

Treatment of Metastatic Melanoma B16F10 by the Flavonoids Tangeretin, Rutin, and Diosmin

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Melanoma is one of the most frequently metastasizing malignant neoplasias. This study examines an experimental model of pulmonary metastasis and the B16F10 cell subline, highly metastatic in the lung. Antimetastatic effects of the flavonoids tangeretin, rutin, and diosmin were analyzed, and at the same time an analysis of the metastatic activity of ethanol was performed, considered to be necessary because it is used as a vehicle for administering the flavonoids. Lentini's model, which complements the macroscopic evaluation of nodule numbers by using a stereoscopic microscope and image analysis at the microscopic level, was used. The greatest reduction in the number of metastatic nodules (52%) was obtained with diosmin; similarly, the percentages of implantation, growth index, and invasion index (79.40, 67.44, and 45.23%, respectively), were all compared with those of the ethanol group, considered to be an effective control group. Rutin- and tangeretin-treated groups also showed reductions of the same index compared with the ethanol group. It would seem that structural factors would better explain these results and the antimetastatic activity of each flavonoid and the respective metabolites.

KEYWORDS: Melanoma; B16F10; metastasis; flavonoid; ethanol; tangeretin; rutin; diosmin

INTRODUCTION

Melanoma is the fastest increasing cancer (3.3% per year growth between 1990 and 1996) among the Caucasian population of the United States (1) and is the 4th most common cancer in Australia and New Zealand and the 10th most common in the United States (2). It is calculated that ~50000 new cases were diagnosed in the United States in 2001 and that at least 10000 people died as a result of metastasis (3). The study of new therapies for melanoma points to the frequent inadequacy of the actual treatments, its steadily growing incidence, and the gradually increasing mortality rates. It is one of the most rapidly metastasizing of malignant neoplasias; after the lymphatic glands, the lung is the most common organ to be attacked, with an incidence of between 12% (4) and 20% (5). Fidler's classical studies dating from 1973 (6) demonstrated the predilection of melanomas to colonize different organs due to the presence of different characteristics: chemical, immunological, or through adhesion to the surface of tumoral cells (7).

Flavonoids are polyphenols that have been shown, in both *in vitro* and *in vivo* test system models, to induce responses with the protective effects of diets rich in fruits and vegetables against degenerative conditions such as cardiovascular disease and cancer (8). Some of these flavonoids, especially rutin and diosmin, are commonly used as active constituents of several pharmaceutical products, mainly against cardiovascular diseases. Diosmin is also used as an antivaricose and vasoprotective agent, whereas rutin has been widely described as a powerful captor of free radicals and as anticarcinogenic (9). Both flavonoids may act in the different developmental stages of malignant tumors by protecting DNA against oxidative damage, inactivating carcinogens, inhibiting the expression of mutagenic genes and enzymes responsible for activating procarcinogenic substances, and activating the systems responsible for xenobiotics detoxification (10). *In vitro*, flavonoids have demonstrated their capacity to modify the activity of enzymatic systems in mammals (kinases, phospholipases, ATPase, lipooxygenases, cyclooxygenases, phosphorodiesterases, etc.), a correlation having been observed in some cases between the flavonoid structure and its enzymatic activity (11–13).

More specifically, preliminary studies on melanoma lines using several flavonoids of a *Citrus* origin showed that the presence of the C2–C3 double bond on the C ring (14),

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conjugated with the 4-oxo function (15), were critical for this biological activity. Subsequent studies showed that the presence of three or more radicals in any of the rings of the flavonoid skeleton significantly increased the antiproliferative activity observed in B16F10 cell cultures, whether these radicals were hydroxyl or methoxyl groups, the flavone tangeretin being of special interest in this respect (12, 13, 16, 17). This finding confirmed previous suggestions (18) that there is no direct relationship between the antioxidant activity of a flavonoid and its antiproliferative and/or antimetastatic activity, although such activity may play a significant role in inhibiting the development of certain cancer lines.

The objective of this study was to analyze the possible antimetastatic effect of the flavonoids tangeretin, rutin, and diosmin and to carry out at the same time an analysis of the metastatic activity of ethanol, considered to be necessary because it is used as a vehicle for administering the flavonoids.

MATERIALS AND METHODS

Cell Line. B16F10, the highly metastatic subline of the murine melanoma B16 (European Collection of Cell Cultures, Salisbury, U.K.), was cultivated with Eagle's minimum essential medium buffered at pH 7.2–7.4 with 10% bovine fetal serum and penicillin (100 $\mu\text{g}/\text{mL}$ and 100 units/mL, respectively). The absence of *Mycoplasma* spp. was confirmed by direct fluorescence with DNA-specific colorant (H33233).

Animals. Fifty female albino Swiss mice, 10–12 weeks old and weighing 28–36 g, were provided by the Laboratory Animals Service of the University of Murcia (license 30030-2AB).

Flavonoids. Three flavonoids were used: diosmin, provided by Nutrafur-Furfural Español S.A. (Murcia, Spain); tangeretin, provided by Extrasynthèse S.A. (Genay, France); and rutin, from Merck (Madrid, Spain). These were dissolved (0.2% w/v) in a solution of water/ethanol (98.8:1.2, v/v) and included in the drink and feed (trituated A04 maintenance feed from Panlab, Barcelona, Spain), both of which were provided ad libitum. The mean dose of flavonoid provided in this way was 20 mg/animal/day, along with 96.8 mg of ethanol.

Reagents. The following reagents were used: Eagle's minimum essential medium (Gibco, Langley, VA), fetal bovine serum (FBS; Gibco, Grand Island, NY), streptomycin and penicillin (Sigma Co., Madrid, Spain), H33233 (Hoescht, Germany), ethanol (Merck, Madrid, Spain), and hematoxylin and eosin (Sigma Co.).

Experimental Procedure. Each animal was inoculated with 5×10^5 cells/200 μL of culture medium in the lateral vein of the tail. The following groups were established: I, control (inoculation with 5×10^5 cells only); II, ethanol solution (98.8 water/1.2 ethanol); III, tangeretin; IV, rutin; V, diosmin. The flavonoids and vehicle (water/ethanol) were administered for 11 days prior to inoculation of the tumoral cells and during the following 21 days in the food and drink.

Twenty-one days postinoculation, the animals were sacrificed by cervical dislocation, and a macroscopic study was made of the lungs, fixing the organs in 10% buffered neutral formol (model I, below). For this, the lungs were processed according to the usual method for inclusion in paraffin, the five lobules of both lungs being placed in the same cassette before making a series of 3 μm sections and selecting 1 of every 14 for staining with hematoxylin and eosin. The quantitative evaluation of the metastatic nodules was made by two observers using the following models: model I, a macroscopic study by stereoscopic magnifying glass (Olympus), counting the metastatic nodules of the pleural surface of the five lobules; model II, quantitative analysis at microscopic level of the five sections of each lobule. Images were captured using an Olympus SZ11 magnifying glass connected to a Sony DXC 151-Ap video camera and a System MIP-4 image analyzer (Digital Image System, Barcelona, Spain), with which we interactively selected the regions to be studied. In accordance with Lentini (19), the initial parameters evaluated were as follows: complete area of lobule at $21\times$ magnification, interactively selecting the lobule to be studied; area of metastasis per lobule; and mean area of metastasis per lobule, obtained by capturing images at $55\times$ magnification and interactively

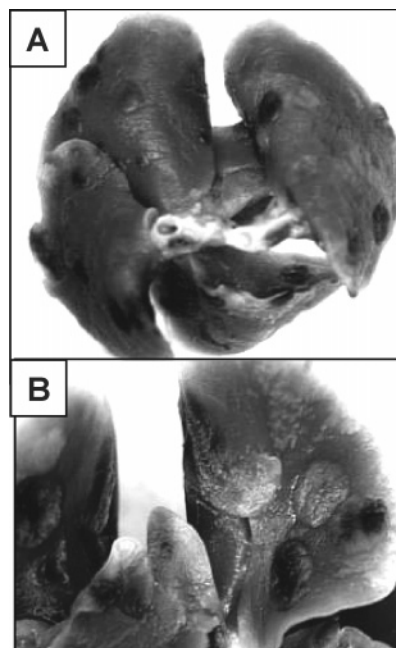


Figure 1. Macroscopic characteristics of the pulmonary metastatic nodules.

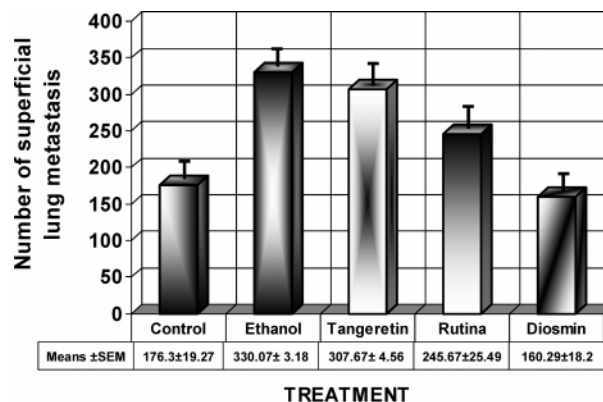


Figure 2. Frequency of the pulmonary metastatic nodules in the control group and groups treated with ethanol, tangeretin, rutin, and diosmin (mean \pm error of the mean).

selecting the different areas of metastasis. The areas were calculated from the maximum and minimum diameters and the mean areas by dividing the total area of metastasis by the number of metastatic nodules.

With these parameters, we calculated (1) implantation percentage = (area of metastasis/total area of lobule) \times 100; (2) growth index = mean area of metastasis/total area per lobule; and (3) invasion index = area of metastasis/mean area of metastasis per lobule.

Statistical Analysis. A descriptive statistical analysis was made by calculating the distribution of frequencies, mean, error of the mean, and typical, maximum, and minimum deviation. Comparison between groups was by one-way analysis of variance on a logarithmic scale. This was complemented by a comparison between means (pairwise *t* test) using least-squares differences (LSD). Values of $p \leq 0.05$ were considered to be significant.

RESULTS

Macroscopic Study. We considered as quantifiable metastatic nodules those structures of a blackish color clearly identifiable on the lung surface and sufficiently separated to be counted individually (Figure 1).

Figure 2 depicts the pulmonary metastatic nodules counted in the different groups. The control (group I) had a mean of 176.30 ± 19.26 metastatic nodules randomly distributed over

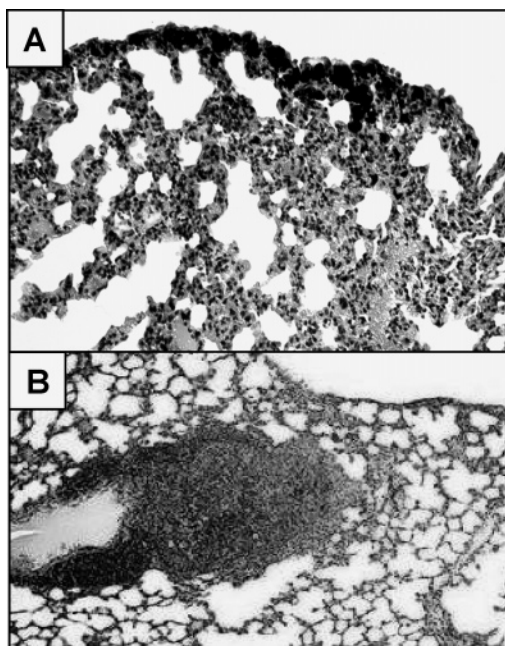


Figure 3. Microscopic characteristics (A) linear pattern at pleural level and (B) metastatic nodules around vessels and bronchi (125 \times).

the lung surface, although it was group II (exclusively administered ethanol solution) that showed the most relevant results, with an increase of 87% metastatic nodules over the control. Indeed, group II was effectively the control group because ethanol was used as vehicle to administer the flavonoids.

In decreasing number of metastatic nodules shown were groups III (tangeretin, nonsignificant decrease compared with ethanol group), group IV (rutin, significant decrease compared with ethanol group, $p < 0.05$), and group V (diosmin, highly significant decrease with respect to ethanol group, $p < 0.00005$). This last group was the only group to show a smaller number of metastatic nodules than the group I control (with a nonsignificant decrease of 9.09%).

Microscopic Study. The localization of the metastasis varied widely, although they were a constant feature at subpleural level, where they took on two basic patterns: linear and solid. At the intraparenchymal level, they appeared mainly around the capillary vessels or veins and bronchioles or bronchi, where they were usually larger than at the subpleural level (Figure 3). Morphologically, the nodules were composed of solid accumulations of neoplastic melanocytes, which, in the largest cases, usually showed small, generally multiple, areas of necrosis in the central parts with frequent, usually peripheral, inflammatory infiltrates, usually peripheral. Cytologically, they showed a moderate degree of cellular and nuclear polymorphism. The mitotic index varied from 6 to 10 mitoses per 10 fields of high magnification ($\times 500$). Melanic pigment was variable and usually arranged in small blackish-brown deposits.

Percentage of Implantation (Figure 4). Group I showed an invasion percentage of the lung parenchyma of between 0.61 and 5.75%, with a mean of $2.64 \pm 0.54\%$. Group II, ethanol, the real group for comparison purposes, showed a 255.09% increase over group I levels of invasion ($p < 0.05$). Compared with group II, the flavonoid-treated groups showed reductions for tangeretin (III) and rutin (IV). The diosmin-treated group (V) showed the greatest reduction in invasion compared with the ethanol group, being statistically significant ($p < 0.05$). Furthermore, it was the only group that showed a reduction with respect to the control (I) (26.8%), although not to a statistically significant degree.

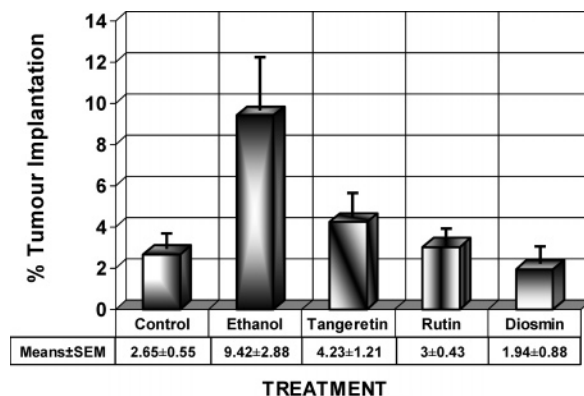


Figure 4. Tumor implantation percentage of control groups and groups treated with ethanol, tangeretin, rutin and diosmin (mean \pm error of the mean).

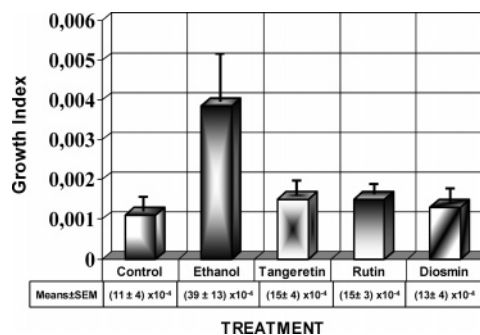


Figure 5. Growth index of control groups and groups treated with ethanol, tangeretin, rutin, and diosmin (mean \pm error of the mean).

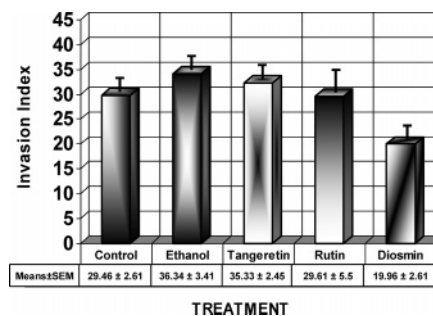


Figure 6. Invasion index of control groups and groups treated with ethanol, tangeretin, rutin, and diosmin (mean \pm error of the mean).

Growth Index (Figure 5). Control group I showed a growth index of 0.0011 ± 0.0004 , whereas ethanol group II showed a 266.57% increase over this group. Of the flavonoids, rutin (group IV) and tangeretin (group III) showed similar indices, whereas diosmin (group V) caused the greatest reduction.

Invasion Index (Figure 6). Control group I showed an invasion index of 29.46 ± 2.6 , the ethanol group (II) showing a 23.31% increase with respect to the control (I), which was not statistically significant. Among the flavonoids, tangeretin (III) showed the highest index (the reduction over ethanol is not statistically significant), followed by rutin (IV). The greatest decrease in the invasion index corresponded to diosmin (V), which showed a significant reduction with respect to ethanol ($p < 0.005$) and with respect to the control (I) (32.28%).

DISCUSSION

As indicated in the Introduction, melanoma, despite representing only $\sim 4\%$ of skin cancers, is responsible for 80% of skin cancer-related deaths, it being one of the most frequently

metastasizing malignant neoplasias (20). For this reason, we chose for this study an experimental model of pulmonary metastasis and the B16F10 cell subline, which has shown itself to be highly metastatic in the lung (21).

Of the many methods that have been proposed for evaluating the effects of treatment (22–27), we chose Lentini's model (19), which complements the macroscopic evaluation of nodule numbers by using a stereoscopic microscope and image analysis at microscopic level, calculating three indices in histological sections: percentage of implantation (percentage of lung tissue area occupied by the metastasis), growth index (size of metastasis), and invasion index (frequency of metastasis foci). These, we felt, gave a more accurate approximation of the real extent of metastasis in the lung.

Studies of subcutaneously injected B16F10 cells have shown that the ethanol diminishes the number of pulmonary nodules compared with the control (28), whereas injection into the lateral vein of the tail significantly increases the metastatic effect of the ethanol (29), as was observed in our study. These apparently contradictory effects of ethanol have been discussed in several studies: for example, *in vitro* studies have described the stimulatory effects of ethanol on cell migration in the case of both B16F10 melanoma (30) and other cell lines, such as breast cancer T47D (31); other studies have described its action on endothelial fibrinolysis (32) and the inhibitory effect on platelet aggregation (33), which may reduce pulmonary metastasis. However, the reasons for which ethanol acts in this way are not fully understood.

The results obtained in the group treated with the ethanol solution (98.8:1.2), namely, a macroscopic (86%) and microscopic (invasion index of 23.31%) increase over the control, suggest tumoral cell transport and invasion of the lung parenchyma. There was also an increase in the index related with the area invaded: a 255.09% increase in the implantation percentage and a 266.57% increase in the growth index. These data suggest an increase in invasion of the lung, especially of tumoral cell proliferation.

Previous studies involving tangeretin, a flavonoid present in citrus fruits, have pointed to a powerful antiproliferative effect on different cell lines, including B16F10 (16) and SK-Mel-1 (17) melanoma, HL-60 leukemia (34), and colorectal cancer (35). Studies relating the chemical structure of flavonoids with their antiproliferative activity showed that the presence of three or more methoxylated radicals in a flavone-type flavonoid skeleton with double C2=C3 bond imparts antiproliferative capacity (12, 36). This would agree with our results, in which tangeretin seems to show a strong antiproliferative effect, reducing the indices related with the area invaded, implantation percentage, and growth index.

Another type of study related with the antimetastatic capacity of flavonoids describes how tangeretin inhibits the mobility of sarcoma cells (37) and platelet aggregation (38) through inhibiting the 12-lipoxygenase activity of the platelets. Similarly, it has been observed that tangeretin diminishes the expression of metalloproteases (MMP-2 and MMP-9) in several cell lines (39), which would also be related with its possible antimetastatic activity. Despite these *in vitro* references, our results pointed to no significant antimetastatic activity, because the reductions observed were not statistically significant with either control (groups I and II), either macroscopically or microscopically (invasion index).

Following tangeretin, the next flavonoid in order of increasing strength was rutin, a powerful free radical scavenger (9) and the basis of some vasoprotective and venotonic pharmaceuticals. Several *in vitro* studies have shown rutin to have antiproliferative effects over the OCM-1 melanoma cell line (40) and some colon

Table 1. Percentage of Reduction on the Macroscopic and Microscopic Evaluations of Tumor Development of Each Flavonoid Treatment Group versus Control Ethanol Group (II)

| parameter | tangeretin (III) | rutin (IV) | diosmin (V) |
|-----------------------------|---------------------|---------------|----------------|
| antiproliferative activity | | | |
| % tumor implantation | 59.2 | 68.1 | 79.4 |
| growth index | 60.3 | 61.1 | 67.5 |
| antimetastatic activity | | | |
| superficial lung metastasis | 7.0 | 25.5 | 52.0 |
| invasion index | 3.0 | 18.8 | 45.2 |

cancer lines (caco-2 and HT-29) (41), carcinoma of human squamous cells (A431) (14), and leukemia HL-60 (36). Recent *in vivo* studies have shown that rutin orally administered at 4% feed intake has a chemoprotective effect on the colon, where it significantly reduces the number of neoplasia foci (42). In our study, rutin reduced the area of invasion, with regard to both the percentage of implantation and growth index, reductions that seem to confirm its antiproliferative effect.

In certain *in vivo* studies (18) rutin seemed to show an antiinvasive capacity (71.2% reduction in surface nodules after oral administration). Our results pointed to a lower degree of reduction, both macroscopically and microscopically (invasion index); however, a degree of antiinvasive action is clear.

The greatest reduction in the number of metastatic nodules was obtained with diosmin; similarly with the percentage of implantation, growth index, and invasion index, all compared with the ethanol group: There was also a reduction in the number of metastatic nodules (9.09%), implantation percentage (26.8%), and invasion index (32.28%) compared with the control (group I). Although several *in vitro* studies have suggested that the antiproliferative effects of diosmin are scant (41, 43), other, *in vivo*, studies have pointed to a degree of antiproliferative action in several tumoral cell lines, including esophagus (44), colon (45), oral (46), and bladder (47). Our results agree with the latter findings, and there was a decrease in the implantation percentage and growth index.

It has been described that diosmin diminishes vein distensibility at microcirculation level, reinforcing capillary resistance, thus perhaps inhibiting the invasion of tumoral cells (48). This antiinvasion effect would be complemented by a capacity to inhibit the release of mediators of inflammation, such as prostaglandins (PGE2) (49), or to act on key enzymes in their biosynthesis (50) and modulating the adhesion of leukocytes, thereby preventing endothelial damage (51). All or any of these mechanisms could explain the significant antimetastatic activity that diosmin evidenced in our study with regard to both the reduced number of nodules at macroscopic level and the invasion index at microscopic level. **Table 1** shows a summary of the data described in **Figures 2** and **4–6** expressed as percentage of reduction on the antiproliferative and antimetastatic index of the different flavonoid treatments versus the control ethanol group (II).

We tried to relate theoretically the results obtained in this study with the plasmatic concentration and structure of each flavonoid, but this activity would be related not only with the flavonoid ingested but also with the metabolites of each flavonoid. Identification and measurement of the physiologic flavonoid conjugates are key prerequisites to an understanding of the role of dietary polyphenols in human health. Most flavonoid glycosides are deglycosylated by β -glucosidases in the small intestine; subsequently, the polyphenols are present as conjugates or glucuronate or sulfate, with or without methylation of the B-ring catechol functional group (8). Ac-

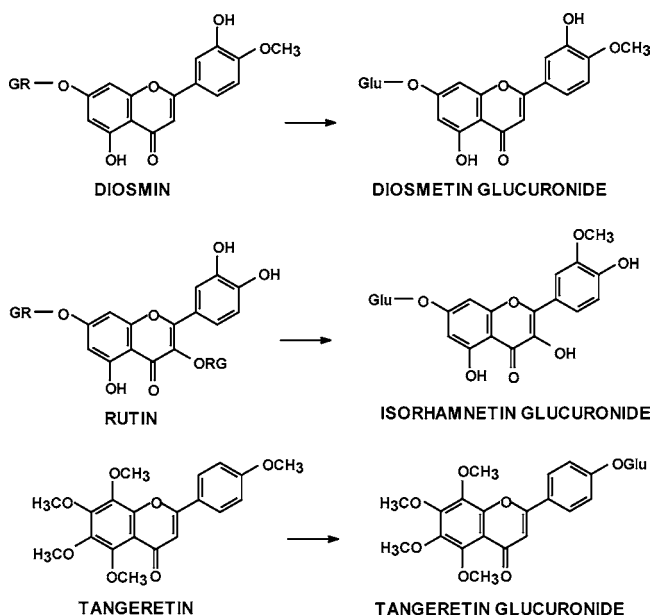


Figure 7. Chemical structures of the flavonoids used in this study and the respective main metabolites according to literature. G = glucose; R = rhamnose; Glu = glucuronide.

cording to several authors, diosmin is metabolized by bacterial flora to form an aglycon, diosmetin, which is then metabolized to form glucuronides and sulfates, the major metabolite being that described in **Figure 7** (52, 53). As in the case of diosmin, the first metabolite formed with rutin is the aglycon quercetin. This subsequently forms conjugates in the form of glucuronides or sulfates, including, in this case, methylation in the 3'-position of the B ring, thus generating as the major metabolite a conjugate of isorhamnetin (**Figure 7**). Finally, with regard to tangeretin, a highly methoxylated compound about which few *in vivo* and *in vitro* references exist, the biotransformation of this flavonoid by rat liver microsomes leads to demethylation of the 4'-position of the B ring, with the possible subsequent formation of the corresponding glucuronide (54, 55).

First, the plasmatic levels or concentrations of these flavonoids should be considered. The literature describes very similar levels for all three at $\sim 15\text{--}60\ \mu\text{M}$ (53–55, 56). To quantitatively justify the effects observed in our results by reference to these cited concentrations would seem to be difficult, and there would, at first sight, seem to be little relationship between the two. It would seem to be more likely that structural factors would better explain the results.

However, these structural factors influence the two activities studied in the present work, antiproliferative and antimetastatic activity, differently and, obviously, as a function of the action mechanisms of each activity. In the first case, the differences in the antiproliferative capacity of the three flavonoids are slight, and evidence of the direct influence of some structural element on these differences does not exist, whether the flavonoids or their major metabolites are taken into consideration. However, this is not the case when the antimetastatic activity of the flavonoids is compared, because the group diosmin–diosmetin glucuronide] showed significantly better results than the groups rutin–quercetin–isorhamnetin glucuronides and tangeretin–tangeretin glucuronide.

This suggests the existence of certain structural factors that regulate the level of antimetastatic activity and that would basically be related with the greater inhibition capacity of certain enzymatic activities or the ability to block receptors responsible for the release of mediators of inflammation (49, 50) or platelet

aggregation (57). In no case would these activities be related with the well-referenced and widely (sometimes inaccurately) acclaimed antioxidant activity, which is lacking in practically all of the mentioned metabolites. Whatever the case, the findings of future quantitative and qualitative studies of these metabolites will be used to carry out *in vitro* studies with the same cell line and *in vivo* studies controlling the dose of metabolites. Identification of the compounds that contribute to the inhibition of cancerous cell lines and of their active concentrations will throw light on the mechanisms behind such inhibition and what the anticarcinogenic properties of these polyphenols are based on.

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