Bioorganic & Medicinal Chemistry 24 (2016) 6004–6011

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Inhibition of the metastatic progression of breast and colorectal cancer in vitro and in vivo in murine model by the oxidovanadium (IV) complex with luteolin

Luciana G. Naso [a], Iker Badiola [b], Joana Marquez Clavijo [b], María Valcarcel [c], Clarisa Salado [c], Evelina G. Ferrer [a], Patricia A. M. Williams [a,*]

[a] Centro de Química Inorgánica (CEQUINOR, CONICET, UNLP), Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 120 N° 1465, 1900 La Plata, Argentina
[b] Department of Cell Biology and Histology, Faculty of Medicine, University of the Basque Country UPV/EHU, Leioa, 48940 Vizcaya, Spain
[c] Innoprot SL, Parque científico y Tecnológico de Bizkaia Edificio 502-P1, 48160 Derio, Spain

ARTICLE INFO

Article history:
Received 18 August 2016
Revised 20 September 2016
Accepted 23 September 2016
Available online 24 September 2016

Keywords:
Oxidovanadium(IV) complex
Luteolin
Antitumor
Antimetastatic
Murine cancer model

ABSTRACT

The anticancer and antimetastatic behavior of the flavonoid luteolin and its oxidovanadium(IV) complex $[VO(lut)(H_2O)_2]Na{\cdot}3H_2O$ (VOlut) has been investigated. Considering that the complex displayed strong anticancer activity on MDAMB231 human breast cancer cell line we herein determined through in vitro assays that the complex would probably reduce breast cancer cell metastasis in a higher extent than the natural antioxidant. In the CT26 colon cancer cell line a stronger anticancer effect has also been determined for the complex (IC_{50} 0.9 μM) and in addition it did not exert toxic effects on normal colon epithelial cells at concentrations up to 10 μM. Working with a murine model of highly aggressive, orthotopic colon cancer model (CT26 cancer cell lines) it has been determined that the complex might prevent metastatic dissemination of the colon cancer cells to the liver. The flavonoid luteolin also exerted anticancer effects (at a low degree, IC_{50} 5.9 μM) on CT26 cell line and produced a 24% reduction of colon cancer liver metastasis.

1. Introduction

Phenolic compounds are natural phytochemicals that are widely present in vegetative foods and nutraceuticals. Cancer chemoprevention, by natural, dietary or synthetic agents that can reverse, suppress or prevent carcinogenic progression, has become an appealing strategy to combat the dogma associated with increasing cases of cancers world. Epidemiological studies point to the fact that long-term consumption of diet rich in fruits and vegetables can reduce the risk of chronic diseases, especially cancer. Such diets can minimize the exposure to deleterious substances, activation of procarcinogens and can maximize the intake of certain beneficial nutrients like isothiocyanates, unsaturated fatty acids, polyphenolic terpenoids (PPT), selenium, terpenes, etc.[1] Natural phytochemicals containing phenolic compounds derived from certain plants with the capability to prevent cancer metastasis have been widely documented[2] and many studies have reported that flavonoids are effective natural inhibitors on cancer invasion and metastasis.[3,4] Moreover, the protective effects of flavonoids against initiation as well as tumor progression have been determined using in vivo studies. For instance, the ability of a 5% blueberries (BB, rich in bioactive substances such as flavonoids and proanthocyanidins) diet to inhibit MDAMB231-luc-D3H2LN metastasis showed that mice developed 70% fewer liver metastasis and 25% fewer lymph node metastasis in comparison to control mice.[5] In other study, the inhibitory effect of luteoloside (luteolin-7-*O*-glucoside, a naturally occurring flavone isolated from the medicinal plant *Gentiana macrophylla*) on hepatocellular carcinoma metastasis has been observed in vivo in

Abbreviations: BSA, bovine serum albumin; CRC, colorectal cancer; Crystal violet, Tris(4-(dimethylamino)phenyl)methylium chloride; DMSO, dimethylsulfoxide; ECM, extracellular matrix; Eosin, 2-(2,4,5,7-tetrabromo-6-oxido-3-oxo-3*H*-xanthen-9-yl)benzoate; FBS, fetal bovine serum; Hematoxylin, 7,11b-Dihydroindeno[2,1-*c*]chromene-3,4,6a,9,10(6*H*)-pentol; Irinotecan, (*S*)-4,11-diethyl-3,4,12,14-tetrahydro-4-hydroxy-3,14-dioxo1*H*-pyrano[3′,4′:6,7]-indolizino[1,2-b]quinolin-9-yl-[1,4′bipiperidine]-1′-carboxylate; Luteolin (lut), 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one; MMPs, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide; PBS, phosphate-buffered saline; RPMI medium, Roswell Park Memorial Institute culture medium; VOlut, $[VO(lut)(H_2O)_2]Na{\cdot}3H_2O$.

* Corresponding author. Tel./fax: +54 0221 4259485.
E-mail address: williams@quimica.unlp.edu.ar (P.A.M. Williams).

http://dx.doi.org/10.1016/j.bmc.2016.09.058
0968-0896/

male BALB/c-nu/nu mouse lung metastasis model.[6] Besides, it has been determined that red wine polyphenols (RWPs) are able to inhibit a 31% tumor growth reducing tumor vascularization and the number of CT26 metastasis in lungs.[7] In regard to the effects of luteolin, its potent proapoptotic effect has been demonstrated on human hepatoma cells both in vitro and in vivo.[8] Moreover, it inhibited proliferation and induced apoptosis of prostate cancer cells (LNCap, DU145 and PC-3 cell lines) in vitro and in xenografts with increased efficacy of cisplatin in gastric cancer cells,[1] and produced a 50% reduction of metastatic colonization of the murine malignant melanoma B16F10 cells in lungs.

Breast cancer is the most common cancer and the leading cause of cancer death in females worldwide. Metastatic spreading occurs in about 50% of cases. In most cases, death results from the dissemination of cancer cells and their proliferation at secondary sites, underlining the importance of controlling and preventing these events.[9] Moreover, cancers of the large and small intestine are major contributors to worldwide cancer morbidity and mortality. Colorectal cancer (CRC) is a multi-step process involving three distinct stages, initiation that alters the molecular message of a normal cell, followed by promotion and progression that ultimately ends up with a phenotypically altered cancer cell. Many signaling pathways are deregulated during the progression of colon cancer. Epidemiological and experimental studies suggest that colon cancer is strongly influenced by nutritional factors, including the amount and composition of dietary fat.[10]

Tumor metastasis occurs by a complex series of events including cell adhesion, invasion, proliferation and vessel formation. Degradation of basement membranes and stromal extracellular matrix (ECM) is crucial for invasion and metastasis of malignant cells. The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases involved in the degradation of the extracellular matrix. The MMPs have been implicated in the processes of tumor growth, invasion and metastasis and are frequently overexpressed in malignant tumors being associated with an aggressive malignant phenotype and adverse prognosis in patients with cancer. MMPs degrade the basement membrane and extracellular matrix, thus facilitating the invasion of malignant cells through connective tissues and blood vessel walls and resulting in the establishment of metastasis.[11] Since prevention of breast cancer cells metastasis is yet unattained, the search of new agents that prevent and inhibit breast cancer cells invasion and metastasis seems to be essential.[12] Although most CRC patients survive curative local resection of the primary tumor, the leading cause of death is metastasis. When detected at an early, localized stage, the five-year survival rate is ca. 90%. However, after metastasis has occurred, the percentage drops to less than 12%. The main target organ is the liver. Almost 20–25% of patients with CRC present liver metastasis at the time of diagnosis, however, the autopsy results reveal that up to 70% of CRC patients had liver metastasis.[13,14]

Platinum drugs are, at present, the only metal-based drugs used in clinical practice for the treatment of cancer but cisplatin cannot be regarded as a drug developed to be an antimetastatic agent. New compounds are continuously being developed and are being pre-clinically studied. These compounds are mostly directed at overcoming cisplatin resistance rather than matching the requirements demanded by antimetastatic chemotherapy. In the literature there exist a few reports of metal-complexes with antimetastatic activity from in vitro and in vivo experiments.[15,16] In a previous study we showed that the structural modification of a natural polyhydroxylated compound, the flavonoid luteolin (lut), produced an enhancement of its anticancer properties.[17] The complexation of the flavonoid with the oxidovanadium(IV) ion, $[VO(lut)(H_2O)_2]Na{\cdot}3H_2O$ (VOlut), showed an increase of the anticancer effects of luteolin when treated on breast MDAMB231 cancer cell lines (from IC_{50} = 88.3 μM to 17.0 μM). We have also shown by bovine serum albumin interaction determinations that the compounds could be distributed and transported in vivo. In the present study, we have determined that the compound VOlut also improved the cancer cell-killing ability of luteolin (IC_{50} 0.9 μM vs 77.9 μM, respectively) in a CT26 colon cancer cell line. We have also found a possible reduction of the spread of breast cancer cells produced by incubation of the cancer breast cell line MDAMB231 with VOlut, studying the in vitro cellular invasion, migration and adhesion processes.

In the knowledge that gastrointestinal metastasis due to breast cancer cell is typically vague and that one of the most common sites of breast and colon cancer metastasis is the liver[18] we hypothesized that VOlut can also suppress metastasis to liver of the colon cancer in vivo. Based in this supposition, the different compounds were directly injected in the bloodstream by the tail of CT26 wearing balb/c mice. In effect, VOlut behaved like an efficient agent inhibiting the colon cancer liver metastatic process in a high degree while luteolin and the oxidovanadium(IV) cation exerted a lesser inhibition (by 24% and 50%, respectively, with respect to the control group).

2. Materials and methods

Luteolin (Nanjing Zelang Medical Technology Co., Ltd) and oxidovanadium(IV) chloride (50% aqueous solution, Carlo Erba) were used as supplied. The tested complex $[VO(lut)(H_2O)_2]Na{\cdot}3H_2O$ (VOlut) was prepared and purified as in Ref. [18]. All other chemicals used were of analytical grade. Anisotropic X band EPR spectrum of the frozen solution was recorded at 140 K, after addition of 5% DMSO to ensure good glass formation. A computer simulation of the EPR spectra was performed using the program WINEPR SimFonia (version 1.25, Bruker Analytische Messtecnik, 1996).

2.1. Cell culture

Human MDAMB231 breast cancer cell lines were obtained from HPA Culture Collection (Salisbury, United Kingdom). The cell line was cultured in endotoxin-free RPMI medium supplemented with 10% fetal bovine serum (FBS) and 100 $U{\cdot}mL^{-1}$ penicillin and 100 $\mu g{\cdot}mL^{-1}$ streptomycin. All reagents were from Sigma–Aldrich (St Louis, MO). Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and passaged according to manufacturer's instructions.

CT26, colon cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The normal colon epithelial cell line was purchased from Sciencell (Carlsbad, CA). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in Roswell Park Memorial Institute (RPMI-1640) culture medium supplemented with 10% FBS and 100 $U{\cdot}mL^{-1}$ penicillin and 100 $\mu g{\cdot}mL^{-1}$ streptomycin. Cells were grown in multi-wells plates and when they reached 70% confluence, the monolayers were washed twice with medium and then incubated with the different compounds.

2.2. Human MDAMB231 breast cancer cell lines studies

2.2.1. Invasion and migration assays

Invasion and migration assays were carried out in the same way and differed only in the membrane filter coating process.[19] Invasion assay was carried out using a membrane invasion culture system (MICS), a bioassay to determine the capability of cancer cells to digest extracellular matrix (ECM) proteins (Matrigel) and migrate through a barrier (filter). Cell motility, a necessary

property of cancer invasion, was assessed by a migration assay that determined the capability of cells to migrate through the filter, when the Matrigel was not included.

2.2.1.1. Invasion assay. The invasion behavior of MDAMB231 cells was determined using a Transwell chamber invasion system (8 μm pore size polycarbonate filters, (Becton–Dickinson). Matrigel (Becton–Dickinson, Bedford, MA) was diluted to 25 μL/100 μL and coated to the top side of the polycarbonate membrane. MDAMB231 cells were trypsinized and suspended at a final density of 3×10^4 cells/well in medium with 10% FBS, and then placed in the upper chamber. Medium with 10% FBS acting as a chemoattractant was placed in the lower chambers. After 3 h, medium was removed and serum-free medium containing the tested compounds, at the required concentrations, were added to the upper compartment of the Transwell chambers. After incubation for 4 h (37 °C, 5% CO_2), the non-invaded cells were removed with a cotton swab and the invaded cells on the lower surface of the membrane filter were fixed with 100% methanol, then stained with 5% crystal violet in 20% methanol and counted microscopically. Data presented refers to the average number of cells attached to the bottom surface from randomly chosen fields.[19,20]

2.2.1.2. Migration assay. The upper side of 8-μm pore size polycarbonate membranes of transwells were coated with (1 μL·mL^{-1}) type I collagen (Sigma–Aldrich). MDAMB231 cells (3×10^4 cells) in 300 μL of culture medium were plated on the upper side of transwell membranes and were allowed to adhere for 3 h. Then, the cells were introduced into 24-well plate and 700 μL of medium with 10% FBS was added to the lower chamber. Cells were treated with 8.5 μM of luteolin, oxidovanadium(IV) cation and VOlut for 4 h (37 °C, 5% CO_2). After incubation, the non-invaded cells were removed with a cotton swab and the invading cells were fixed with 100% methanol and then stained with 5% crystal violet in 20% methanol. The invaded cells on the lower surface of the membrane filter were counted microscopically. Data are presented as the average number of cells attached to the bottom surface from randomly chosen fields.[21]

2.2.2. Wound healing migration assay

MDAMB231 cells were grown in 24-well plates until confluence in conditioned medium. A scrape was made through the confluent monolayer with a sterile plastic micropipette tip to create a denuded zone of constant width. Afterwards, the dishes were washed twice with medium and incubated at 37 °C in fresh medium in the presence or absence of luteolin, oxidovanadium(IV) cation and VOlut (8.5 μM). Cell migration into the wound was monitored and photographed with an inverted microscope (Nikon Eclipse TS 100). In order to quantify the migrated cells, pictures of the initial wounded monolayer were compared with the corresponding pictures of cells at the end of the incubation.[22]

2.2.3. Adhesion assay

Plates of 96-well were coated with 2 μg/100 μL/well of fibronectin (Science cell) and incubated at 4 °C overnight, and then non-specific binding sites were blocked with 0.5% bovine serum albumin (BSA) for 2 h at 4 °C. Then they were washed three times with phosphate-buffered saline (PBS). After a pre-treatment with or without luteolin, oxidovanadium(IV) cation and VOlut (8.5 μM) for 24 h, MDAMB231 cells were trypsinized and suspended at a final concentration of 2×10^5 cells·mL^{-1} in serum-free medium. 100 μL of cell suspension was seeded to each coated well. The cells were incubated at 37 °C for 40 min, and the non-adherent cells were removed by PBS. The colorimetric MTT-assay was used to determine the number of remaining adherent cells.[20]

2.3. CT26 colon cancer cell lines studies

Stock complex solutions were prepared dissolving the compounds in DMSO with a manipulation time of 15 min. CT26 cell line was seeded at a density of 5000 cells/well in 96 well plates, grown overnight and treated with either vehicle, luteolin, VOlut and oxidovanadium(IV) ion at different concentrations in FBS free medium. The dissolution vehicle, DMSO, yield a maximum final concentration of 0.5% in the treated well. After 24 h of incubation, 3-(4,5-methyl-thiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich, St. Louis, MO) was added at 100 μg/well for 2 h. The formazan products generated by cellular reduction of MTT were dissolved in DMSO and the optical density was measured at 560 nm. All experiments were done in triplicate. Data were presented as proportional cell viability (%) of the treated group compared with the untreated cells (control) which viability is assumed to be 100%.

2.4. In vivo studies

All animals received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' published by the NIH. Balb/C mice were divided into four groups of 5 animals. For the CT26 liver metastasis model, syngeneic Balb/c mice were anaesthetized with isoflurane (Abbot Laboratories Ltd., 100% isoflurane, Maidenhead, Berkshire, UK). After that, left upper quadrant laparotomy and splenic exteriorization were performed. Then, 75 μl of the tumor cell suspension (2×10^5 cells/mouse) were injected beneath the splenic capsule. The spleen was returned to the peritoneal cavity followed by two-layer closure of the peritoneum and skin using 6-0 nylon sutures. Next, the mice were injected by vein tail with luteolin, VOlut and oxidovanadium(IV) cation at 20 mg/kg mouse body weight, and the control animals were injected with PBS (control group) on alternate day. Treatment was initiated 1 day after tumor inoculation and mice were killed after 11 days.

2.5. Histological analyses. Hematoxylin and Eosin staining of paraffin-embedded sections of liver

For histologic analysis of liver metastasis, fixed liver tissues were embedded in paraffin and pairs of 7 μm sections were cut at three levels with 500 μm between them. Next, hematoxylin and eosin staining was prepared using a standard method[23] and the liver metastatic tumor area quantified by ImageJ Software developed by NIH (Bethesda, MD).

3. Results and discussion

To characterize the bioactive solution species as a result of the dissolution process and to determine its stability at physiological pH, the EPR spectrum of the complex in culture medium (Fig. 1) has been measured. This solution spectrum showed one single EPR signal. The rhombic deformation of the coordination sphere of the oxidovanadium(IV) cation is evidenced form the following calculated spin Hamiltonian parameters: $g_z = 1.954$, $g_x = 1.986$, $g_y = 1.978$ and hyperfine coupling constants values of $A_z = 156 \times 10^{-4}$ cm^{-1}, $A_x = 44 \times 10^{-4}$ cm^{-1} and $A_y = 52 \times 10^{-4}$ cm^{-1}. Considering the additivity rule used in the determination of the identity of the equatorial ligands of VO(IV) complexes ($A_z = \sum n_i A_{z,i}$, with n_i: the number of equatorial ligands of type i and $A_{z,i}$: contribution to the parallel hyperfine coupling from each of them),[24] the A_z value could be calculated as 154.6×10^{-4} cm^{-1} and this value agree with the experimental one. The calculation of A_z has been performed taken into account a coordination sphere with two

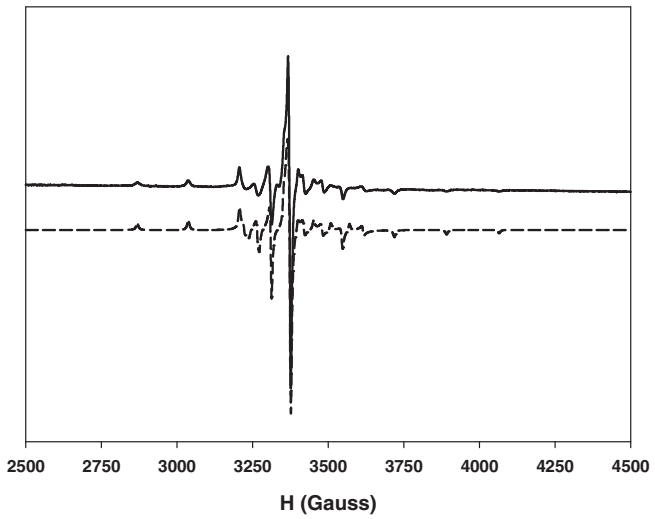

Figure 1. Experimental (solid line) and calculated (short dash line) electron paramagnetic resonance (EPR) spectrum of a RPMI culture media solution of VOlut (140 K, 1 mM) at X-band (9.49 GHz).

ArO^- groups of luteolin ($2 \times 38.6 \times 10^{-4}\ cm^{-1}$) and two OH^- ions ($2 \times 38.7 \times 10^{-4}\ cm^{-1}$). Then, by EPR measurements of a dissolution of $[VO(lut)(H_2O)_2]Na{\cdot}3H_2O$ (VOlut) in RPMI medium we could determine that the complex remained stable at the pH value of the culture media. The only effect observed in this medium is the water dissociation giving rise to the $[VO(lut)(OH)_2]^{3-}$ species.

3.1. On human MDAMB231 breast cancer cell lines

3.1.1. Invasion assay

The effect of the compounds on the invasion of MDAMB231 cells was examined using Matrigel-coated chambers. As shown in Figure 2a and b, luteolin and oxidovanadium(IV) cation solutions showed a slight inhibitory effect on in vitro invasion, resulting in about 15% inhibition. On the other hand, a 64% reduction on cell invasion can be observed when cells were treated with 8.5 μM of VOlut. It has to be noted that the concentrations of the compounds used in these assays did not displayed any cytotoxic effect on the MDAMB231 cells.[17]

3.1.2. Migration assays

To determine whether luteolin, VOlut and oxidovanadium(IV) cation can affect the migration ability of tumor cells in vitro, transwell migration assays (oriented migration) and wound healing assay (non-oriented migration) were performed. As shown in Figure 3a and b (transwell migration assays) VOlut showed significant inhibition of migration (54%) which was somewhat lower than the extent of inhibition of invasion (64%) measured at the

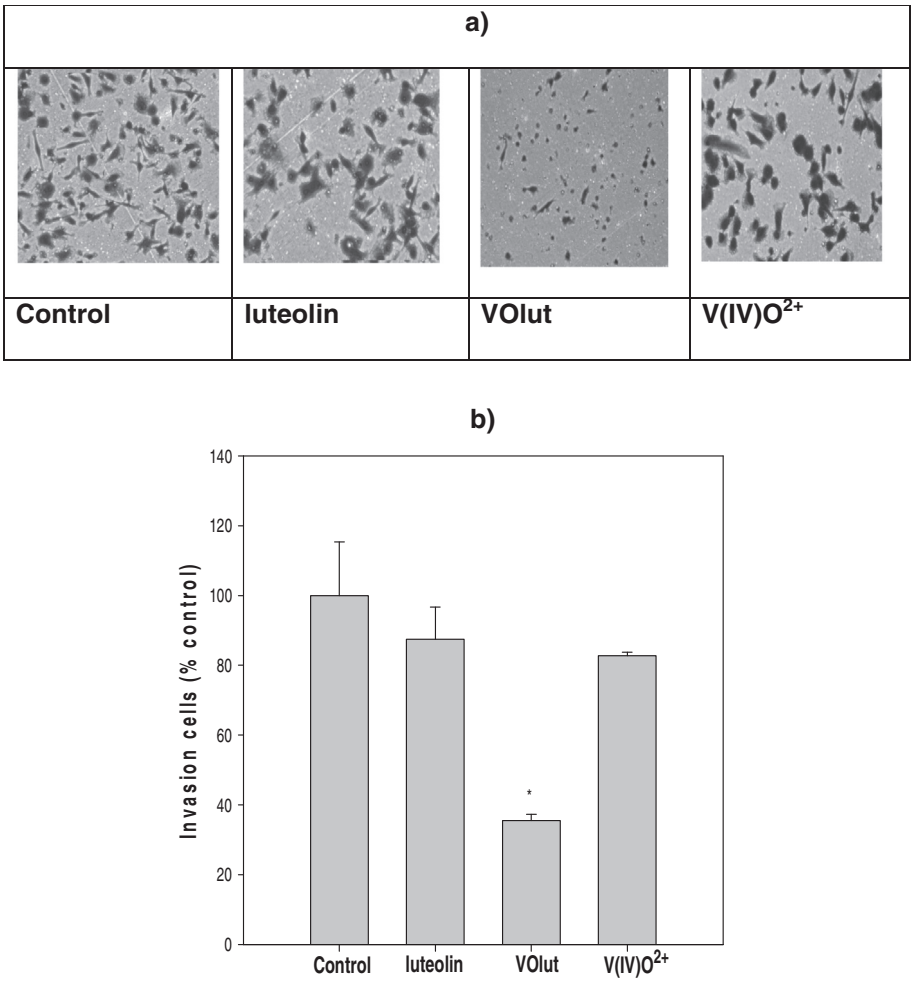

Figure 2. Effect of the compounds on MDAMB231 cell invasion in vitro at 0 (control) and 8.5 μM of luteolin, oxidovanadium(IV) cation and VOlut. (a) Photographs of the cell invasion through the polycarbonate membrane stained by crystal violet. (b) Quantification of the invasion assay. Asterisks: significant values in comparison with the basal level (P <0.05). Data are presented as the mean ± SEM of three separate experiments.

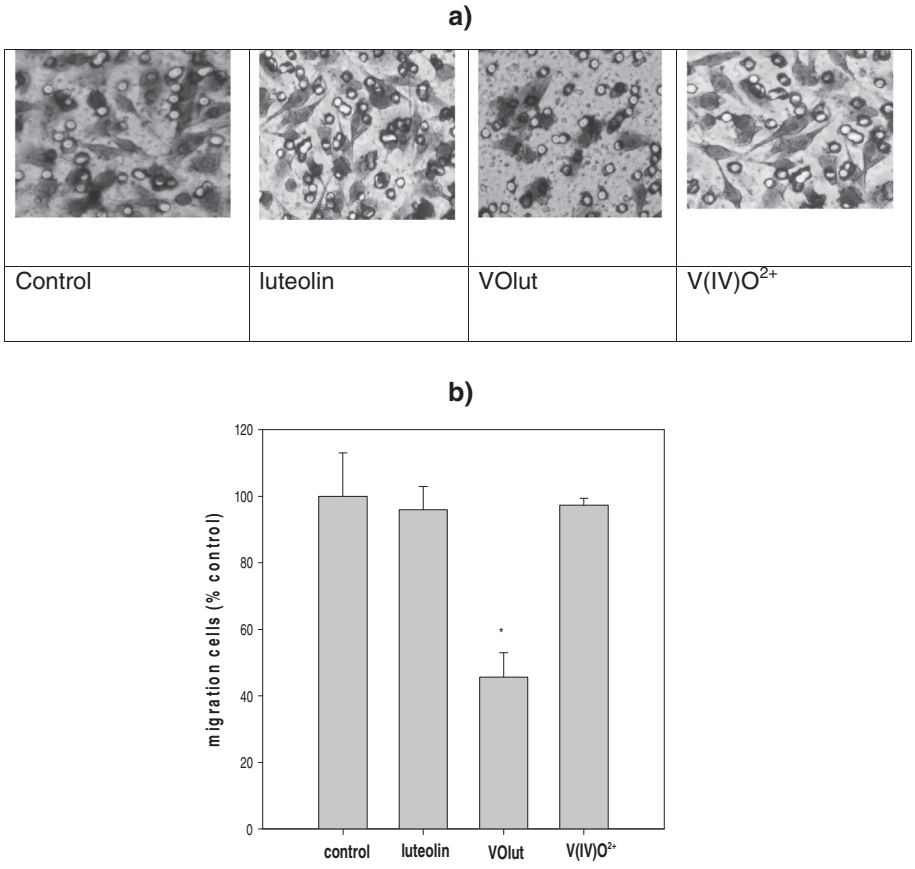

Figure 3. Effect of the compounds on MDAMB231 cell migration in vitro at 0 (control) and 8.5 μM of luteolin, oxidovanadium(IV) cation and VOlut. (a) Photographs of the cell invasion through the polycarbonate membrane stained by crystal violet. (b) Quantification of the migration assay. Asterisks: significant values in comparison with the basal level (P <0.05). Data are presented as the mean ± SEM of three separate experiments.

same concentration. Luteolin and oxidovanadium(IV) behaved like the control cells (untreated cells).

In the other migration assay, the standard wound healing test, a wound through a confluent cell monolayer was created with a pipette tip, and the migration of cells to fill up the wound was recorded by microscopic observation at 0, 5 and 24 h. After 24 h, the wound had almost completely filled in the cleared region in untreated MDAMB231 cells (Fig. 4). VOlut was found to be effective in reducing cellular migration in the MDAMB231 cells in a time-dependent manner. On the contrary, luteolin and oxidovanadium(IV) cation were not efficient in decreasing the migration of the breast cancer cells.

3.1.3. Adhesion assay

Tumor cell adhesion to extracellular matrix and basement membranes is considered to be an initial step in the invasive process for metastatic tumor cells[11] We examined the influence of luteolin, VOlut and oxidovanadium(IV) cation on the adhesion of MDAMB231 cells to the substrates precoated with fibronectin, which is a basement member component. As shown in Figure 5, the adhesion ability of MDAMB231 cells treated with 8.5 μM VOlut decreased 29%, compared with the control cells treated with DMSO (solvent). Luteolin and oxidovanadium(IV) cation showed not significant differences with the control.

3.2. CT26 colon cancer cell lines

3.2.1. Viability assay

The cytotoxicity of VOlut on CT26 colon carcinoma cells viability in vitro has been compared with the effects of luteolin and the oxidovanadium(IV) cation. Luteolin (Fig. 6) displayed a biphasic behavior with a slight stimulation of the viability in the low range of concentrations and was an inhibitory agent at higher doses (IC_{50} = 77.9 μM). On the other hand, both VOlut and the oxidovanadium(IV) cation behaved as efficient anticancer agents being its IC_{50} values 0.9 μM and 5.9 μM, respectively.

Among drugs frequently used in the treatment of colorectal cancer metastasis, irinotecan and oxaliplatin have been selected to make comparisons of the effects of the complex on the viability of CT26 cell line with those of these commercial anticancer drugs. Irinotecan ((*S*)-4,11-diethyl-3,4,12,14-tetrahydro-4-hydroxy-3,14-dioxo1*H*-pyrano[3′,4′:6,7]-indolizino[1,2-*b*]quinolin-9-yl-[1,4′bipiperidine]-1′-carboxylate, a water-soluble semisynthetic derivative of camptothecin, is a key component of first and second-line treatment regimens for metastatic CRC but this drug produced common complications such as diarrhea and neutropenia.[25] Oxaliplatin (a diaminocyclohexane (DACH) platinum derivative) inhibits DNA replication and transcription through the formation of intra and interstrand DNA adducts but caused adverse reactions that narrowed its therapeutic index.[26] We have found that both displayed cell killing effects on the CT26 cell line showing IC_{50} values higher than 100 μM (data not shown). Previous studies were somehow different (50% reduction of cell viability at a concentration of 4 mM for oxalilplatin[27] and an IC_{50} value of 85 μM for irinotecan)[28] under different experimental conditions. Therefore, the comparison performed using the same experimental conditions, allows us to determine that the flavonoid luteolin and its complex produced a more deleterious effect on the colon cancer cell line than the pharmaceutical drugs.

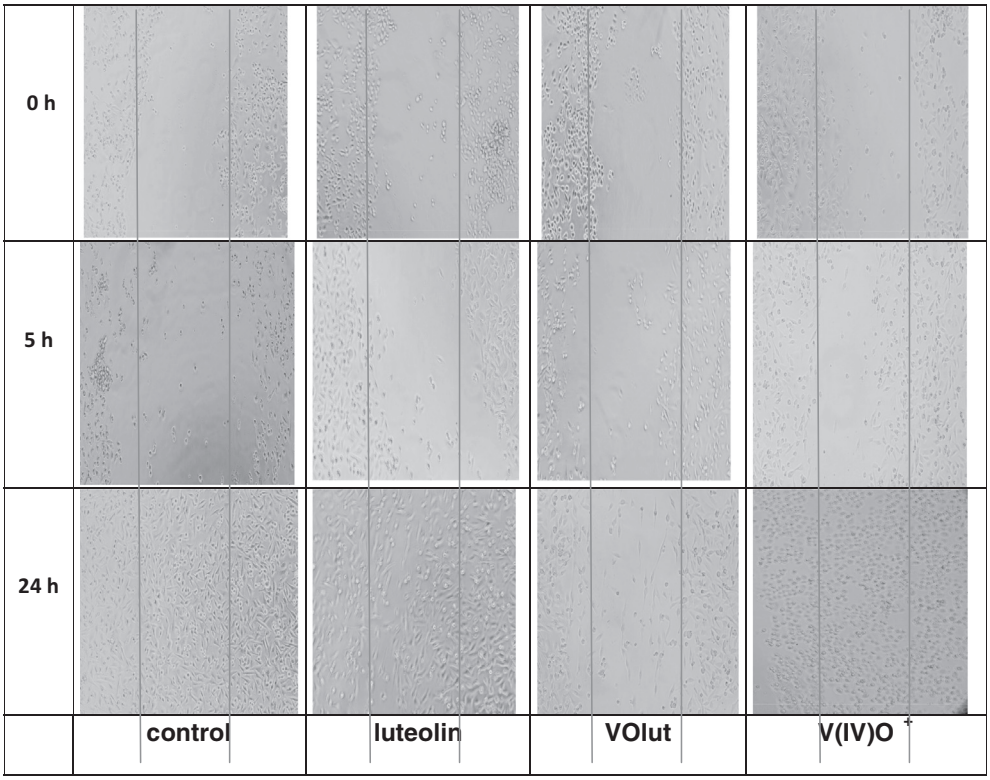

Figure 4. Effect of the different added compounds on the motility of MDAMB231. Cell monolayer was scraped by a sterile micropipette tip, and the cells were treated with 8.5 μM of luteolin, oxidovanadium(IV) and VOlut for 24 h. The number of cells in the denuded zone was quantified by inverted microscopy.

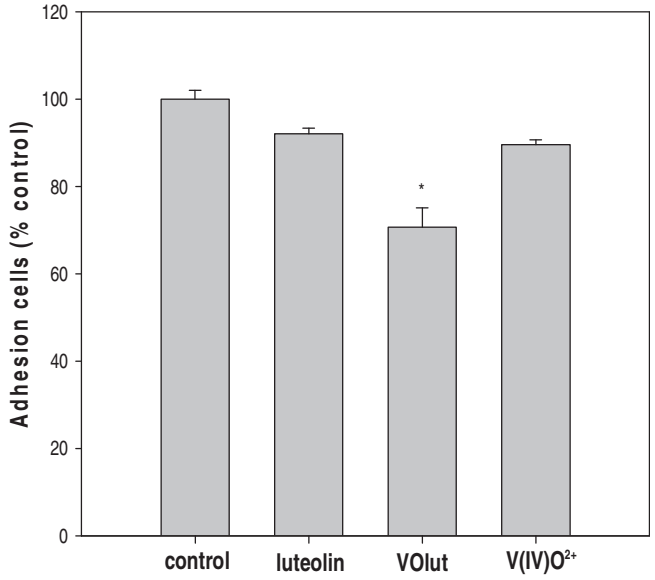

Figure 5. Effect of luteolin, VOlut and oxidovanadium(IV) on the adhesion to fibronectin of MDAMB231 cells. 2×10^5 cells·mL^{-1} were added to the wells coated with fibronectin and the plates were incubated at 37 °C for 40 min. Medium was then carefully suctioned out of each well. Each well was washed three times with PBS. Then the MTT assay was used to determine the number of adherent cells. Asterisks: significant values in comparison with the basal level (P <0.05). Data are presented as the mean ± SEM of three separate experiments.

To compare the toxicity of the compounds on normal cells, their effects on the colon epithelial cells viability were evaluated. It can be observed in Figure 6 that luteolin and the oxidovanadium(IV) cation exerted a low inhibitory effect on epithelial cells (IC_{50} >100 μM) but at high concentrations the metal cation resulted more cytotoxic (44% inhibition of cell viability at 100 μM). VOlut exhibited an IC_{50} value of 80.5 μM but at concentrations lower than 10 μM it had minimal toxic effects on normal cells.

3.3. In vivo studies

The effects of luteolin, VOlut and oxidovanadium(IV) cation on the metastasis of CT26 colon cancer cells from the spleen to the liver of mouse are shown in Figure 7. The compounds were administrated by vein tail considering that previous studies on the interaction of the complex with bovine serum albumin indirectly showed that the complex could be transported and stored in blood by albumin.[17] The presence of the metastatic nodules when the mice were treated with luteolin, the oxidovanadium(IV) cation or PBS (control group) can be observed in Figure 7a. Surprisingly, there is no evidence of metastatic nodules in the liver of VOlut mice-treated. Hematoxylin and eosin staining revealed the high cell density in the metastatic tumors. The mouse section with metastasis is shown in Figure 7b. From Figure 7c it can be seen that luteolin and oxidovanadium(IV) cation produced the reduction of colon cancer liver metastasis by 24 and 50%, respectively, with respect to the control group. Therefore, it can be seen that VOlut behaved like an efficient agent strongly inhibiting the metastatic process.

4. Discussion

Various studies have shown that different flavonoids and polyphenols exerted antimetastatic effects on several cells lines.[3,11,29,30] In particular, there are some reports about the antimetastatic effects of luteolin on cancer cells lines. It has been

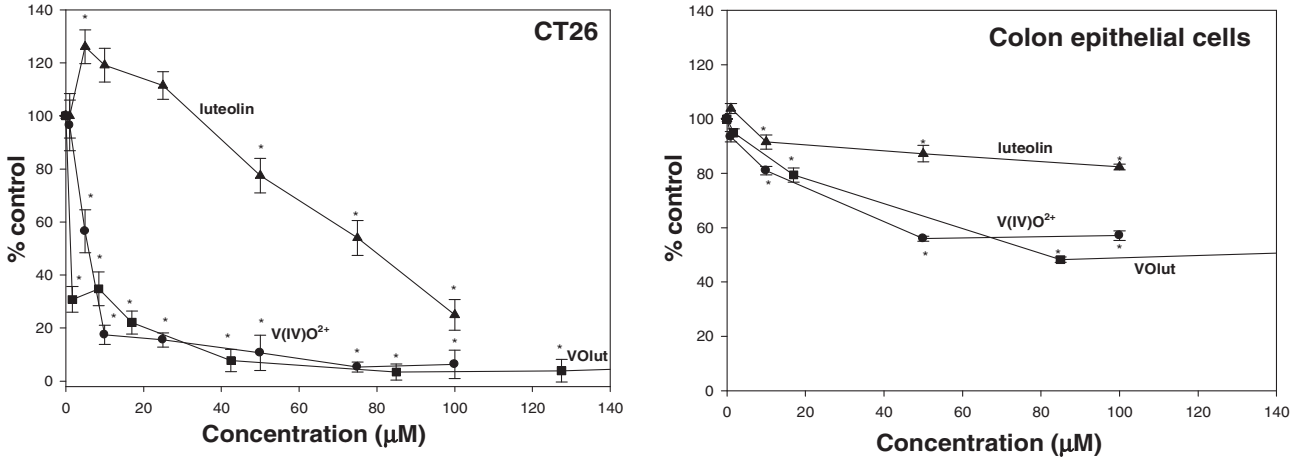

Figure 6. Effects of luteolin, VOlut and the oxidovanadium(IV) cation on CT26 colon carcinoma and colon epithelial cells viability (MTT assay). Asterisks: significant values in comparison with the control (P <0.05). Data are presented as the mean ± SEM of three separate experiments.

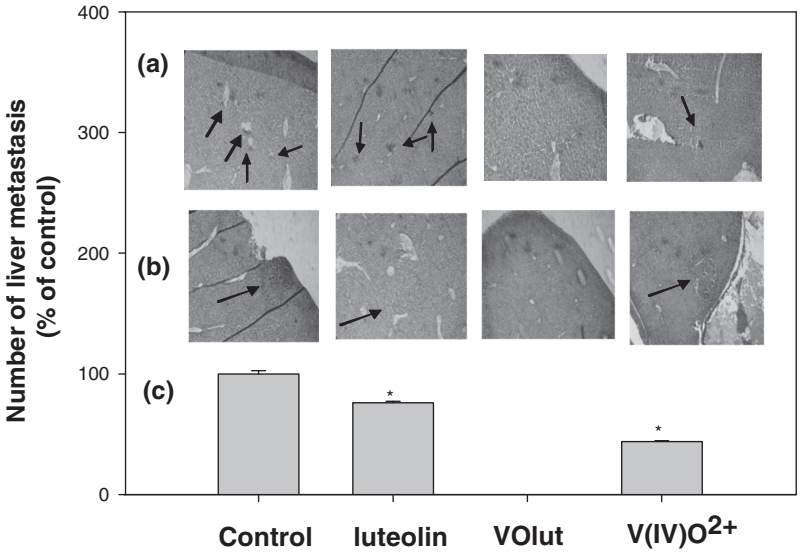

Figure 7. Hematoxilin and eosin staining of liver tissue of mouse models of colon liver metastasis treated with PBS, luteolin, VOlut and oxidovanadium(IV) cation. Arrows indicate (a) micrometastasis (b) macrometastasis. (c) Effects of compounds on liver metastasis induced by intrasplenic injection of CT26 cells into Balb/c mice. Asterisks: significant values in comparison with the control group (P <0.05).

determined that luteolin, in concentrations that are not cytotoxic to the cells, exerted an inhibitory effect on the invasive phenotype of LNM35, MCF7/6 and MDAMB231-1833 cancer cells.[22] Luteolin inhibited the invasion of prostate cancer PC3 cells through E-cadherin[31] increasing the expression of E-cadherin while reducing the expression of vimentin and integrin on malignant melanoma cells.[32] As it was mentioned above the development of metastasis is the most critical parameter for determination of survival in breast cancer patients. Therefore, it is interesting to find new compounds to prevent the growth and the spread of the cancer. In the current report we evaluated the effects of luteolin, oxidovanadium(IV) cation and a new compound, VOlut on the steps involved in the metastasis (invasion, migration and adhesion) of MDAMB231 cell line. Our data suggest that VOlut inhibits the invasion, migration and adhesion of MDAMB231 cells but luteolin and oxidovanadium (IV) cation are not effective. Herein we have determined that luteolin did not affect the normal colon epithelial cells viability but inhibited the viability of the CT26 cells at relatively high concentrations. VOlut and the oxidovanadium(IV) cation behaved as efficiently anticancer agents and both compounds had a slight effect on the viability of normal colon epithelial cells at low concentrations. We have also demonstrated that the treatment with VOlut strongly suppressed transplanted CT26 cancer cell growth in the liver of mice used as a liver metastasis model. A similar effect has been found for the drug regorafenib, but it behaved as a more toxic agent.[33] Luteolin and the oxidovanadium(IV) cation reduced to a lesser extent the metastatic nodules. These data shows that in addition to the inhibitory effect of tumor growth, the complex VOlut also produced a great inhibition of the metastatic dissemination of the colon cancer cells to the liver, improving the action of the parent drug.

5. Conclusions

In the search of the production of new antitumor agents derived from natural products we have previously synthesized the luteolin coordination complex VOlut. In view of its marked anti-cancer

effects we have studied its antimetastatic effect in vitro and in vivo. In the MDAMB231 human breast cancer cell line VOlut produced a reduction of cellular invasion, migration and adhesion (in a lesser extent) showing that the complex is able to reduce breast cancer metastasis.

Animal model of liver metastasis has been induced by intrasplenic injection of CT26 cells into Balb/c mice. Luteolin and the oxidovanadium(IV) cation produced a reduction of colon cancer liver metastasis in mice but surprisingly the complex inhibited liver metastasis. Considering that at low concentrations the complex has a deleterious effect on the CT26 colon cancer cell line and does not affect normal colon epithelial cells and further it prevents the metastatic dissemination of the colon cancer cells to the liver, this compound appears to be a promising candidate to improve the clinical treatment efficiency in patients with advanced CRCs. However, more controlled studies are needed in order to investigate the efficacy and safety of the complex.

Acknowledgements

The authors would like to thank Mrs. Eva Ferrandez (INNOPROT) and Dr. Luis Lezama (Departamento de Química Inorgánica, Facultad de Ciencia y Tecnología, Universidad del País Vasco) for their valuable help during the breast cancer cell studies and EPR spectrum simulations, respectively. This work was supported by UNLP, CICPBA (PICyT 813/13) and by ANPCyT (PICT13 0569) of Argentina. E.G.F. and L.G.N. are Research Fellows of CONICET. P.A. M.W. is a Research Fellow of CICPBA, Argentina.

References and notes

1. Batra, P.; Sharma, A. K. *Biotech* **2013**, *3*, 439.
2. Weng, C. J.; Yen, G. C. *Cancer Metastasis Rev.* **2012**, *31*, 323.
3. Chien, S. T.; Shi, M. D.; Lee, Y. C.; Te, C. C.; Shih, Y. W. *Cancer Cell Internat.* **2015**, *15*, 1.
4. Dai, Z. J.; Wang, B. F.; Lu, W. F.; Wang, Z. D.; Ma, X. B.; Min, W. L.; Kang, H. F.; Wang, X. J.; Wu, W. Y. *Molecules* **2013**, *18*, 934.
5. Adams, S.; Kanaya, N.; Phung, S.; Liu, Z.; Chen, S. *J. Nutr.* **2011**, *141*, 1805.
6. Fan, S.; Wang, Y.; Lu, J.; Zheng, Y.; Wu, D.; Li, M.; Hu, B.; Zhang, Z.; Cheng, W.; Shan, Q. *PloS One* **2014**, *9*, e89961. DOI: 10.1371.
7. Walter, A.; Etienne-Selloum, N.; Brasse, D.; Khallouf, H.; Bronner, C.; Rio, M.; Beretz, A.; Schini-Kerth, V. B. *FASEB J.* **2010**, *24*, 3360.
8. Selvendiran, K.; Koga, H.; Ueno, T.; Yoshida, T.; Maeyama, M.; Torimura, T.; Yano, H.; Kojiro, M.; Sata, M. *Cancer Res.* **2006**, *66*, 4826.
9. Zheng, H.; Li, Y.; Wang, Y.; Zhao, H.; Zhang, J.; Chai, H.; Tang, T.; Yue, J.; Guo, A. M.; Yang, J. *Toxicol. Appl. Pharmacol.* **2014**, *280*, 10.
10. Pandurangan, A. K.; Esa, N. M. *Asian Pac. J. Cancer Prev.* **2014**, *15*, 5501.
11. Wang, L.; Ling, Y.; Chen, Y.; Li, C. L.; Feng, F.; You, Q. D.; Lu, N.; Guo, Q. L. *Cancer Lett.* **2010**, *297*, 42.
12. Dastpeyman, M.; Motamed, N.; Azadmanesh, K.; Mostafavi, E.; Kia, V.; Najafabadi, A. J.; Shokrgozar, M. A. *Med. Oncol.* **2012**, *29*, 2512.
13. Zhang, Y.; Davis, C.; Ryan, J.; Janney, C.; Peña, M. M. O. *Clin. Exp. Metastasis* **2013**, *30*, 903.
14. Kuppusamya, P.; Yusoffa, M. M.; Pragas Maniama, G.; Ichwanb, S. J. A.; Soundharrajanc, I.; Govindan, N. *Acta Pharm. Sinica B* **2014**, *4*, 173.
15. Bergamo, A.; Sava, G. *Chem. Soc. Rev.* **2015**, *44*, 8818.
16. Bergamo, A.; Masi, A.; Peacock, A. F. A.; Habtemariam, A.; Sadler, P. J.; Sava, G. *J. Inorg. Biochem.* **2010**, *104*, 79.
17. Naso, L. G.; Lezama, L.; Valcarcel, M.; Salado, C.; Villacé, P.; Kortazar, D.; Ferrer, E. G.; Williams, P. A. M. *J. Inorg. Biochem.* **2016**, *157*, 80.
18. Zhou, X. C.; Zhou, H.; Ye, Y. H.; Zhang, X. F.; Jiang, Y. *World J. Surg. Oncol.* **2012**, *10*, 256.
19. Lirdprapamongkol, K.; Sakurai, H.; Kawasaki, N.; Choo, M.; Saitoh, Y.; Aozuka, Y.; Singhirunnusorn, P.; Ruchirawat, S.; Svasti, J.; Saiki, I. *Eur. J. Pharm. Sci.* **2005**, *25*, 57.
20. Li, F.; Li, C.; Zhang, H.; Lu, Z.; Li, Z.; You, Q.; Lu, N.; Guo, Q. *Toxicol. Appl. Pharmacol.* **2012**, *261*, 217.
21. Wang, L.; Ho, J.; Glackin, C.; Martins-Green, M. *Translat. Oncol.* **2012**, *5*, 344.
22. Attoub, S.; Hassan, A. H.; Vanhoecke, B.; Iratni, R.; Takahashi, T.; Gaben, A. M.; Bracke, M.; Awad, S.; John, A.; Kamalboor, H. A.; Al Sultan, M. A.; Arafat, K.; Gespach, C.; Petroianu, G. *Eur. J. Pharmacol.* **2011**, *651*, 18.
23. Khine, M. M.; Aung, W.; Sibbons, P. D.; Howard, C. V.; Clapham, E.; Mc Gill, F.; Van Velzen, D. *Lab. Invest.* **1994**, *70*, 125.
24. Chasteen, N. D. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum: New York, 1981; Vol. 3,.
25. Fuchs, C.; Mitchell, E. P.; Hoff, P. M. *Cancer Treat. Rev.* **2006**, *32*, 491.
26. Alcindor, T.; Beauger, N. *Curr. Oncol.* **2011**, *18*, 18.
27. Tan, S.; Peng, X.; Peng, W.; Zhao, Y.; Wei, Y. *Oncol. Lett.* **2015**, *9*, 2056.
28. Keese, M.; Gasimova, L.; Schwenke, K.; Yagublu, V.; Shang, E.; Faissner, R.; Lewis, A.; Samel, S.; Löhr, M. *Int. J. Cancer* **2009**, *124*, 2701.
29. Wenga, C. J.; Yen, G. C. *Cancer Treat. Rev.* **2012**, *38*, 76.
30. Lee, W. J.; Chen, W. K.; Wang, C. J.; Lin, W. L.; Tseng, T. H. *Toxicol. Appl. Pharmacol.* **2008**, *226*, 178.
31. Zhou, Q.; Yan, B.; Hu, X.; Li, X. B.; Zhang, J.; Fang, J. *Mol. Cancer Ther.* **2009**, *8*, 1684.
32. Ruan, J.; Liu, Y.; Zhang, L.; Yan, L.; Fan, F.; Shen, C.; Wang, A.; Zheng, S.; Wang, S.; Lu, Y. *Acta Pharmacol. Sinica* **2012**, *33*, 1325.
33. Elkacem, L. A.; Arns, S.; Brix, G.; Gremse, F.; Zopf, D.; Kiessling, F.; Lederle, W. *Mol. Cancer Ther.* **2013**, *12*, 1322.