (Aditya, Shim, Yang, Lee, & Ko, 2014). It increases the expression of BRCA gene in androgen-sensitive (LNCaP) and insensitive (DU-145) prostate cancer cells in a dose- and time-dependent manner (Fan, Meng, Auborn, Carter, & Rosen, 2006). Another report on PCa cells revealed that genistein directly inhibits Akt and NF-κB pathways, which leads to activation of apoptosis (da Silva et al., 2013). These reports positively reveal the pleiotropic effect of genistein on prostate cancer cells.

6. Molecular targets of genistein in colon cancer: *in vitro* studies

The WHO reports that, in 2012, there were approximately 694,000 deaths from colorectum cancer, which represents the third and the second most common type of cancer diagnosed in 2012 among men and women, respectively (World Health Organization, 2015b). Epigenetic studies revealed that increased cell proliferation and loss of normal cell cycle regulation are responsible for colon cancer growth and progression (Baylin & Ohm, 2006).

Moreover, recent studies report that complicated clusters of regulatory factors, such as ERKs, tumor suppressor gene p53 and cell cycle regulators, play a vital role in the progression of colon cancer (Raskov, Pommergaard, Burcharth, & Rosenberg, 2014).

Zhang et al. elucidated the anti-carcinogenic mechanism of genistein on HCT-116 and SW-480 human colon cancer cells (Zhang et al., 2013). These findings revealed that genistein (25–100 μM for 48 h) showed growth inhibitory activity and promoted apoptosis in a dose-dependent manner. Genistein causes p53-dependent G2/M phase cell cycle arrest in colon cancer cells. Moreover, genistein activates ATM/p53, p21 and GADD45α and down-regulates the expression of CDK1 and cdc25A, which are mainly involved in the regulation of cell cycle and apoptosis.

Genistein treatment in human colonic cancer HT-29 cells has revealed that genistein promoted FOXO3 activity and inhibited EGF-induced proliferation in HT-29 cells. Moreover, genistein targeted (upstream) the PI_3K/Akt pathway and stimulated downstream FOXO3 interaction with tumor suppressor p53mutant. The increased FOXO3 activity facilitates the expression of p27, which leads to cell cycle arrest (Qi, Weber, Wasland, & Savkovic, 2011).

7. Molecular targets of genistein in liver cancer: *in vitro* studies

The WHO reports that, in 2012, there were approximately 745,000 deaths from liver cancer. (World Health Organization, 2015b). Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer death in men and eighth in women worldwide. HCC is chemoresistant to several currently available chemotherapeutic drugs (Gu, Zhu, Dai, Zhong, & Sun, 2009). Surgical removal of the tumor is the most efficient method currently followed to treat HCC patients. The high incidence of distant metastasis is reported to be the major cause of HCC patients' mortality. Hence, development of potent anti-metastatic compounds is the hallmark of HCC chemotherapy, which can increase the survivability of HCC patients. The mechanism of action of genistein on HCC cells has been recently reviewed (Dai et al., 2015). This report showed that genistein exhibits antitumor activity by modulating cellular motility and migration. HCC cells treated with genistein significantly enhanced the epithelial markers, E-cadherin and α-catenin, but decreased the mesenchymal markers, N-cadherin and vimentin, at both mRNA and protein levels *in vitro* and *in vivo*. This study positively reveals that genistein inhibits the process of TGF-β-induced epithelial–mesenchymal transition (EMT), which is thought to be associated with tumor metastasis. Genistein reversed the EMT phenotype in HepG2, Bel-7402 and SMMC-7721 cells. Genistein has been shown to target the nuclear factor of activated T-cells (NFATs), which is very important for various cellular functions. In the NFAT signaling pathway, the activated NFAT translocates to the nucleus and exerts its function at target transcription sites. As a transcription factor, NFATs are important for various cellular processes and play a major role in malignancy and tumor progression.

Furthermore, NFATs are reported to be involved in the activation of EMT processes. In HCC cells, genistein inhibited NFAT1 and repressed the development of EMT. This report positively elucidates the anti-metastatic efficacy of genistein, which could be used for liver cancer therapy (Dai et al., 2015).

Gu et al. demonstrated the anti-metastatic activity of genistein, using a HCC model cell line, MHCC97-H (Gu et al., 2009). These cells, when treated with genistein (at 10 and 20 μM), showed induced cell cycle arrest at G2/M phase. Moreover, decreased S-phase cells were observed, when the incubation times were increased to 48 and 72 h. Although genistein induces apoptosis in MHCC97-H cells, genistein has been shown to target cell adhesion molecules such as integrin and thereby reduces the adhesion property of MHCC97-H cells. Moreover, genistein targets focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase which plays a major role in integrin-mediated signal transduction pathways. FAK is closely associated with cell growth, cell adhesion and motility. Upregulation of FAK is linked with oncogenesis and low expression of FAK is mainly associated with decreased cell migration, loss of cell

attachment and induction of apoptosis. FAK over expression has been reported in HCC cells which facilitate the invasion and metastasis of HCC. The HCC cells treated with genistein blocked the FAK signaling process by significantly down-regulating the expression and phosphorylation of FAK. This report positively reveals that genistein inhibits the metastatic potential of HCC by inhibiting FAK over expression. Moreover, it inhibits HCC progression by cell cycle arrest and apoptosis.

8. Molecular targets of genistein in lung cancer: *in vitro* studies

The WHO reports that, in 2012, there were approximately 1.59 million deaths from lung cancer, which, among men and women, represents the first and the third most common type of cancer diagnosed in 2012, respectively (World Health Organization, 2015b). Lung cancer is mediated mainly by carcinogens from tobacco smoke and other smoke effluents. Carcinogens activate common cell survival signaling pathways and inflammatory cytokines, which play a major role in cancer development (Chen, Li, Bai, & Lin, 2011). Inhibitors of EGFR, e.g., Erlotinib and Gefitinib, show a significant clinical benefit to the non-small cell lung cancer (NSCLC) patients. The antitumor activity of these drugs is correlated with the down-regulation of Akt, which is one of the primary anti-apoptotic pathways activated by EGFR. The Akt pathway activates NF-κB through EGFR-independent mechanisms which lead to transcription of several genes, such as survivin, cyclooxygenase 2, Bcl-xl, and Bcl-2. These genes play a major role in various stages (cellular growth, invasion, angiogenesis and apoptosis). Genistein has been hypothesized to enhance the activity of EGFR inhibitors in NSCLCs through inhibition of NF-κB. When combined with the EGFR inhibitors, genistein enhanced growth inhibition and induced apoptosis in several NSCLC cell lines: H3255 with EGFR mutation L858R; H1650 with EGFR deletion E746-A750; H1781 carrying wild-type EGFR (Gadgeel, Ali, Philip, Wozniak, & Sarkar, 2009). Combination of genistein and EGFR inhibitors significantly down-regulated NF-κB expression and prevented the NF-κB-DNA binding. Moreover, genistein and EGFR inhibitors greatly reduced the expression of pAkt, EGFR, PGE2 and COX-2, which is consistent with the inactivation of NF-κB. These studies suggest that the enhanced antitumor activity of genistein and EGFR inhibitors combination in NSCLC cell lines may be due to a synergistic effect on NF-κB inhibition (Fig. 2).

9. Molecular targets of genistein in ovarian cancer: *in vitro* studies

Ovarian cancer is one of the gynaecologic malignancies related to hormonal and reproductive events. The frequency of ovarian cancer incidents is much less in Asian countries and this is correlated with the high dietary intake of soy isoflavones.

Genistein inhibits cellular proliferation in the ovarian cancer cell SK-OV-3, in which it causes cell cycle arrest at the G2/M phase in a dose- and time-dependent manner (Choi, Kim, & Lee, 2007). The molecule also inhibits the proliferation of HO-8910 cells by altering the levels of proteins associated with the cellular checkpoint pathway (Ouyang et al., 2009). In addition, genistein inhibits the expression of VEGF and VEGF receptor (VEGFR) which are the most promising targets for ovarian cancer therapy. VEGF and VEGFR play a major role in angiogenesis and metastasis of ovarian cancer. Genistein has a greater down-regulation effect on VEGF protein secretion than have other isoflavones (Luo, Jiang, King, & Chen, 2008).

The protective mechanism of genistein is concentration-dependent: high concentrations induce apoptosis and cell death in ovarian cancer cells, whereas, at lowest concentrations, genistein shows antioxidant activity without causing any genotoxic or cytotoxic effect (Lee, Kim, & Song, 2012). This represents an additional example of "good" (high dose) versus "bad" (low dose) effects of genistein in relation to the concentrations applied to a specific cellular model.

Treatment of the ovarian carcinoma cell SKOV3 with genistein showed that genistein down-regulates the expression of the miR-27a, which is an important regulator in various types of cancer; the down-regulation of miR-27a was also accompanied by the increase in an expression of the miR-27a target gene Sprouty2 (an intracellular regulator of receptor tyrosine kinase signaling). These results suggest that the inactivation of miR-27a by genistein can have a protective role in ovarian cancer by blocking ovarian cancer cell growth and migration (Xu et al., 2013).

10. Molecular targets of genistein in bladder cancer: *in vitro* and *in vivo* studies

Bladder cancer, together with lung cancer and mesothelioma, is one the most common occupational cancers, which represent 19% of all cancers and are caused by external environmental situations, such as air pollution, UV radiation and indoor radon (World Health Organization, 2011). Bladder cancer is the fifth most common type of malignancy in the western hemisphere. The highest rate of mortality was observed in European countries and it was lower in Asian countries. The effect of genistein against bladder cancer has been revealed earlier by both *in vitro* and *in vivo* studies. The results showed that genistein exerts growth inhibitory activity in 253J B–V human bladder cancer cells *in vitro* in a time- and dose-dependent manner and causes the arrest of the cell cycle at the G2-M phase. It has been reported that genistein down-regulated the NF-κB pathway and induced apoptosis. The *in vivo* orthotopic tumor mouse model antitumor potential of genistein revealed that genistein reduced tumor volume by inducing tumor cell apoptosis and it also inhibits angiogenesis (Singh, Franke, Blackburn, & Zhou, 2006). These results suggest that genistein is a potent anticancer and antimetastatic agent, which could be used for bladder cancer therapy. Fig. 3 illustrates the molecular targets of genistein in apoptotic and NF-κB signaling pathways.

11. Molecular targets of genistein in neuroblastoma: *in vitro* studies

Neuroblastoma, together with nephroblastoma, medulloblastoma and retinoblastoma, is one of the most common solid cancers in infancy, which arises in children before the age of 15 years (World Health Organization, 2015c). This extracranial malignant tumor causes deregulation of the apoptotic pathway and plays a major role in the progression of neuroblastoma (Schleiermacher, Janoueix-Lerosey, & Delattre, 2014).

Genistein (10 μM) down-regulated the expression of Bcl-2 mRNA and protein level in SK-N-DZ neuroblastoma cells. The highest percentage (93%) of Bcl-2 protein knockdown was observed when the cells were transfected with Bcl-2 siRNA and co-treated with genistein. In SK-N-DZ neuroblastoma cells, genistein up-regulates the expression of death factors and death domains, such as TNFR-1, Fas, TNF-α, FasL, TRADD, FADD, and effectively activates caspase-8. The Bcl-2 siRNA knockdowns the expression of Bcl-2, which leads to activation of the mitochondria-mediated apoptotic pathway. This investigation reveals that genistein activates both receptor- and mitochondria-mediated apoptotic pathways and inhibits the growth of human neuroblastoma SK-N-DZ cells *in vitro* (George, Banik, & Ray, 2010).

12. Molecular targets of genistein in brain tumor: *in vitro* studies

The American Cancer Society's estimates report that about 22,850 malignant tumors of the brain or spinal cord (12,900 in males and 9,950 in females) will be diagnosed in 2015 and about

15,300 deaths will occur in 2015 in the United States (The American Cancer Society, 2015b). The antitumor property of genistein was studied in four brain tumor cell types: KNS60, U251MG (KO), A172 and ONS76 cells, which have TP53 mutations at different codons. In the radiosensitive A172 and ONS76 cells, genistein treatment (at 50 μM concentration) induced a noticeable increase in the expression of p21. In these cells, the increased expression of p21 works together with the decreased expression of cyclin B1 and CDK1 in inducing cell cycle arrest. However, in radiosensitive KNS60 and U251MG (KO) cells, the levels of endogenous p21 were not registered in detectable amounts, and hence could not induce cell cycle arrest. The results suggest that genistein treatment is effective in radiosensitive cells (Khaw, Yong, Kalthur, & Hande, 2012).

Inhibition of telomerase enzymes is specifically targeted for tumor treatment because telomerase enzymes are exclusively present in tumor cells and absent in a normal somatic cells (Jagadeesh, Kyo, & Banerjee, 2006). Genistein (at 50 μM concentration) caused a decrease in the expression of TERT (telomerase reverse transcriptase; a catalytic subunit of telomerase) and TR (telomerase RNA template) and a consequent decrease in the activity of telomerase. Though telomerase activity is affected by genistein, it did not result in shortening of the telomere, which shows that genistein causes cytostatic rather than cytotoxic effects in brain tumor cells (Khaw et al., 2012).

13. Molecular targets of genistein in gastric cancer: *in vitro* studies

The WHO reports that gastric cancer, which accounts for nearly one million deaths per year (Yuasa, 2003), represented the fifth most common site of cancer diagnosed in 2012, among women (World Health Organization, 2015b). Genistein treatment enhanced the chemosensitivity of gastric cancer cells to drugs by down-regulating the activity of the transporter proteins involved in multi-drug resistance, e.g., ABCC1, ABCCD5, ABCG2 and ERK 1/2. Activation of the signaling cascade by ERK 1/2 occurs commonly in many types of cancer. Studies on gastric cancer cells suggest that genistein enhances the chemosensitivity by suppressing the activity of ERK 1/2 (Huang, Wan, Luo, Huang, & Luo, 2014).

14. Conclusion

Genistein is a phytoestrogen that inhibits growth in various cancer cells *in vitro* and *in vivo* by targeting different cellular processes (Fig. 2) which are regulated by well-known signaling pathways, as emerges from Table 1, where we summarize the main molecular targets of genistein here reviewed. Oncogenic activation of cellular signaling pathways plays a major role in cell growth, metastasis and angiogenesis. Molecular and docking studies have revealed that the primary and most likely effects of genistein targets are the inhibition of PTKs (EGFR/VEGFR/Her2) and the interaction with ERαβ. Other signaling pathways (e.g., NF-κB) triggered by genistein can often be a result of these primary targets.

Certainly, many questions remain unsolved when we analyze the molecular mechanisms of genistein, as well as of other polyphenols. Are the "first" hits of genistein common to several cancer cells or do they differ? The substrates which are directly targeted by genistein must be carefully determined. For this reason, it is necessary to determine the exact intracellular concentration of genistein and its stability in a given cellular model. Of course, we cannot exclude a rapid metabolism of the molecule after cellular uptake and the generation of active metabolites. In this case, well designed metabolomics studies are welcomed.

It is noteworthy that the key issues emerging from this review, fundamental to discriminate between the "good" and the "bad" effects of genistein, are essentially twofold: doses applied and experimental model considered. Important information is expected from ongoing clinical trials based on the administration of genistein to cancer patients. A search in the http://clinicaltrials.gov/ with keywords "genistein" in October 2014 showed that there are more than 53 clinical studies on genistein. Therefore, it is easy to ascertain the most effective doses for future studies on anticancer effects of genistein.

In summary, genistein acts as a potent anticancer agent which prevents, retards or blocks carcinogenesis by its pleiotropic mechanisms. However, for specific cancers, such as BC, careful attention must paid to the doses applied and to the "molecular signature" of this tumor in single patients. In this respect, chemotherapy and/or chemoprevention, based on genistein administration, will benefit greatly from the progress of personalized medicine.

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