UNIVERSITÀ DEGLI STUDI DI CATANIA

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DOTTORATO DI RICERCA IN BIOTECNOLOGIE
XXIII CICLO

Enhancement of efficacy and selectivity of chemopreventive compounds in human breast cancer cells by using Immunoliposomes

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A papà e mamma

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Introduction

1. The cancer burden

Cancer is the leading cause of death in economically developed countries and the second one in developing countries after cardiovascular diseases. Globally, there were an estimated 12.4 million incident cases of cancer in 2008 (6 672000 in men and 5 779000 in women) and 7.6 million deaths from cancer (4 293 000 in men and 3 300 000 in women) (**Fig.1**).

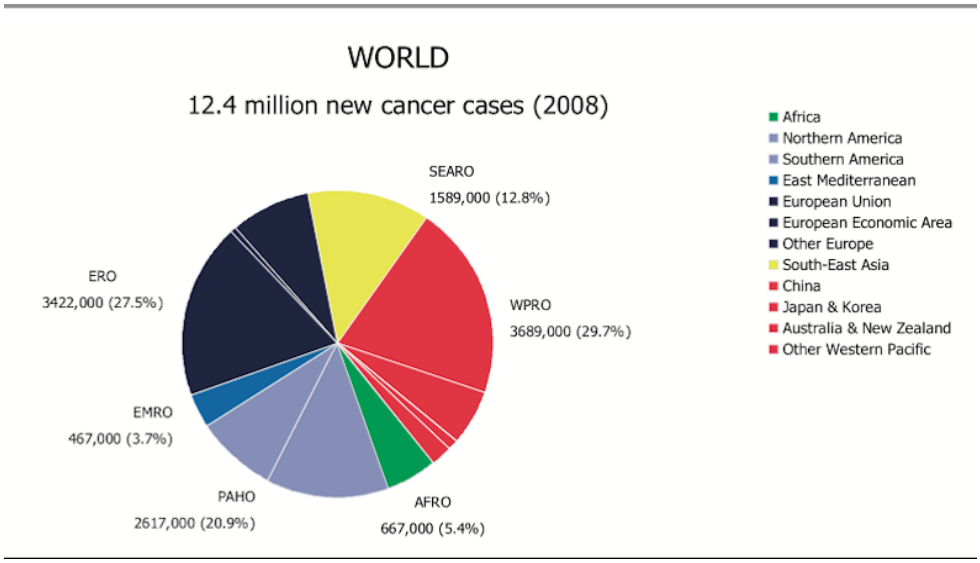


Figure 1 Distribution of Global Cancer cases by World Health Organization Region (2008)

In the European Region (EURO), as in the rest of the world, the commonest incident cancer for men is lung cancer followed by prostate, colorectal, bladder and stomach cancer. They are also the commonest forms of cancer death in men (**Fig. 2 and 3**). Among women breast cancer is the commonest form of cancer and it is also the main cancer cause of death in women, followed by colorectal cancer, lung cancer and stomach cancer (**Fig. 2** and **3**).

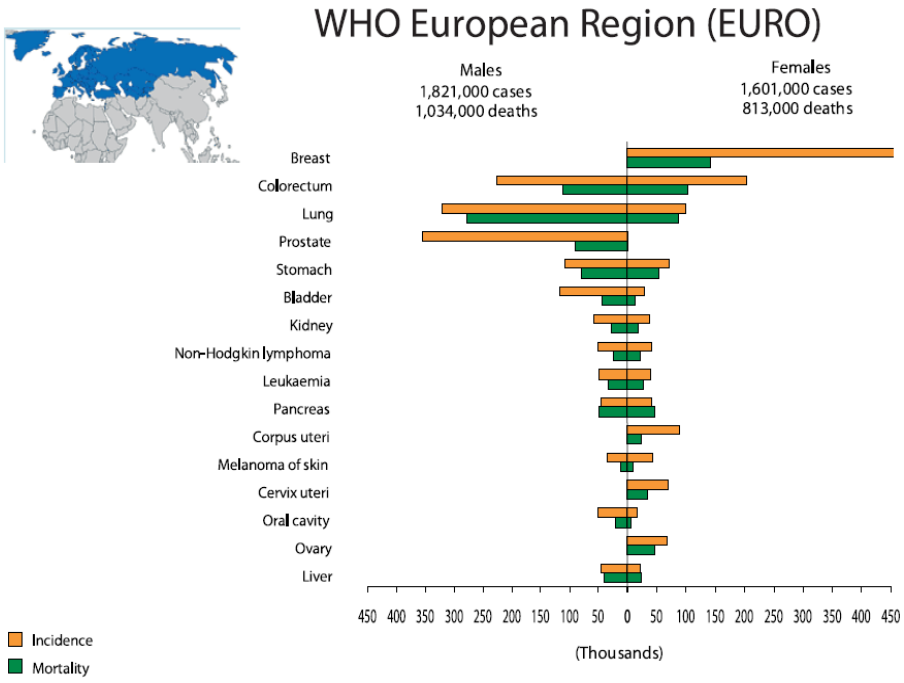
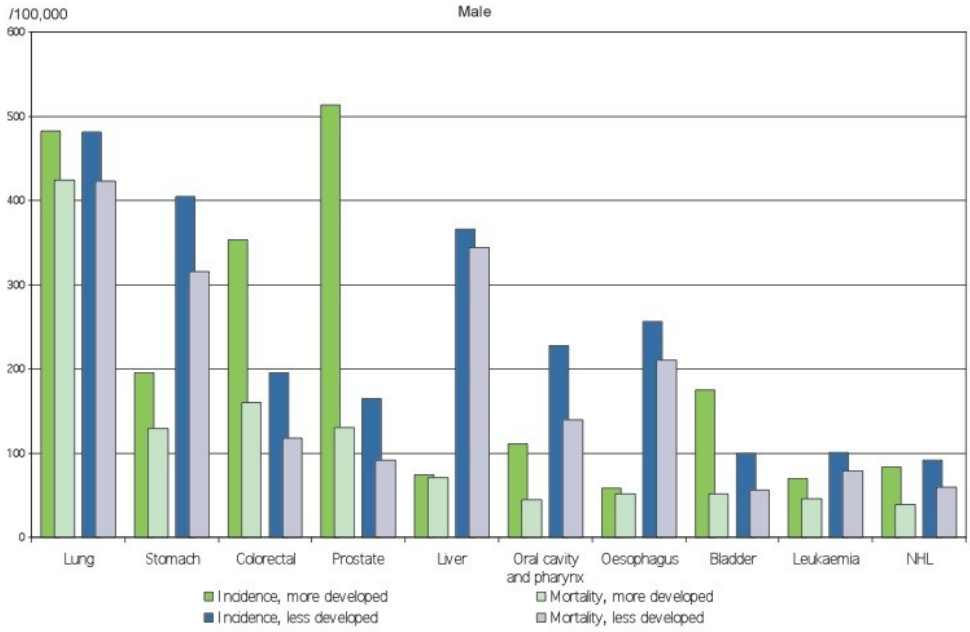


Figure 2 Cancer Incidence and Mortality in World Health Organization European Region (EURO) (Data from World Cancer Report 2008)



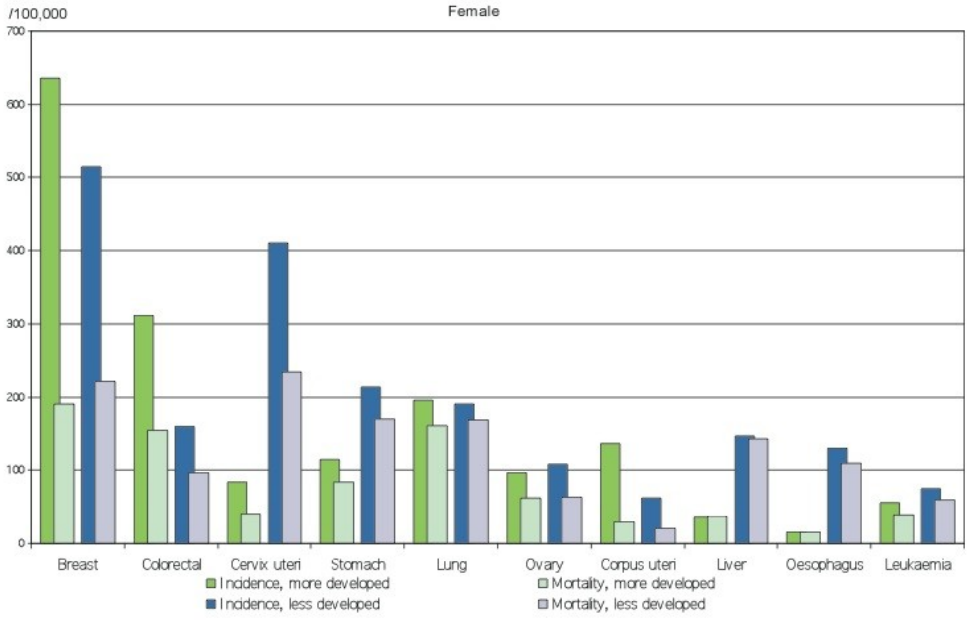


Figure 3 Incidence and mortality of the most common cancers in males and females in more developed and less developed countries. (Data from World Cancer Report 2008)

Globally, breast cancer comprises 23% of all female cancers that are newly diagnosed in more than 1.1 million women each year [1]. Breast cancer already is an urgent public health problem. Its incidence has grown rapidly during the last decades in many developing countries by up to 5% per year [2,3,4] and slowly in developed countries. Mortality rates have remained fairly stable between 1960 and 1990 in most of Europe and the Americas, then showed appreciable declines, which have reached 25-30% in northern Europe [5]. A plausible explanation could be that the screening programs in developed countries has increased the number of new detected cases and in parallel, the technical and pharmacological advances has reduced the mortality.

2. Distinctive features of cancer

The growth and development of breast cancer is similar to that of the other cancer types.

Cancer is a multi-step process through which cells undergo profound metabolic and behavioural changes, leading them to proliferate in an excessive and untimely way, to escape surveillance by the immune system, and at last to invade distant tissues to form metastases [6].

These changes arise through the accumulation of modifications in the genetic programmes that control cell proliferation and lifespan, the relationships with neighbouring cells and the capacity to escape the immune system. This process results in the formation of a mass of deregulated cells, which don't respect the rules that control normal cell growth and behaviour. A cancer cell is a cell that escapes the laws governing cell community life, attaining an independent survival advantage. In doing so, cancer cells try to adapt and fight off the defence systems of the organism and progressively adopt an aggressive and invasive behaviour. They become able to travel within the body and to home preferentially in hospitable organ environments as metastases. Metastatic cancer cells have become so good to adapt themselves to new conditions that they resist many attempts at killing them, including cytotoxic drugs or radiation treatments. This is the reason why most cancers are best treated at an early stage, i.e. at a time when cancer cells still have limited adaptive capacity and consequently are unable to bypass the effects of treatment. Recent experimental studies have identified the minimum number of steps needed to develop a fully cancerous cell [7] (**Fig. 4**).

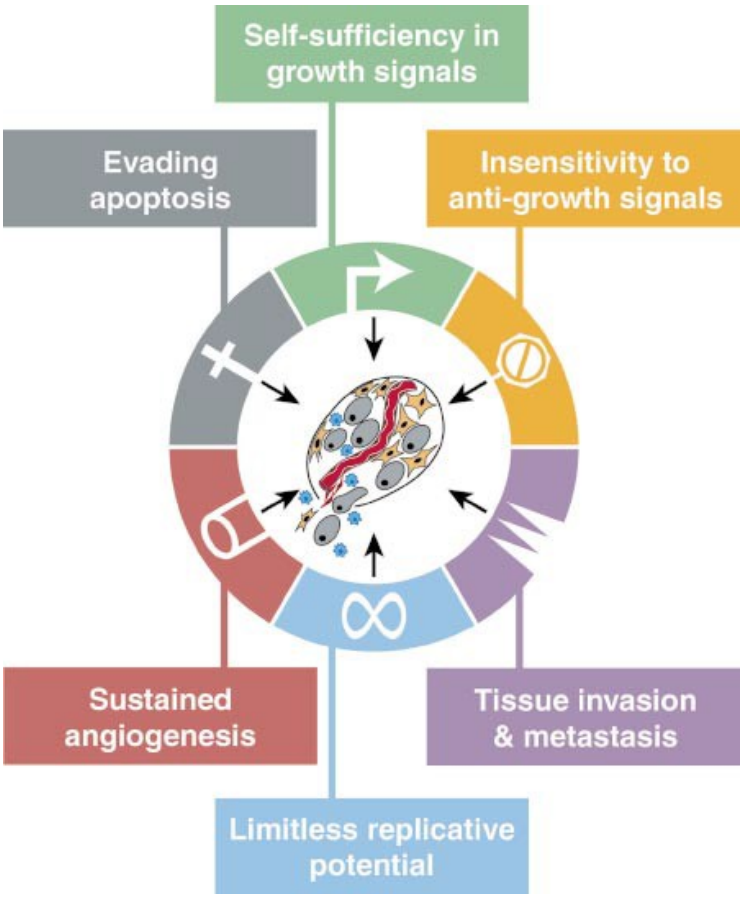


Figure 4 Acquired Capabilities of Cancer [7]

Physiologic changes and novel capabilities acquired during tumour development that represent the successful breach of an anticancer defence mechanism into cells and tissues.

Some fundamental rules must be violated:

- **Self sufficiency in grow**: cells should proceed to divide only when they receive appropriate signals; in the tumour this rule is broken and cells have permanently activated cell division by switching on the circuits that are normally activated when cells are stimulated by a hormone or a growth factor.
- **Evasion from apoptosis**: when confronted by stressful or improper conditions for DNA (Deoxyribonucleic acid) replication, cells activate self destruction programmes rather than allowing DNA replication to

proceed in conditions in which genes may become damaged. In cancer cells also these auto-destruction programmes are bypassed. To do that, cells have to get rid of its safety brakes, which normally should prevent aberrant cell divisions. These brakes are controlled by two master genes: RB (Retinoblastoma gene) and TP53 (Tumour Protein_53) which produces the p53 protein, a stress sensor that normally prevents cells from dividing when their environment is disturbed. When these two brakes are released by mutations, cells can not only divide themselves but also avoid entering programmed cell death, thus allowing the formation of a tumour mass.

- **Insensitivity to antigrowth signals**: another parameter eluded by cancer cells concerns cell division. Normal cells divide only a limited, fixed number of times, they are able to replicate their DNA and divide only for a finite number of times, thanks to particular structures at the end of each chromosome called telomeres. The telomeres are made of small DNA repeated sequences which become eroded each time the cells divide. When all repeats are gone, a cell cannot divide any longer and becomes a senescent cell. In a cancer cell, the activation of an enzyme called telomerase allows the addition of new repeats at the end of chromosomes, thus allowing the cell to divide an infinite number of times. Achieving these functional changes is enough for the cell to become cancerous.
- **Angiogenesis**: cancer cells to improve tumour supply in oxygen and nutrients promote angiogenesis, that is, the synthesis of new blood vessels dedicates to tumour vascularisation. They are able to do that by releasing of vascular endothelial growth factor (VEGF), which stimulate the growth and invasion of new blood vessels accelerating its proliferative capacity.
- **Tissue invasion and metastasis**: one of the main features that distinguish benign from malignant cancers is the ability of tumour cells to spread from their original location to invade and colonise distant organ sites. As long as the tumour remains confined to one specific

location, it remains curable and it can be removed surgically. Once tumour cells start to spread into the organism, they become more difficult to control. They may reach distant organ sites and form secondary tumours, called metastases; these tumour cells able to spread, have acquired special properties that make them more resistant to treatments and to destruction by the immune system. “Metastasis” is a term that origin from the combination of two Greek words, “meta”, meaning “beyond”, and “stasis”, that meaning “location”. A metastasis is a lesion that has changed position. It consists of a series of steps by which growing tumours disturb the architecture of the tissue where they arise, take the space and place of normal cells, infiltrate into healthy areas and cross vessel barriers to enter the lymphatic or blood circulation. They acquire the capacity to become independent from their organ of origin, to invade other organs, to travel in the body and to form colonies.

Expression of various Matrix Metallo proteinases (MMPs) has been found to be up regulated in every type of human cancer and correlates with advanced stages and metastatic properties [8, 9]. Up-regulation of MMP expression determinates degradation of basement membrane components, allowing the tumour cells to invade into the adjacent stroma and to break down the basement membranes associated with capillaries and lymphatic vessels allowing tumour cells to enter the circulation **(Fig. 5)**. MMPs are also involved in cell migration by removing sites of adhesion, exposing new binding sites, cleaving cell-cell or cell-matrix receptors and releasing chemoattractants from the extracellular matrix (ECM)[10].

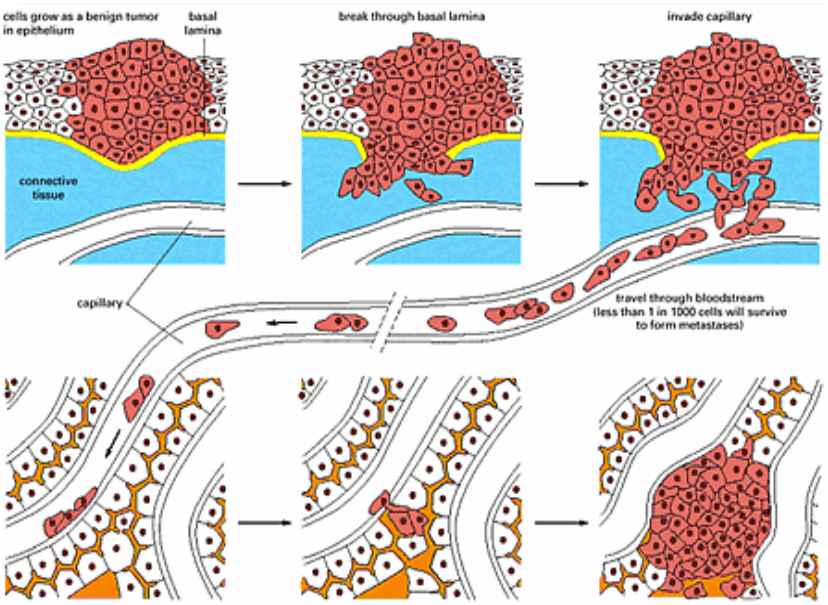


Figure 5 Process of metastasis

Cells grow as benign tumor, break through the basement membrane, travel through the blood stream invading other tissues, adhere to capillary wall, escape from blood vessel and proliferate to form metastases.

3. Breast cancer

3.1 Detection and staging of breast cancer

Breast cancer (BC) is the most commonly diagnosed cancer among women. More than 1 in 4 cancers in women (about 28%) are breast cancers. The incidence is very low in females below the age of 15, and increases very steeply by the age of 45. After menopause, the production of estrogens and progesterone from the ovaries ceases, and the increase in breast cancer incidence rates with age slows down compared to premenopausal women. This suggests a significant implication of hormones in the etiology/development of breast cancer. In vitro experiments have shown that estrogen increases mammary cell proliferation, and also in vivo experiments in animals have demonstrated it [11]. In addition to estrogen exposure, there are other known risk factors for the development of the disease, they include: ethnicity, radiation exposure, family history, genetic predisposition such as mutations in Breast Cancer genes 1 and 2 (BRCA1 and BRCA2), and lifestyle factors, such as obesity, alcohol consumption, and lack of exercise [12].

The mammary gland is the functional structure of the female breast. In adults, each mammary gland is composed of fifteen to twenty lobes, divided by adipose tissue. Each lobe is subdivided into lobules, which contain the glandular alveoli that secrete the milk into a series of secondary tubules. These tubules converge to form a series of mammary ducts, which in turn converge to form a lactiferous duct that drains at the tip of the nipple (**Fig. 6**).

It is generally believed that most breast cancers (approximately 75%) occur within the terminal ductal-lobular unit (TDLU), consisting of the lobule and its adjacent ducts [12]. However, there is still not complete agreement as to whether breast cancer originates as a disease of a single cell, whose progeny spread through a single duct system and accumulate multiple genetic changes toward malignant transformation, or as a cluster of genetically unstable cells and ducts that are simultaneously involved [13].

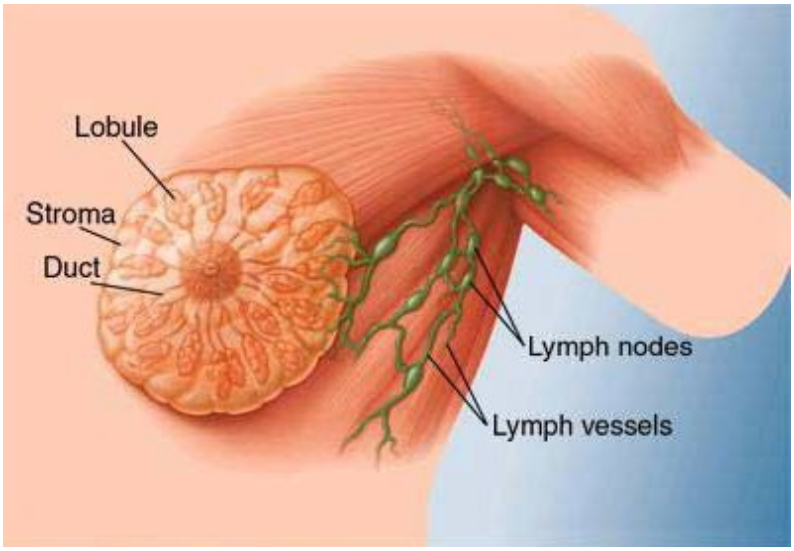


Figure 6 Anatomy of the breast

Over time, cancer cells can invade nearby healthy breast tissue and make their way into the underarm lymph nodes, small organs that filter out foreign substances in the body. If cancer cells get into the lymph nodes, they then have a pathway into other parts of the body.

When cancer cells break away from the primary (original) tumor and travel through the lymph system or blood to other places in the body, another (secondary) tumor may form. The secondary tumor is the same type of cancer as the primary tumor. For example, if breast cancer spreads to the bones, the cancer cells in the bones are actually breast cancer cells; the disease is metastatic breast cancer, not bone cancer.

Tumour cells that are capable of spreading have acquired special properties that make them more resistant to treatments and to destruction by the immune system. Therefore, detection of distant spread and metastases is often an indicator of poor prognosis for the patient. This is reflected in the

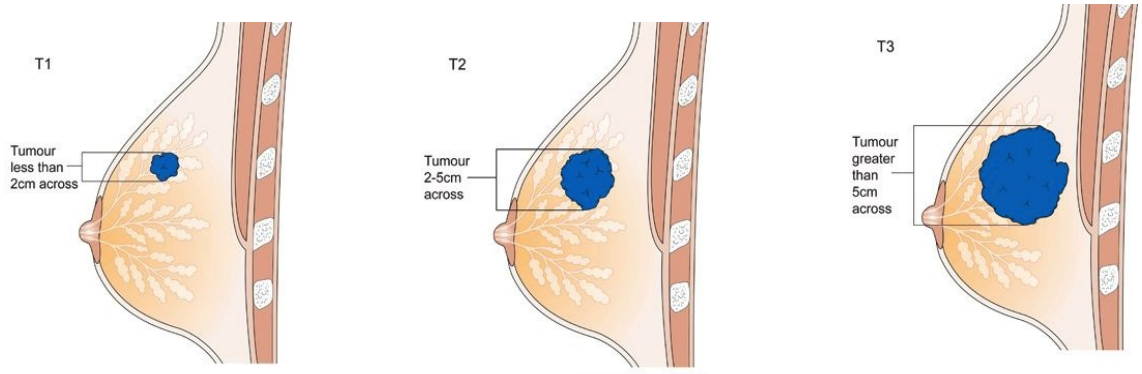
TNM classification system, which provides a universal system to describe the anatomic extent of a cancer.

The staging system normally used to classify the stages in breast cancer is called TNM, which stands for ‘tumour, node, metastasis’. So TNM staging takes into account the size of the tumour, whether the cancer has spread to nearby lymph nodes, and whether it has spread to other parts of the body (metastasis).

The T stages are numbered 1 to 4 and describe the size of the tumour (**Fig. 7a**, **7b**, **7c** and **7d**). The N stages are numbered 0 to 3 and describe which lymph nodes are affected, if any.

The M stages are M0 (no sign of cancer spread) and M1 (cancer has spread to another part of the body).

A B C



D

Figure 7 Tumor size stages

a), b) and c) illustrate T1, T2 and T3 stages of breast cancer; in d) pea, peanuts, walnut and lime show tumor size.

3.2 Expression of estrogen receptor

Many breast tumors have feature that distinguishes them from most other tumors: the estrogenic hormone-dependence for their development and growth [14-19]. Breast tissue requires input from estrogens to grow, consequently tumors that arise from this tissue, show estrogenic dependence for their proliferation [18].

Hormone receptors, such as the estrogen receptor (ER) and progesterone receptor (PR), are determinants of breast tumor behavior and may suggest etiologic pathways [19,20]. ERs bind estrogen and facilitate protein synthesis, cell division and breast cell proliferation [21-23]. PRs, in turn, are regulated by circulating levels of estrogen [19,20].

Expression of ER has long been known to be one of the most important prognostic factors in breast cancer [24,25]. The clinical investigation of tamoxifen, a competitive antagonist of the estrogen receptor in breast tissue used as an anti-cancer treatment in the 1960's, has had a big effect on survival of women with estrogen positive breast cancer. The survival benefit of ER positivity per se, coupled with the beneficial effects of tamoxifen, means that women who have cancer which expresses ER, have the best chance to survive their breast cancer in a shorter term. However, the risk of recurrence and death vary over time in patients with ER positive and ER negative disease. A data analysis of 2006 generated from three clinical trials of chemotherapy showed that the risk of recurrence in ER negative patients was highest in the first two to three years after treatment, which then decreased dramatically with increasing time from diagnosis. In patients with ER positive disease, the risk of an event in the first few years after diagnosis was low, but in a long term, the hazards were slightly higher for patient with ER positive disease when compared with ER negative disease [26]. This demonstrates that, with the development of modern chemotherapeutic regimens, patients with ER negative cancer, who survive the first 5 years after diagnosis, have at least as good long term survival as patients with ER positive cancer.

3.3 The biology of HER2 and its importance in breast cancer

The human epidermal growth factor receptor 2 (HER2) also known as HER-2/neu or ErbB2, is a member of the ErbB family of cell surface receptor tyrosine kinases (RTKs) involved in the transmission of signals controlling normal cell growth and differentiation [27]. The family consists of 4 receptors sharing a high degree of identity; HER1 (EGFR, ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). The HER2 receptor, a 185 kDa transmembrane glycoprotein, is encoded by the HER2 gene, a proto-oncogene located on the long arm of chromosome 17q21 [28]. The erbB receptors share a similar structure comprised of a cysteine-rich extracellular region, a lipophilic transmembrane segment, an intracellular domain with tyrosine kinase activity and a carboxy terminal domain that is autophosphorylated upon receptor activation [29] (**Fig. 11**).

The receptors exist as monomers which upon ligand binding associate to form of homo- or hetero-dimers, resulting in the activation of intrinsic kinase activity, ultimately leading to stimulation of intracellular signaling cascasdes [30]. Notably, HER2 is the preferred dimerization partner of the other HER-family receptors [31] and HER2-containing heterodimers result in potent mitogenic signaling, especially HER2:HER3 heterodimers [30,32,33]. The preference for HER2-containing dimers is likely due to the open conformational state of HER2 compared with the other receptors. Crystallographic studies have revealed that HER1, HER3 and HER4 exist in a closed conformation with the dimerization domain (domain II) tethered to domain IV preventing the formation of dimers with other HER receptors [34-36]. Upon ligand binding to domains I and III the receptors undergo a significant conformational rearrangement exposing the previously concealed dimerization arm for interaction with other HER receptors also in the active state [34,36,37].

In contrast, HER2 is an “orphan receptor” with no known endogenous ligands, however, this receptor is constitutively in a open conformation with the dimerization arm exposed, resembling the ligand-bound state of EGFR [34,37]. This open-conformation facilitates dimerization with other HER family

receptors defining a key role for HER2 in the signal transduction of ligand-driven heterodimeric complexes.

HER2 is normally expressed at low levels in adult cell types including the breast epithelium, the central nervous system, bone, muscle, skin, heart, lungs and intestinal epithelium [28,38]. The function of HER2 in adult tissues is not fully understood, however the receptor appears to play a role in the proliferation and differentiation of epithelial cells [30] and in the protection of cardiomyocytes against apoptosis [39]. In fetal tissues, however, HER2 is widely expressed and is critical to normal development [38]. The clinical significance of HER2 in cancer was first discovered in the early 1980s following the identification of the *neu* onocogene, the mutationally active rat homologue of HER2 [40]. The human homologue was soon identified [28,41] and found to be overexpressed in a mammary carcinoma cell line [42]. On the basis of these findings, Slamon and co-workers examined HER2 expression in a series of primary human breast tumours and reported a significant association between HER2 overexpression, relapse and patient survival [43]. HER2 is now known to be overexpressed in approximately 20-30% of BCs [44], and overexpression is also common in ovarian, prostate, lung, gastric and oral cancers [45].

Overexpression of HER2 is a combined result of increased transcription and protein translation. Indeed, breast cancer cells may have as many as 100 copies of the gene per cell compared with two copies of the HER2-gene in normal cells [46]. Moreover, there are approximately 20 thousand receptors per cell on normal cells, but breast cancer cells may contain as many as 500 thousand to 2 million HER2 receptors per cell [47]. At this high level of HER2 receptor expression, the kinase activity of HER2 becomes constitutively activated which appears to exert potent mitogenic and transforming effects on cells [47].

This high density of HER2 promotes the formation of HER2 heterodimers as well as the formation of ligand-independent constitutively active HER2 homodimers [32,48,49]. HER2-containing dimers persist longer on the cell surface due to their slower rate of internalization, resulting in overactive cell

signaling and leading to dysregulated cell growth, proliferation and malignant transformation [32].

Pathologically, HER2 overexpression is associated with large tumor size, lack of ER/PR expression, presence of nodal metastasis, high nuclear grade and ductal histology [50]. Clinically, HER2 is associated with aggressive disease, increased risk of relapse and poor long-term survival [43,50].

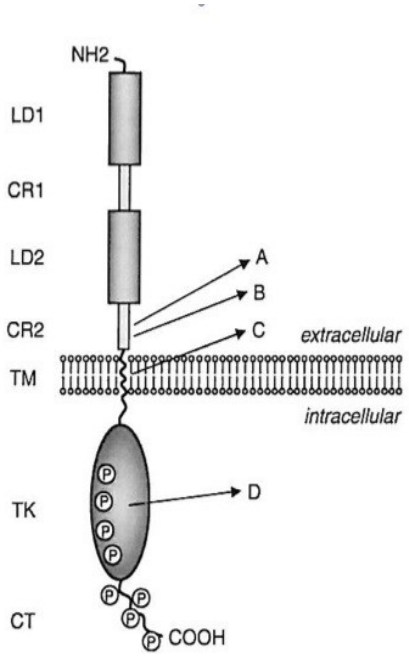


Figure 8 Structure of HER2

The protein structure consists in two ligand-binding region (LD1 and LD2), two cysteine regions (CR1 and CR2), a short transmembrane domain (TM), a catalytic tyrosine kinase domain (TK) and a carboxy terminal tail (CT). Numerous sites of tyrosine phosphorylation within the TK and CT domains are indicated by circled P. Letters on the right (A-D) indicate areas that are altered or mutated in certain naturally occurring or experimentally induced cancer.

3.4 The monoclonal antibody trastuzumab (Herceptin®)

Given the critical importance of HER2 in some forms of breast cancer, extensive research has focused on HER2 inhibitors as potential anticancer agents. Trastuzumab (Herceptin®, Genentech, Inc., San Francisco, CA), is currently the only HER2-targeted therapeutic agent that has received marketing clearance from the U.S. Food and Drug Administration (FDA) for use in the treatment of patients with HER2- overexpressing breast cancer [47]. Trastuzumab is a humanized mAb that binds specifically to HER2 on the C-terminal portion of the extracellular domain (ECD) near the juxtamembrane region in domain IV of the receptor [51]. Trastuzumab was constructed by grafting the complementary-determining regions (CDRs) from the murine mAb 4D5 into a human kappa IgG1 to avoid eliciting a human anti-mouse antibody (HAMA) response in patients [52].

3.4.1 The mechanisms of action

The effectiveness of trastuzumab appears to be correlated with the level of HER2 expression in breast cancer, and with the accessibility of tumors to the drug [53]. However, the mechanism by which trastuzumab induces regression of HER2-overexpressing tumors is not completely understood, but several molecular and cellular effects have been observed in experimental *in vitro* and *in vivo* models. Several of the proposed mechanisms are described below.

3.4.1.1 Immune-mediated response

One of the proposed mechanisms of trastuzumab anti-tumor action is through antibody-dependent cellular cytotoxicity (ADCC) [54-56]. Specifically, the natural killer (NK) cells are important for the ADCC response to trastuzumab [47]. These cells express the Fcγ receptor that binds the Fc domain of the IgG1 trastuzumab antibody, and promotes lyses of trastuzumab-bound cancer cells. The importance of this immunological effect was revealed by Clynes et al. [57], who achieved a tumor regression rate of 96% in mice bearing HER2-overexpressing BT-474 xenografts treated with

trastuzumab, but only a 26% reduction in tumor growth in mice lacking the Fcγ receptor.

3.4.1.2 Inhibition of angiogenesis

Both primary and metastatic breast cancer are dependent on angiogenesis for tumor growth [54]. Trastuzumab can modulate different pro- and antiangiogenic factors to achieve angiogenesis suppression [55,56,58].

3.4.1.3 Inhibition of HER2 extracellular cleavage

The ECD of HER2 can be released by proteolytic cleavage from the full-length receptor, yielding a 110-kDa fragment that can be detected *in vitro* in cell culture medium, and a 95-kDa amino-terminally truncated membrane-associated fragment with increased kinase activity [51]. The HER2-ECD can also be detected *in vivo* in serum, and is currently measured in the clinic with an FDA approved ELISA-based test to follow-up and monitor patients with metastatic breast cancer [59]. Molina et al. [60] demonstrated in HER2-overexpressing SK-BR-3 and BT-474 human breast cancer cells that trastuzumab can block metalloprotease-mediated cleavage of the HER2-ECD. Moreover, several clinical studies have demonstrated that a decline in serum HER2-ECD during trastuzumab treatment correlates with improved tumor responsiveness and progression-free survival [61,62], which indirectly supports the hypothesis that trastuzumab may act by inhibiting HER2 cleavage [63].

3.4.1.4 Inhibition of PI3K pathway

Overexpression of HER2 receptor tyrosine kinases leads to ligand-independent homodimerization and autophosphorylation of tyrosine residues on the cytoplasmic domain of the receptors [64]. Phosphatidylinositol 3' kinase (PI3K) associates with these phosphorylated tyrosine residues and activates downstream effectors, which ultimately leads to enhanced protein synthesis, cell proliferation, survival and motility [64]. Trastuzumab can inhibit the PI3K pathway.

3.4.2 The resistance to trastuzumab

Despite the fact that trastuzumab-based treatment strategy has established a milestone in the therapy of HER-2 positive breast cancer with attractive clinical benefits, either as a single agent or in combination settings, one of the major drawbacks of the trastuzumab-therappy is trastuzumab resistance, even in highly selected HER-2 overexpressed patients. In fact, only about 30% of HER-2 positive metastatic breast cancer patients respond to trastuzumab and approximately 70% of patients with overexpressed HER-2 receptor may have primary resistance [65]. Additionally, the majority of those patients who achieve initial efficacy tend to develop secondary trastuzumab resistance within one or two years [66].

Several mechanisms have been postulated in an attempt to explain both intrinsic and acquired resistance to trastuzumab but it is not completely understood.

a) Cleavage of HER-2 extracellular domain to form the truncated HER-2 receptor and the overexpression of membrane associated mucin MUC4 to mask or block the trastuzumab binding site, which can interrupt the interaction between HER-2 receptor and this antibody [67-70].

b) Although trastuzumab reduces HER2-mediated signaling, it might not reduce signaling mediated through other HER receptors [47]. For example, heterodimerisation of HER2 with other receptors of the erbB2-family can be induced by ligands of HER1, HER3 and HER4, and in the presence of an excess of ligands, the resulting heterodimers may initiate mitogenic signaling even in the presence of trastuzumab [47]. Indeed, increased levels of the ErbB family ligands EGF and heregulin blocked trastuzumab-mediated growth inhibition of HER2-overexpresssing breast cancer cell lines, and this inhibition was associated with increased signaling from HER2/HER1(EGFR) and HER2/HER3 complexes [71,72]. Valabrega et al. compared tumor tissue from patients with advanced HER2-positive breast cancer before and after trastuzumab treatment, and observed a strong increase in TGF-α production upon disease progression, suggesting a possible role

of EGFR signaling by TGF-α as mediator of acquired resistance to trastuzumab. Indeed, these authors found that trastuzumab was less efficient at inhibiting the growth of HER2-positive SK-BR-3 cells engineered to overexpress Tumor Growth Factor alpha (TGF-α compared to the parental cells [73].

c) bypass signalling through the non-EGFR family growth factor receptor insulin-like growth factor-1 receptor (IGF-1R), enables activation of the downstream signal cascades without the participation of HER-2. Therefore, the further understanding of trastuzumab action and resistance mechanisms highlights the need of novel targeted drugs aiming at HER-2 overexpression [74,75].

4. Cancer and Bioactive compounds

Recently, attention has been focused on identifying bioactive compounds able to inhibit the process of carcinogenesis.

The interest of the medicine on natural compound rises at the time of Hippocrates, the father of modern medicine, who said two and a half thousand years ago, “Let food be the medicine and medicine be the food.” Galen of Pegamon (129–199 A.D.), a Greek physician and follower of Hippocrates’ teachings was said to have prescribed various foods, including peeled barley, milk, and various vegetables for the treatment of cancer [76].

Approximately 35 years ago, the National Cancer Institute (NCI) initiated a Diet and Cancer Program to elucidate the role of food and its nutrients in cancer prevention and therapy. These studies were among the first to show that consumption of fruits, vegetables, and whole grains are associated with a decreased risk of many types of cancers [77]. Subsequently, numerous case-control studies were conducted which yielded several potential dietary constituents as possible chemopreventive and therapeutic agents. In another study, the National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity [78]. Of the 92 anticancer drugs commercially available prior to 1983 in the US and among worldwide approved anticancer drugs between 1983 and 1994, 60% are of natural origin [78]. Natural products discovered from medicinal plants have played an important role in the treatment of cancer. Natural products or their derivatives comprised 14 of the top 35 drugs in 2000 based on worldwide sales [79]. Two plant derived natural products, paclitaxel and camptothecin were estimated to account for nearly one-third of the global anticancer market or about $3 billion of $9 billion in total annually in 2002 [80].

The beneficial effects of fruits and vegetables have been attributed to, among other things, the high content of bioactive compounds that are non-nutrient constituents commonly present in food [81].

Among bioactive compounds that showed an interesting therapeutic activity

against cancer stand out curcumin and resveratrol that were the subject of our research [82-97].

4.1 Cucumin

4.1.1 Origin of curcumin and its analogues

Curcumin (Curc) is the yellow spice derived from the roots, rhizome, of the plant *Curcuma longa*. It is native of India and Southeast Asia and it has been used to treat a broad range of ailments in Ayurvedic medicine for at least 4000 years. The powdered extracts of dried roots, often called turmeric, may contain volatile and nonvolatile oils, proteins, fat, minerals, carbohydrates, moisture and curcuminoids. As shown in **Fig. 9** the curcuminoids constitute approximately 5% of most turmeric preparations and they are a mixture of three principal compounds: curcumin (sometimes referred to as curcumin I), demethoxycurcumin (curcumin II) and bisdemethoxycurcumin (curcumin III). The majority of commercially available curcumin contains the following composition: curcumin I (77%), curcumin II (17%) and curcumin III (3%) [98] (**Fig. 10**).

Curcumin was first isolated in 1815 by Vogel [99] and its chemical structure was confirmed by Lampe and Milobedezka in 1910 [100]. It is an oil-soluble coloring compound, readily soluble in alkali, ketone, acetic acid, and chloroform, while insoluble in water at acid or neutral pH.

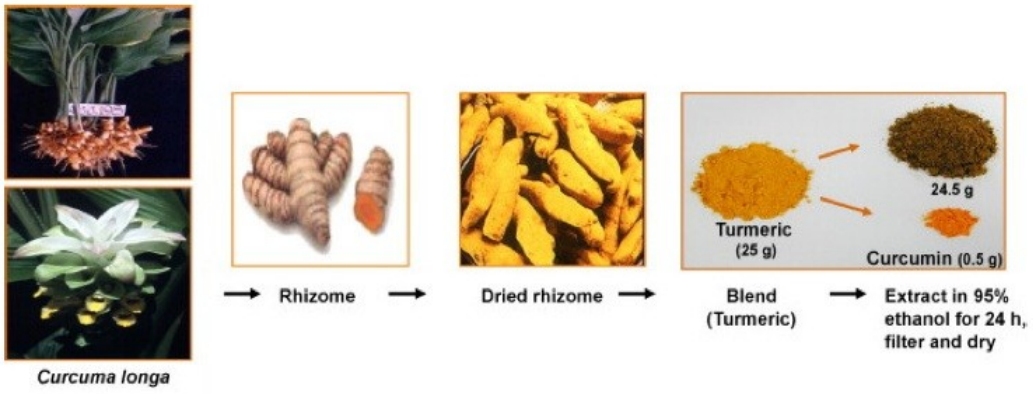


Figure 9 The extraction of curcumin

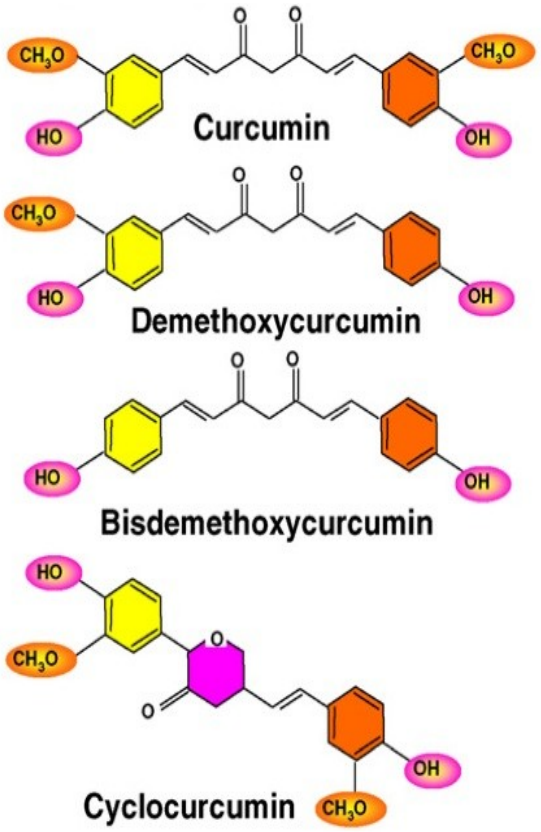


Figure 10 Structures of curcumin and curcuminoids

4.1.2 Molecular targets of curcumin

As shown in **Fig. 11** many molecular targets have been proposed for curcumin. It modulates many regulatory proteins including transcription factors, enzymes, cytokines, adhesion molecules, grow factors and this suggests that curcimin is able to act upon a wide number of biochemical and molecular cascades.

Curcumin shows anti-cancer activity both *in vitro* and *in vivo* through a wide variety of mechanisms. It acts over different cancer cell lines *in vitro*, such as breast, lung, prostate, pancreas, bladder, ovary, kidney, cervix, brain, bone marrow and skin cancer [101]. It has also been shown to potentiate the effect of chemotherapeutic agents [102-105] and that of gamma-radiation [106] in cell culture.

The most accepted curcumin activities are described below and resumed in **Fig 11**:

- Curcumin has been responsible for the induction of apoptosis, due to the production of reactive oxygen species (ROS) that are known as mediators of intracellular signaling pathways. Excessive production of ROS leads to oxidative stress, loss of cell function, and ultimately apoptosis [107]. ROS production leads to depolarization of the mitochondrial membrane and releases pro-apoptotic molecules from mitochondria into the cytosol, which may act to induce apoptosis [108]. In addition, cytochrome-c release from the mitochondrial membrane results in an increased level of cytochrome c at the cytoplasm and nucleus, which induces apoptosis through mitochondrial permeability transition [109]. ROS generation by curcumin also releases apoptosis inducing factor (AIF) and endonuclease G (EndoG) into the cytosol and nucleus where they induce chromatin condensation and DNA fragmentation [110]. The curcumin mechanism of ROS-triggered cell death also involves the p53 tumor suppressor gene. Many reports suggested that p53 is a potential mediator for ROS-dependent apoptosis [111]. The p53 is a tumor suppressor protein, which not only plays

a central role in the cellular stress response pathways but also promotes apoptosis through a variety of mechanisms. The p53 is involved in cell signal transduction, cellular response to DNA damage, genomic stability, cell cycle control, and apoptosis.

- Moreover, curcumin has the ability to protect lipids, hemoglobin, and DNA against oxidative degradation.
- Several studies demonstrated that curcumin also inhibits cyclooxygenase (COX) activity in rat peritoneal neutrophils and human platelets [112]. It is believed that over-expression of COX-2 is related with a wide variety of diseases including colon, lung, and breast cancers. COX is a key enzyme responsible for the conversion of arachidonic acid to prostaglandins and thromboxanes. COX-2 is the inducible form of COX, which is over-expressed at inflammatory sites, and research evidence has indicated the critical role of COX-2 in tumor promotion and carcinogenesis. Furthermore, curcumin inhibits the metabolism of arachidonic acid to prostaglandin D2 in mouse epidermis [113]. Curcumin has the ability to inhibit COX-2, but not COX-1 gene expression in colon carcinogenesis [114].
- Curcumin showed also inhibitory effects on nuclear factor-kappa β (NF-κβ), mitogen-activated protein kinases (MAPK), and several cytokines, and was found to reduce the expression of TNF-α-induced Interleukin (IL)-1b, IL-6, IL-8, and TNF-α itself [115,116]. MAPK play a key role in inflammatory stimuli and environmental stresses. Three sub-groups of MAPK cascades (ERK, JNK, p38) have been identified in mammalian cells. The ability of curcumin to regulate the MAPKs signaling pathway might contribute to the suppression of inflammation in many cancer cell lines.
- Curcumin also modulates MMPs; they are members of zinc-dependent endopeptidases, which are over-expressed in tumor infiltration. MMP2 and MMP9 are often involved in the tumor angiogenesis, mainly through their degradation of extracellular

matrix [117]. It has been reported that curcumin reduces the invasion and metastasis of cancer cells, suppressing the MMPs expression and inhibiting the TPA-induced activation of ERK and transcriptional activation of NF-kB in human breast cancer epithelial cells [118]. NF-κβ is a transcription factor that widely acts as a regulator of genes that control cell proliferation and cell survival. The NF-κβ proteins exist in the cytoplasm in an inactive state, but translocates to the nucleus by the activation of various kinases, phosphorylation, and degradation of the I-κβ, the NF--κβ cytoplasmic inhibitor [119]. It was observed that NF-kB promotes or inhibits apoptosis depending on the phenotype, but activation of the NF-κβ is reported to protect cells against apoptotic stimuli in the majority of tumor cell lines via the initiation of cell survival genes [120]. Curcumin inhibited the I-κβ kinase (IKK) signaling complex responsible for the phosphorylation of IkB, thereby blocking improper activation of NF-κβ and induce apoptosis [121]. Bcl-2 and Bcl-XL are anti-apoptotic proteins that are regulated by NF-κβ. Several studies have shown that curcumin efficiently induces apoptosis in various cancer cell lines through the suppression of these anti-apoptotic proteins [122].

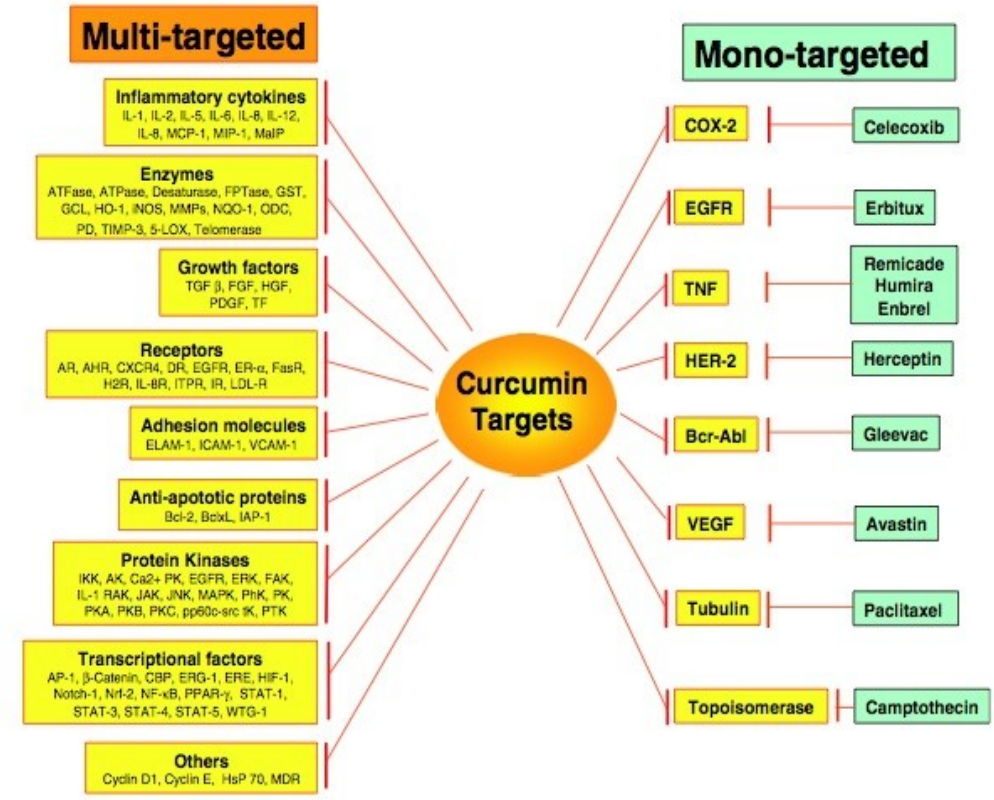


Figure 11 Multiple molecular targerts of curcumin [105]

4.2 Resveratrol

4.2.1 Origin and chemistry

Resveratrol (RESV) (3,5,49-trihydroxystilbene) was first discovered by Michio Takaoka more than 60 years ago, in the resin of *Veratum grandiflorum* (false hellebore). Then, RESV was also detected in grapevines (*Vitis vinifera*) in 1977 by Langcake, who found that the compound was synthesized by leaf tissues in response to fungal infection or exposure to ultraviolet light. This property classifies it as a phytoalexin, compounds produced by plants in

response to damage, stress or infection [123]. RESV is a polyphenol and a member of the stilbene family. It can be found in the *cis*- or *trans*-configuration and in a glycosylated form [123]. It has been found in many plants, including grapes, peanuts, berries, *Polygonum* roots, and other traditional oriental medicine plants [124]. RESV has been reported to be a phytoestrogen due to its structural similarity to the estrogenic agent diethylstilbestrol (**Fig. 12**).

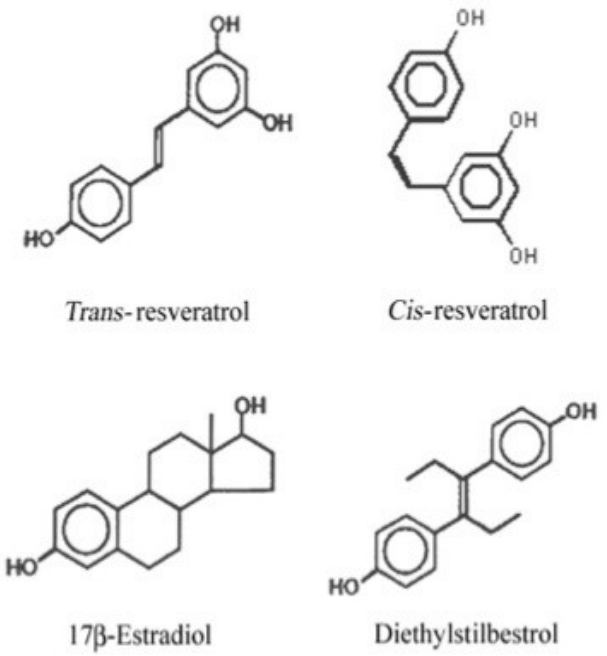


Figure 12 Chemical structure of *cis*- and *trans*-resveratrol, diethylstilbestrol (synthetic estrogen), and 17ß-estradiol

4.2.2 Biological activity

In recent years, research on RESV has described several beneficial effects of this compound on human health. It has been reported to have both anticarcinogenic and cardioprotective activities, which could be attributed to its antioxidant and anticoagulant properties [125]. RESV has been reported

to be effective in inhibiting platelet aggregation and lipid peroxidation, altering eicosanoid synthesis, modulating lipoprotein metabolism [126,127], and exhibiting vaso-relaxing and anti-inflammatory activities [128,129]. For its anticarcinogenic activities, potential mechanisms of RESV action have been studied extensively, though there is no clear consensus on the matter. RESV has been reported to inhibit the three major stages of carcinogenesis: initiation, promotion, and progression (**Fig13**).

Anti-initiation activity was related to its antioxidant and antimutagenic effects and induction of phase II drug-metabolism enzymes.

Anti-promotion activity was indicated by anti-inflammatory effects and inhibition in vitro of cyclooxygenase and hydroperoxidase.

Anti-progression activity was related to its capability to induce human promyelocytic leukemia cell differentiation. It also inhibits the development of preneoplastic lesion in carcinogen-treated mouse mammary glands in culture and inhibits tumorogenesis in a mouse skin cancer model [130].

Many *in vitro* studies have addressed the RESV activities in breast cancer cells. RESV exhibits its action in both hormone-sensitive and hormone-resistant breast cancer cells. It has also been reported to exhibit anti-initiation, anti-promotion, and anti-progression activities in breast cancer cells, where these properties seem to be related to regulation of xenobiotic carcinogen metabolism and anti-inflammatory, antiproliferative, and proapoptotic effects [130-132].

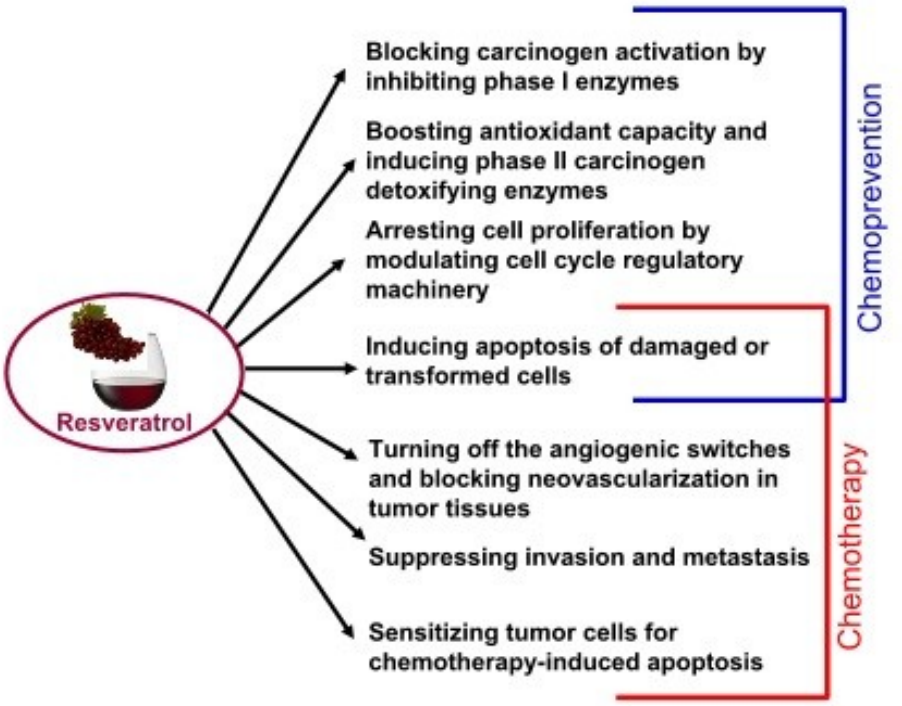


Figure 13 Biochemicals mechanisms responsible for chemopreventive and chemotherapeutic potential of resveratrol

4.3 Limits in the use of bioactive natural compounds

Although, curcumin and resveratrol have shown significant efficacy in cell culture studies, they elicited limited efficacy in various clinical studies. Their introduction into the clinical setting is hindered largely by their poor absorption, rapid metabolism, or a combination of both, ultimately resulting in poor bioavailability upon oral administration [133,134].

Therefore, to circumvent these limitations and to ease their transition to clinics, alternate strategies should be explored. Drug delivery systems such as nanoparticles, liposomes, microemulsions, and polymeric implantable devices are emerging as one of the viable alternatives that have been shown to deliver therapeutic concentrations of various potent chemopreventives such as curcumin, resveratrol and other bioactive compound into the systemic circulation.

5. Drug Delivery Systems

5.1 Features and advantages of DDSs

Drug delivery systems (DDSs) can improve the pharmacological properties of traditional drugs by altering drug pharmacokinetics and biodistribution [135,136]. DDS can include liposomes, nanospheres, micelles, dendrimers, as well as various polymeric-based systems [137-139]. Among the many DDS available, liposomes became very popular (gained popularity) in recent years thanks to their clinical success.

Due to their small size (about 100 nm or less), they readily extravasate from circulation through vascular gaps or defects attributed to ongoing angiogenesis that is typical of tumour sites [140], which have been reported to be about 200 nm or greater [141]. DDS retention within these sites is generally high due to the poor lymphatic drainage observed within tumors [142,143]. Furthermore, their lower size limit of diameter ensures that these vehicles do not randomly penetrate normal vessel walls. In cancers, an imbalance in factors that regulate angiogenesis, such as overexpression of , results in both increased vascular permeability and chaotic tumour-vessel architecture. In combination, these effects cause enhanced permeation and retention (EPR), resulting in high local drug concentrations.

5.2 Liposomes

Liposomes are spherical, self-closed structures formed by one or more concentric lipid bilayers containing an aqueous phase inside and between the bilayers [144] (**Fig. 14**). The lipids used in the formation of liposomes are usually comprised of a hydrophilic headgroup and two hydrophobic fatty acyl chains. These amphiphilic molecules spontaneously assemble into aggregates in an aqueous environment. Water-soluble molecules occupy the aqueous compartment, whereas molecules of a more lipophilic character occupy the lipid bilayers. Liposomes can vary substantially in size and lamellarity.

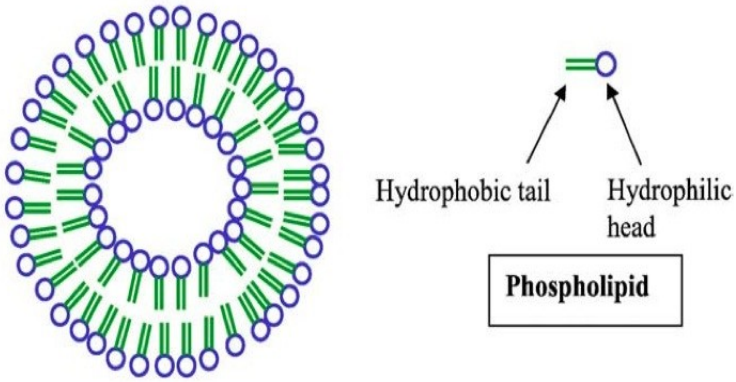


Figure 14 Schematic illustration of a liposome

They are subdivided into multilamellar vesicles (MLVs) consisting of several concentric bilayers, large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) (**Fig. 15**).

Liposomes can be formed by a variety of methods. When they are prepared by hydration of the dried lipid mixture, they spontaneously form MLVs. Other procedures, such as prolonged exposure to ultrasound or pressure-driven filtration through small-pore filters, cause MLVs to form SUVs or LUVs.

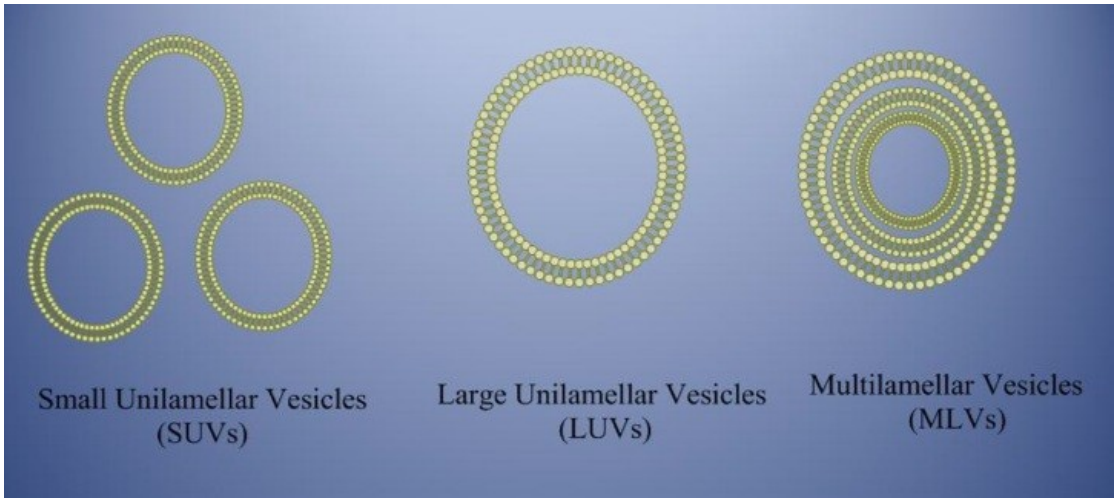


Figure 15 Classification of liposomes

The clinical success of liposomes has also made them very popular drug carriers for various chemotherapeutics. For example, the clinically approved drugs DaunoXome and Doxil are liposomal formulations that encapsulate the commonly used chemotherapeutic agents daunorubicin and doxorubicin respectively.

The pharmacokinetics of liposomes depend on their physiochemical characteristics such as size, surface charge, membrane lipid packing, steric stabilization, dose and route of administration [145]. In general, larger liposomes are eliminated from the blood circulation more rapidly than smaller ones [146]. Binding of opsonins to liposomes depends on liposome size; consequently, the reticuloendothelial (RES) uptake of liposomes by the liver is size-dependent [147]. The action of the reticuloendothelial system results in rapid removal from the blood and accumulation in tissues such as liver and spleen. In general, for a given liposome composition, the larger the liposome, the faster the clearance by the RES [148–150].

Optimal liposome size depends on the tumor target. In tumor tissue, the vasculature is discontinuous, and pore sizes vary from 100 to 780 nm. By

comparison, normal vascular endothelium is < 2 nm in most tissues, 6 nm in postcapillary venules, 40–60 nm for the kidney glomerulus, and up to 150 nm for sinusoidal epithelium of the liver and spleen. Most liposomes are 100 nm [151].

Negatively charged liposomes were believed to be more rapidly removed from circulation than neutral or positively charged liposomes; later studies have indicated that the type of negatively charged lipid affects the rate of liposome uptake by the RES. For example, liposomes containing negatively charged lipids that are not sterically shielded (phosphatidylserine, phosphatidic acid, and phosphatidylglycerol) are cleared more rapidly than neutral liposomes of similar composition. However, liposomes containing sterically shielded lipids (for example ganglioside-GM1 and phosphatidylinositol) are cleared even more slowly than neutral liposomes [152].

Thus, one way to reduce liposomal uptake by the RES is by creating sterically stabilized liposomes. “Steric stabilization” refers to the colloidal stability resulting from attachment of hydrophilic polymers or glycolipids on the liposomes (**Fig. 16**). The best-studied stabilizers are polyethylene glycol and ganglioside GM1. Sterically stabilized liposomes (Stealth) showed prolonged lifetimes in the circulation as compared with nonstabilized liposomes [153–157]. Sterically stabilized liposomes are also less reactive toward serum proteins and less susceptible to RES uptake than nonstabilized liposomes [153]. The mechanism by which sterically stabilized liposomes are thought to decrease RES-mediated uptake is that the stabilizer occupies the space immediately adjacent to the liposomal surface, excluding other macromolecules from this space. Consequently, access to and binding of blood plasma opsonins to the liposome surface are hindered, preventing interactions with RES macrophages. However, although sterically stabilized liposomes prolong circulation time and decrease liposomal uptake by the RES, they do not actively target the liposome to the tumor.

Polyethylene glycol (PEG) is the most widely used polymeric steric stabilizer. PEG is a linear polyether diol with many useful properties. It is highly soluble

in aqueous and organic media and possesses very low immunogenicity and antigenicity [158] and is non-toxic. It can be attached to the liposome surface in various ways, but the most widely used method is to anchor the polymer in the liposome membrane via a cross-linked lipid (for example PEG-DSPE) [19]. It was shown that steric stabilisation of liposomes with PEG increases their longevity in the circulation [160]. A supplementary method to ensure long circulation times involves adding cholesterol (Chol) to the lipid bilayer. Cholesterol acts as a spacer between phospholipids of the liposome membrane because of its inflexible structure, preventing demixing of the lipids and reduces PEG chain-chain interactions. This results in improved steric stabilization of the liposomes.

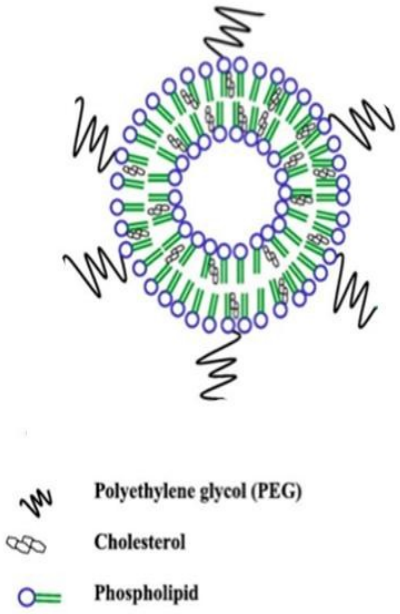


Figure 16 Sterically stabilized PEGylated liposome

5.3 Immunoliposomes

5.3.1 Definition and advantages

A large variety of therapeutically active molecules (e.g. antitumor drugs, oligonucleotides, DNA, enzymes, peptides and hormones) have been successfully incorporated in liposomes. Especially in the field of cancer chemotherapy, much effort has been invested to successfully achieve site-specific drug delivery with liposomal systems. Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells [161].

Several types of ligands have been used for this purpose, including antibodies, antibody fragments [159,162-164], vitamins [165], glycoproteins [166,167], peptides (RGD-sequences) [168,169], and oligonucleotide aptamers [170].

The first report on antibody-targeted liposomes came from Torchilin et al. about two decades ago [171]. These antibody-targeted liposomes (further referred to as immunoliposomes) were shown to be able to specifically bind to the antigen that is expressed on the target cells. Targeted delivery systems of this type have two basic advantages: because the drug is released at the tumor site instead of circulating widely through the body, it should be more effective for a given dosage; it should also have fewer harmful side effects because smaller amounts of the drug come into contact with healthy tissue. Several coupling techniques have been described for conjugating antibodies or their fragments to liposomes, each with their own advantages and drawbacks [163,172,173]. Many *in vitro* experiments have demonstrated highly specific binding of immunoliposomes to target cells. However, despite the excellent targeting properties *in vitro*, successful results on targeting of immunoliposomes in tumor models are scarce up to now.

5.3.2 Clearance of immunoliposomes from the circulation

The most important barrier limiting the usefulness of immunoliposomes for targeted drug delivery has been the rapid recognition and removal from the blood by cells of the mononuclear phagocyte system (MPS), particularly the macrophages in liver and spleen [174,175]. In addition, the presence of antibodies conjugated to the liposomal surface makes immunoliposomes highly susceptible to Fc-receptor-mediated phagocytosis and, as a result, even more prone to rapid clearance [174,176]. The Fc-receptor family, which is expressed by different cells of the MPS, binds the constant region (Fc) of antibodies resulting in internalization of antibody-opsonized complexes [177]. Similarly, immunoliposomes bearing whole antibodies are cleared rapidly due to exposed Fc parts [174,176,178].

The advent of so-called long-circulating liposomes produced by coating the liposome surface with the polymer polyethylene glycol, has revived interest in targeted drug delivery [179,180]. As previous mentioned, PEG-coating sterically stabilizes the liposomal membrane by decreasing interactions with destabilizing and opsonic factors *in vivo*. As a consequence, PEG-coated liposomes show longer circulation times and reduced uptake by the MPS.

There are different methods available for coupling antibodies to PEG-liposomes [181]. For attaching antibodies to the surface of PEG-liposomes, two main strategies have been followed: those in which the ligand is coupled directly to the liposome bilayer (**Figure 17 A** and **B**) and those in which the ligand is attached to the terminal end of PEG (**Figure 17 C**) [159,164,182]. The latter strategy yields protein present at the surface of the PEG coating. Indeed, it has been shown that the clearance rate of PEG-immunoliposomes is dependent on the antibody density at the liposome surface [159,164,183].

At low antibody density (below 50 µg mAb/µmol PL), the PEG-immunoliposomes are cleared at rates only slightly more rapid than those few minutes [159]. Likely, clearance is mediated by the exposed Fc-region of the whole antibodies conjugated to the PEG-terminal ends.

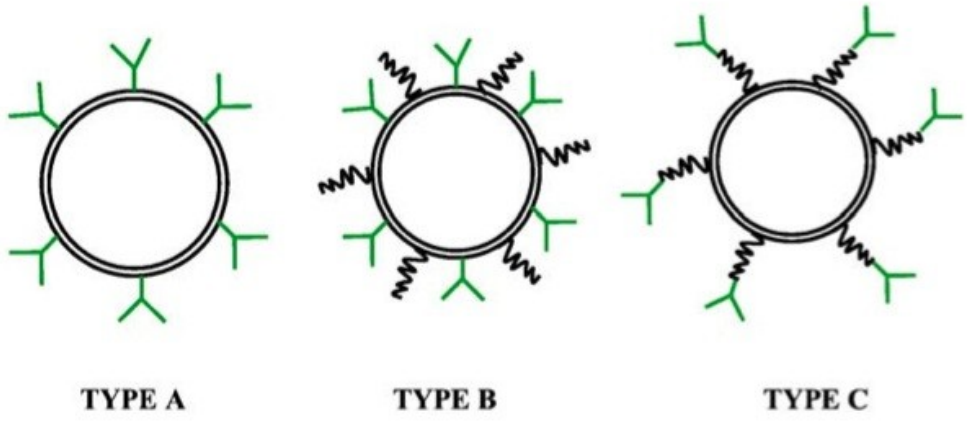


Figure 17 Illustration of antibodies' conjugation to liposomes
Type A: 'PEG-free' immunoliposome with antibody directly linked to the lipid; Type B: PEG-immunoliposome with antibody directly linked to the lipid; Type C: PEG- immunoliposomes with antibody conjugated to the distal end of the PEG chain.

5.3.3 Tumor cell binding: the importance to choice a target epitope

For the successful delivery of antineoplastic drugs by targeted liposomes, it is required that the drugs are delivered to every malignant cell. Therefore, liposomes should be targeted to surface molecules that are present on tumour target cells and, most importantly, that are not expressed at similar levels by normal cells. Since tumour cells are known for their heterogeneity with respect to phenotype, expression of the target epitope on all malignant cells is very unlikely. Targeted drug delivery will mainly affect those cells expressing the target epitopes at high densities whereas target epitope-negative cells or those with low-density expression are likely to become less susceptible to the treatment. For our research we have chosen HER2 receptor as target, which, as previously mentioned, is expressed in about 20-30% of breast cancer cells, while its expression is only at low level in normal tissues [44,45].

5.4 Therapeutic availability

After tumour cell binding, the encapsulated drug should become therapeutically available. In principle, the delivery of encapsulated compounds to tumor cells can take place via four different mechanisms:

- release of encapsulated compounds from cell surface-bound immunoliposomes with subsequent uptake of free drug by the tumor cells [184],
- transfer of lipophilic drugs from the immunoliposomal bilayers to the plasma membrane of tumor cells [185]
- endocytosis of cell-surface receptor-bound immunoliposomes with subsequent intracellular release of encapsulated compounds [6or fusion of the immunoliposomal membrane with the target cell-membrane or endosomal membrane [187].

The first mechanism aims for extracellular release of liposome-encapsulated compounds, whereas in the latter three mechanisms, the drug will be released onto or inside the cell. Intracellular release of antitumor drug has as main advantage that it may overcome multidrug resistance, which is a common mechanism for cancer cell adaptation or survival [188-190]. In general, tumor cells circulating in the bloodstream require intracellular delivery as extracellular delivery will result in fast diffusion and redistribution of the drug over the blood compartment [191]. In case of solid tumors, the extracellular release of drug at the tumor site seems preferable as this may lead to diffusion of drug within the tumor mass allowing the drug to reach also those tumor cells that do not express the targeted antigens or that are out of reach for the relatively large immunoliposome carriers. This effect may also occur after intracellular delivery of certain drugs which have physico-chemical characteristics that promote the leakage or active transport of a fraction of these drugs out of the target cells

Research’s Aim

Research's Aim

Curcumin and resveratrol are two of the most studied bioactive dietary compounds. They have therapeutic activities for a large spectrum of the most common diseases, including cancer, since they modulate multiple cellular pathways, but they show severe limitations in their bioavailability due to their hydrophobic character that prevent their success in chemotherapy. Most common problems are poor absorption, low half-life, due to rapid clearance and inactivation by metabolic enzymes. The development of suitable drug delivery systems and, in particular, of liposomes, may be an appropriate strategy for the effective administration and of these compounds.

Liposomes increase drugs' half life and reduce their clearance, but they don't discriminate among the different cellular types, then this is a factor limiting their effectiveness and suitability to be used in cancer therapy.

For this reason, in the last decade the research against cancer is oriented towards 'targeted therapy', which is based on the possibility to deliver drugs to specific tumor sites, narrowing the field of action compared to conventional chemotherapy and decreasing side effects. The specific aims were:

1. To develop liposomal formulations of curcumin, resveratrol, or curcumin+resveratrol in order to improve their bioavalability and their cytotoxic effects on human breast cancer cellular models.
2. To design and optimize immunoliposomes containing the anti-HER2 antibody to target breast cancer cells expressing that surface antigen;
3. To evaluate the cytotoxic effects of the compounds in three different systems: free compounds, compounds incorporated into regular liposomes and incorporated into anti-HER2 immunoliposomes on two breast cancer cell lines showing different HER2 expression levels;
4. To demonstrate the specificity and selectivity of the immunoliposomes' systems in the cytotoxicity studies compared to regular liposomes or free compounds;
5. To evaluate the cellular uptake of the compound and the liposome cell binding in the optimum system by a novel image-based flow cytometry approach.

Materials and Methods

1. CELL CULTURES

1.1 Cell lines

Two breast cancer cell lines, JIMT1 and MCF7, have been used for this study.

JIMT1 cell line was derived from a patient diagnosed with breast cancer at the age of 62 years. The tumor was a grade 3 invasive ductal breast cancer. It's a relatively novel cell line, commercially available since 2004. As a cell line, JIMT1 carry phenotypic hallmarks of HER-2-positive breast cancer that is, histologically, representing a high-grade invasive ductal carcinoma lacking expression of estrogen and progesterone receptors [192]. Although it is a HER2 positive cell line, it is resistant to the conventional therapy with trastuzumab [193].

MCF7 is one of the most used cell lines to study breast cancer. It is a hormone responsive breast cancer cell line with differential sensitivities to estrogens and anti-estrogens, positive expression of estrogen receptor, progesterone receptor and differences in tumorigenicity and proliferation rates with JIMT1 [194], but the main difference between both cell lines is that, differently to JIMT1, MCF7 cells present a very low HER2 expression.

Both cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) with Glutamax (GIBCO), supplemented with 50 units/ml penicillin, 50mg/ml streptomycin (GIBCO), and 10% of heat-inactivated fetal bovine serum (FBS) (GIBCO). The cell were maintained in monolayer in T25 or T75 flasks (SARSTEDT, Spain), and incubated at 37°C in an atmosphere of 95% air and 5% CO2. All cell work was carried out in a tissue culture hood (equipped with a laminar flow cabin), using only sterile equipment in direct contact with the cells.

1.1.1. Thawing of JIMT1 and MCF7 cells

Cells were kept in liquid nitrogen in cryotubes. Tubes were removed from the liquid nitrogen tank and put into a 37°C water bath for about 30-60 sec. The cells were transferred to a 15 mL tube using 5 mL pre-heated DMEM and centrifuged at 1500 rpm for 5 min. Media was removed by aspiration and the cell pellet was “flipped” and resuspended in 5 mL DMEM before transferred to a T25 flask.

1.1.2 Passaging of JIMT1 and MCF7 cells

Cells were passaged every 3rd or 4th day, when they had reached 100% confluence. Media was removed by aspiration using sterile Pasteur pipette and a vacuum line. 5 mL phosphate buffered saline (PBS) was added twice to the opposite side of where the cells were attached. The flask was gently rocked about 5 times to wash away media residues from the cells. PBS was removed by aspiration. 1 mL Trypsin/EDTA (Ethylenediaminetetraacetic acid) (GIBCO) (in case of T25 flask use), or 2 mL Trypsin/EDTA (in case of T75 flask use) was added directly to the cells and the flask was incubated for 5 min in 37°C, 5% CO2. Cells were detached by gently tapping the flask. To neutralize trypsin's effect an amount of DMEM five times more of total trypsin volume was added to the flask; that is 5 mL of DMEM for 1 mL of trypsin/EDTA or 10 mL of DMEM for 2 mL of trypsin/EDTA. Cells were suspended in the media, transferred to a 15 mL tube and centrifuged at 1500 rpm for 5 min. Supernatant was removed by aspiration and the cell pellet was “flipped” to separate the cells. The cells were resuspended in 10 mL DMEM and passaged into T75 flasks containing 5 mL DMEM yet.

2. MTT Assay

Traditionally, the determination of cell growth is done by counting viable cells after staining with a vital dye. One of this approaches is trypan blue staining, which is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death), but the method is not sensitive and cannot be adapted for high-throughput screening. Measuring the uptake of radioactive substances, usually tritium-labelled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances.

The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method is simple, accurate and yields reproducible results. The key component of this method is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTT. Solutions of MTT, dissolved in medium or balanced salt solutions without phenol red, are yellowish in colour. Yellow MTT enters the cells and passes into the mitochondria where mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding dark purple formazan crystals which are insoluble in aqueous solutions (**Fig.18**).

The crystals can be dissolved in a organic solvent such as Dimethylsulfoxide (DMSO) or isopropanol and the released, solubilised formazan reagent is spectrophotometrically measured at 570 nm. The assumed advantage of this cell assay system is that this reaction can only take place in living cells with functional mitochondria. It is also assumed that the amount of formazan formed during a given exposure period is directly proportional
in the number of viable cells per well.

When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance.

The MTT method is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors, cytotoxicity studies, so it's

possible derivate cell growth curves.

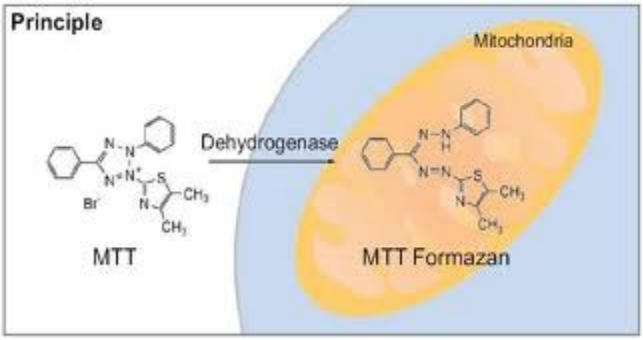


Figure 18 Cleavage of the yellow-colored tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) into a purple-colored formazan by the mitochondrial enzyme succinate-dehydrogenase

2.1 Experimental procedure

Cell suspensions were seeded into 96-well microplates. They were left one day in order to reach a confluence of 80%. Then medium were aspirated and the plates were incubated at 37°C for 72 hours with different drugs' concentrations resuspended in the medium, wells with only medium served as blanks.

After 3 days, medium and consequently drugs were aspirated and MTT was added (100 μL of a solution of 5%MTT in DMEM). After 3-5 hours of incubation in 37°C in dark, MTT was removed by aspiration, plates were washed with PBS and 100 μL of DMSO were added in each well; to allow the solubilisation of formazan's crystal, plates were mixing on an agitator for 10 minutes and cellular viability was quantified using an absorbance spectrophotometer microplate reader (SPECTROstar Omega, BMG LABTECH) measuring absorbance at 570nm with background correction at 620nm.

The absorbance measured was proportional to the number of living cells in each well.

The cytotoxic IC_{50} values (inhibitory concentration 50%) for the drugs were determined from log concentration-effect curves in GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA), using nonlinear regression analysis. Data are presented as the means ± standard error of the mean.

3. Protein extraction and analysis

3.1 Preparation of cell lysates

Protein extracts were made from cells when approximately 80% confluent. Cells were washed twice with PBS and were scraped with a cell scraper, the lysate were transferred to a 1.5 ml tube and centrifuged at 1500 rpm for 5 min. The cellular pellets were lysed in a lysis buffer containing 50 mM Tris pH 7.4, 1% Igepal CA-630, 150 mM NaCl, 5 mM EDTA and 10 mg/mL of protease inhibitor cocktail (Sigma–Aldrich, Europe).
Cells were kept on ice for 20 min and, after a freezing/thawing cycle, lysates were centrifuged at 12,000 rpm for 5 min to remove particulate matter and cleared lysate was placed in a fresh 1.5 ml tube. Samples were then processed immediately, or frozen and stored at -20°C.

3.2 Determination of protein concentration

Protein concentrations were estimated using the Bradford colorimetric assay (Bio-Rad, Richmond, CA, USA). Protein-containing solutions were mixed with Bradford assay solution and the absorbance at 595nm measured. The protein concentration was then determined based on a standard curve derived from protein standards of known concentration.

3.3 Separation of proteins by polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins for Western blot analysis were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The required amount of lysate to give 50 µg protein was mixed with 5 µl sample buffer and 2 µl sample reducing agent, and diluted to a total volume of 30 µl with lysis buffer. Samples were then heated at 95°C for five min, briefly centrifuged to collect the whole sample, and resolved on polyacrylamide gels by electrophoresis in gel tanks

containing 1X running buffer (15 g Tris HCl pH 8.3, 72 g Glycine, 5 g SDS in a final volume of 1 L).

The gels were run at 120V for approximately 1 hr 30min. When the bromophenol blue dye front was seen to have reached the end of the gels, electrophoresis was stopped.

3.4 Western blotting

Following electrophoresis, the acrylamide gel was placed over the nitrocellulose membrane and sheets of pre-soaked filter paper were placed on top of the gel. Excess air was again removed by rolling a universal tube over the filter paper. The proteins were transferred from the gel to a nitrocellulose membrane at a current of 34mA at 15V for 2 hours.

The nitrocellulose membranes were blocked for 2 hours at room temperature with fresh 5% non-fat dried milk in Tris-buffered saline (TBS) with 0.5% Tween (Sigma–Aldrich, Europe). After blocking, the membranes was washed 3 x 5 min using 1X TBS/PBS. The membrane was then incubated with primary monoclonal anti-bodies against HER2 (sc-284, Santa Cruz Biotechnology, INC) for 1hr. The membrane was again was washed 3 x 5 min using 1X TBS/PBS and then incubated with horseradish peroxidase-linked secondary antibodies (Sigma–Aldrich, Europe). Finally the membrane was washed 3 x 5 min using 1X TBS/PBS. Bound antibody was detected using enhanced chemiluminescence (ECL) (Amersham International, Buckinghamshire, UK).

3.5 Enhanced chemiluminescence detection

Protein bands were developed using the Enhanced Chemiluminescence Kit (Amersham International, Buckinghamshire, UK) according to the manufacturer's instructions. The blot was removed to a darkroom for all subsequent manipulations. A sheet of parafilm was flattened over a smooth surface, e.g. a glass plate, making sure all air bubbles were removed. The

membrane was placed on the parafilm, and excess fluid removed. 1.5 mls of ECL detection reagent 1 and 1.5 mls of reagent 2 were mixed and covered over the membrane. Charges on the parafilm ensured the fluid stayed on the membrane. The reagent was removed after one minute and the membrane covered in cling film. The membrane was exposed to autoradiographic film in an autoradiographic cassette for various times, depending on the signal. The autoradiographic film was then developed. Densitometric analyses were performed using the Sigma-Gel gel analysis software (Jandel Scientific, Chicago, IL, USA).

4. LIPOSOMES

4.1 Chemicals

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol) 2000] (D2000M) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Lissamine rhodamine B 1,2-dihexa-decanoyl-sn-glycero-3-phosphoethanolamine (Rhod-PE) was obtained from Invitrogen (Europe). Natural lipid egg yolk phosphatidylcholine (EYPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG-2000 (DSPE-PEG) were obtained from Lipoid GmbH (Ludwigshafen, Germany). All lipids were dissolved in chloroform/methanol (1:1) and stored at -20°C. Herceptin^{TM} (Trastuzumab) was obtained from ROCHE.

4.2 Liposomes preparation: thin film method

Two type of lipid composition were used in the present study, one containing only EYPC for simple liposome preparation and another one used for the immunoliposome preparation containing adequate amounts of each lipid combined in a molar ratio of 74.5:20:5:0.5 (EYPC:Chol:DSPE-PEG:D2000M), previous studies demonstrated that this was the optimal composition [195].
The organic solvent was removed by evaporation under a stream of nitrogen (N_2) and a thin film of lipid was formed inside of the test tubes. The tubes were further vacuum-dried for 3-4 h to remove any residual organic solvent. Subsequently the lipid film was hydrated using Histidine buffer (THIS) (1.2 g/L histidine–HCl, 0.78 g/L histidine, pH 7.4). The resultant liposomal suspension was agitated by vortexing at 37°C and a homogeneous milky suspension of MLVs was obtained.

4.3 Size Reduction of Liposomes

The liposomal suspension was filter-extruded through a 100 nm polycarbonate membrane Track-Etch Nuclepore membrane (Whatman, UK)

in order to obtain large unilamellar vesicles (LUVs). Size reduction was done by 15 extrusion cycles by hand with a syringe extruder Liposofast™ (Avestin Inc., Canada) [196] or by LIPEX™ Extruder (Northern Lipids Inc) for more concentrated preparations and bigger volumes unable to pass by liposofast **Fig. 19**.

The resultant products were stored at 4 °C until use.

A **B**

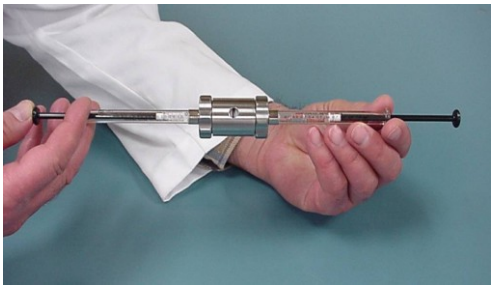


Figure 19 (A) Syringe extruder Liposofast™ (B) LIPEX™ Extruder

4.4 Immunoliposomes preparation: antibody derivatization and conjugation to liposomes

Chemical strategy for this immunoliposome preparation is based on:

1. use of free amino groups present in the antibody molecules;
2. modification of this functional groups by Traut's reagent (derivatization);
3. use of a PEG derivate (D2000M) which acts as a linker between liposomes and antibodies.

Perham and Thomas [197] originally prepared an imidoester compound containing a thiol group, methyl 3-mercaptopropionimidate hydrochloride. The imidoester group can react with amines to form a stable, charged linkage, while leaving a sulfhydryl group available for further coupling. Traut et al. [198] subsequently synthesized an analogous reagent containing one additional carbon, methyl 4-mercaptobutyrimidate. Later, this compound was found to cyclise as a result of the sulfhydryl group reacting with the intrachain imidoester, forming 2-iminothiolane. The cyclic imidothioester still can react with primary amines in a ring-opening reaction that regenerates the free sulfhydryl. Traut's reagent is fully water-soluble and reacts with primary amines in the range of pH 7–10. The cyclic imidothioester is stable to hydrolysis at acid pH values, but its half-life in solution decreases as the pH increases beyond neutrality [199]. **Fig. 20** describes the thiolation of antibody using Traut's reagent in the preparation of immunoliposomes.

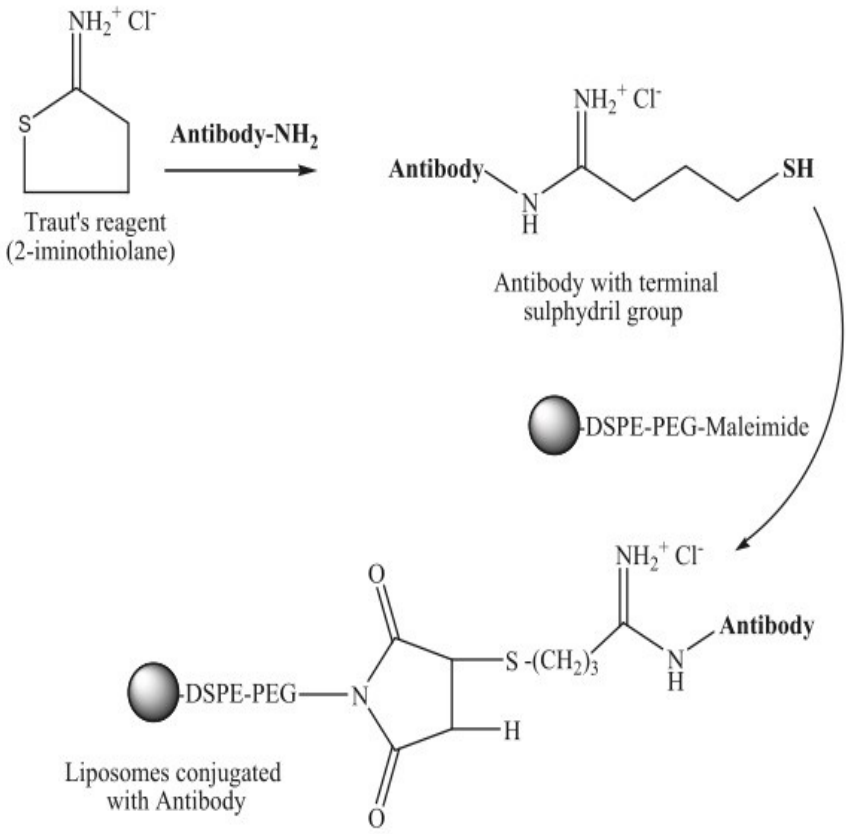


Figure 20 Thiolation of trastuzumab using Traut's reagent and conjugation of thiolated antibody to maleimide groups on liposome

4.4.1 Experimental protocol

Lipids, were combined in a molar ratio of 74.5:20:5:0.5, and liposomes were formed by thin film method as described in the section 4.2; then liposomes' size was reduced in order to obtain LUVs. At the same time, trastuzumab was derivatized as previously described [195-200-201]. Briefly, the antibody was thiolated by reaction with 2-iminothiolane (Traut's reagent) for 2 hours and then incubated with the unilamellar vesicles in an Argon inert atmosphere for 12 h at room temperature with gentle agitation. During the reaction, the thiol group of the derivatized antibody reacted with the maleimide group of the D2000M lipid present in the liposome yielding the

immunoliposome with the antibody covalently attached (the technique is thoroughly reviewed in [202]). After the derivatization, the unbound antibody was separated by size exclusion chromatography and finally the drugs (curcumin and/or resveratrol) in 5% of DMSO, were added and incubated at 37 °C for 1h with gentle agitation to obtain the final immunoliposome suspension. Then the Immunoliposomes were chromatographied again for further purification.

4.5 Separation of no-encapsulated compounds and unbound antibody from liposomes

4.5.1 Separation of no-encapsulated compounds from liposomes by Ultrafiltration

To separate no-encapsulated compounds from liposomes, ultrafiltration was used.

Ultrafiltration (UF) is the process of separating extremely small particles from fluids. Ultrafiltration can only separate molecules which differ by at least an order of magnitude in size.

Normally the membranes for the ultrafiltration are rated according to the nominal molecular weight limit (NMWL) or the molecular weight cut-off (MWCO). The membrane used for the experiments has a MWCO of 30,000; in this case free curcumin with a 368 MW, free resveratrol with 228 MW or both can be easily separated from encapsulated fraction.

In this study, the liposomal preparation was transferred to 15mL cellulose membrane ultrafiltration tubes (Amicon Ultra-15 Centrifugal Filters). The samples were then centrifuged using Heraeus Biofuge PRIMO centrifugator (Heraeus, Europe). The centrifugation was done at speed of 1500 rpm for 15 minutes.

4.5.2 Separation of no-encapsulated compounds and unbound antibody from immunoliposomes by Size Exclusion Chromatography

Immunoliposome's formulation was added to a glass tube packed with Sepharose CL-4B (Sigma– Aldrich) to approximately 20 mL (in a glass column 20 cm height and 2 cm in diameter).

THIS buffer was used for the elution and it was added repeatedly to insure continues flow and prevent the column from drying. The flow rate was estimated to be 2 mL/min. To detect when immunoliposome preparation passed through the column and collected it, the column was connected to an UV detector. Many organic compounds absorb UV light of various

wavelengths; the passage of immonoliposomes and drugs through the detector results in a change of the signal.

In experiments we performed, it was also possible discriminate the immunoliposome preparation and the unbound antibody, obtaining two different peaks, the second one, referring to free antibody, very more less (**Fig. 34** in Results section).

4.6 Quantitative analysis of lipid concentration

Fiske's assay is a quantitative method to determinate phosphorus content and it is used to quantify the phospholipid solutions concentration. First, a standard solution of dipotassium hydrogen orthophosphate (K_2HPO_4) was prepared to a final concentration of 0.387 mM. Aliquots of phosphate stock solution were transferred to six glass tube in order to have a standard curve with different concentrations of phosphorus were 20, 40, 80, 120, 160 and 200 nmoles, respectively (**Fig. 21**).

A volume of 0.4 mL of perchloric acid ($HClO_4$) at 60% was added in the standard tubes and in the samples and incubated at 185°C for 30 minutes. After cooling at room temperature, a volume of 4 mL of ammonium molybdate solution and 0.5 mL of ascorbic acid 10% were added and mixed. Standard tubes and samples were incubate into sand bath at 180-200°C for 15 minutes. Ammonium molybdate reacted with the acid producing a coloured compound that can be measured spectrofometrically at 812 nm quantifying phosphorous content and indirectly lipid concentration.

All the sample's measurements were made in triplicate.

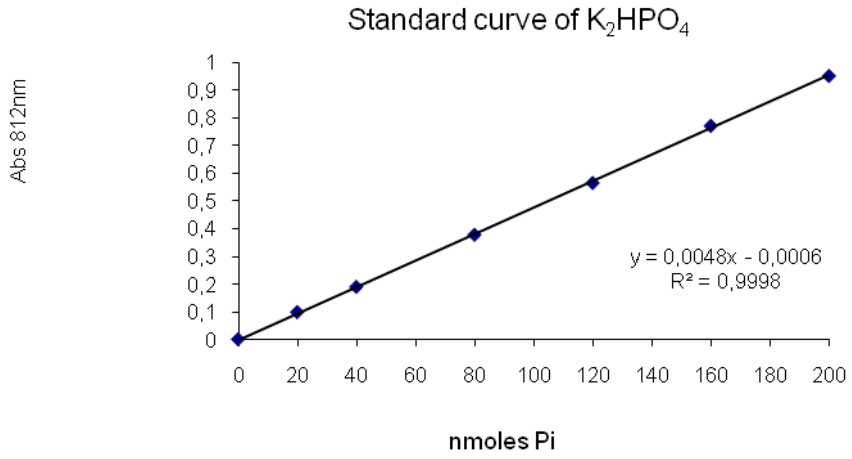


Figure 21 Standard curve of dipotassium hydrogen orthophosphate used for quantification of lipid content

4.7 Size determination of liposomes

Liposomes' s particle size was measured using the technique of dynamic light scattering (DLS) also known as photon correlation spectroscopy (PCS).
The principle behind this technique is that when a light beam hits a particle which is in random motion or ‘Brownian’ motion, it causes a Doppler shift in the wavelength of the incoming light and this shift relates to the size of the particle. In this way, the larger is the particle the slower is the motion, while the smaller particles will move faster.
This technique requires a very short time to measure the particle size.
Zetasizer Nano ZS (Malvern Instruments Ldt, UK) was used to measure the particle size of the liposomes. The particle size was measured using a He/Ne 4mW laser. As sample void liposomes, encapsulated liposomes and immunoliposomes were used.
Results were obtained from three series of measurements in which each measure has at least 12 replies. It was considered as accepted only the results that showed a polydispersity index (PDI) less than 0.2; PDI is a measure of samples’ homogeneity, more homogeneous is the sample, lower is PDI.
The software of the instrument determinated the size of LUVs (Z, diameter in nm) and estimated the wideness of distribution (polyispersity index) in function of the intensity of the light scattered from particles.

5. Quantification of encapsulated compounds by High Performance Liquid Chromatography

5.1 High Performance Liquid Chromatography (HPLC): theory

High performance liquid chromatography (HPLC) is a separation technique that uses differences in distribution of compounds in two phases, called stationary and mobile phase. The stationary phase may be a porous solid, usually in a thin layer of particles or a substance bound to a solid support. The mobile phase designates the liquid (a solvent or a mixture of solvents) flowing over the particles. Under certain dynamic conditions, each component in a sample has a different distribution equilibrium depending on affinity for the different phases and/or molecular size. As a result, the components move at different speeds over the stationary phase and are thereby separated from each other. This is the principle behind affinity chromatography.

A HPLC system is basically composed of 1) a pump, 2) an injector, 3) a column, 4) a detector and 5) data acquisition system (**Fig. 22**).

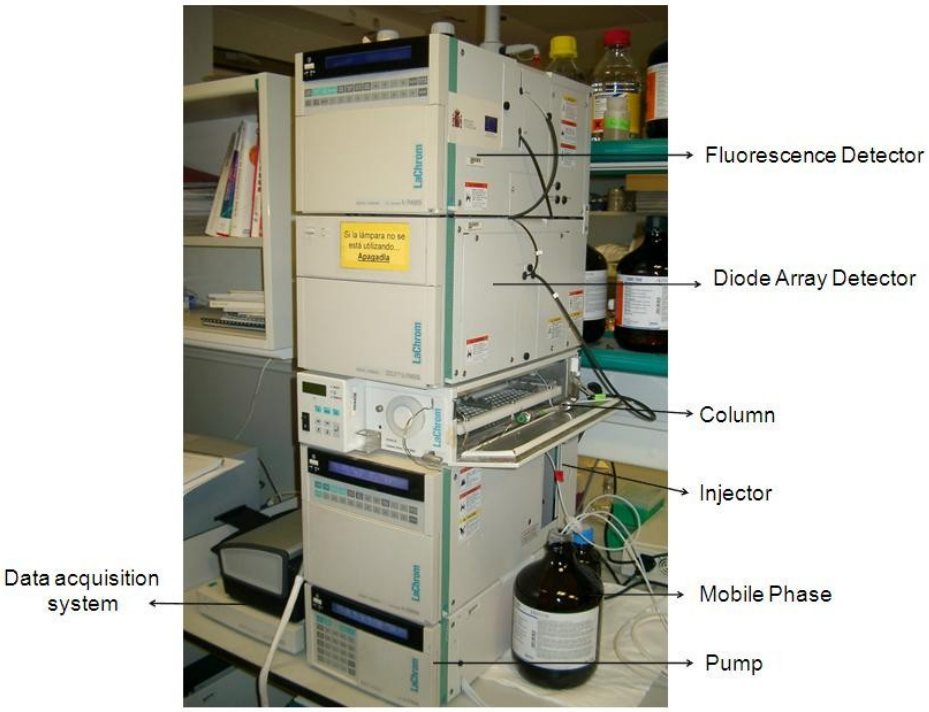


Figure 22 HPLC components

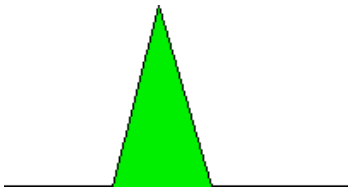
The column is a stainless steel tube which is packed with spherical solid particles of stationary phase. Mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. The sample is injected from a sample injector, located near the column inlet. The injected sample enters in the column with the mobile phase and the components in the sample migrate through it.. Compounds that tend to be affined to the mobile phase therefore migrate faster through the column while compounds that tend to be affined to the stationary phase migrate slower. If the sample it is not a pure compound, but an extract, as in the case of turmeric, its components will distribute between the two phases separately in so that those who have more affinity with the mobile phase, are not retained by the stationary phase and elute for first. In opposition to those with higher affinity for the stationary phase will advance more slowly through the column and elute later. In this way, each

component is separated on the column and sequentially elutes from the outlet. Each compound eluting from the column is detected by a detector connected to the outlet of the column.

When the separation process is monitored by a recorder starting at the time the sample is injected, it's possible to obtain a graph. This graph is called a chromatogram. The time required for a compound to elute (called retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristics of the compound. Retention time is therefore used as an index for qualitative determination and area under curve (AUC) as an index for quantitative determination. In this sense, the smaller the AUC, the less concentrated the compound is, as shown in the simplified diagram in **Fig. 23 A** and **B**.

The retention time of the target compounds and the concentration for each unit of peak area are based on data obtained by analyzing samples with known quantities of the reference standards. Normally, the reference standards are highly purified target compounds.

A



B

Figure 23 Schematic diagrams of preparation at different concentrations. (A) At left the more concentrated, (B) at right the less concentrated.

5.2 Quantification of Curcumin and Resveratrol through HPLC

Curcumin and resveratrol were analyzed and quantified according the methods previously developed respectively by C.E. Green [203] and Lei Chen [204], using an Agilent LC 1100 series (Agilent Technologies, Inc., Palo Alto, CA, USA) controlled by the Chemstation software and equipped with a pump, autosampler, column oven and Fluorescence (FL) and UV–diode array detector. All solvents used were of HPLC grade (Sigma–Aldrich, Europe); before usage they were sonicated in order to degass them, reducing the possibility to block the column by air bubbles solved on them. The column was stored in methanol and had to be washed thoroughly with distilled water before changing to the mobile phase.

For curcuminoids an isocratic method was used; the mobile phase consisting of methanol, isopropyl alcohol, water and acetic acid in the proportions 20:4:27:48:5 v/v. The flow rate was 0.5 mL min^{-1}. The isocratic elution was monitored with fluorescence detector at a wavelength of excitation of 420 nm and emission of 540 nm. The screening samples and the standard were eluted for separation with a run time of 25 min for peak identification.

For trans-resveratrol the analysis was carried out through a linear gradient method using two mobile phases: 10% methanol in water (solvent A) and 90% methanol in water (solvent B). The flow rate was 0.6 ml min^{-1} with a linear gradient from 0% B to 90% B in 25 min. Diode-array detection was set at 304 nm.

The software ChemStation for LC 3D (Agilent Technologies Life Sciences and Chemical Analysis, Waldbronn, Germany) was used for quantization purposes. Quantitative evaluation of the compounds was performed by means of a six-point regression curve (R^2 > 0.996) in a concentration range between 0.02 mM and 0.8 mM, using external standards at known concentrations.

5.2.1 Experimental protocol:

Standard of curcumin and resveratrol (Sigma–Aldrich, Europe) were dissolved in methanol and then quantified by measuring absorbance at 420 nm for curcumin and 304 nm for resveratrol. It was possible obtain the concentration by using the law of Lambert & Beer, knowing the value of the molar extinction coefficient of the compounds (ε_{420nm}= 87 600 M^{-1} cm^{-1} for curcumin; $\varepsilon_{304\ nm}$ = 30 335 M^{-1} cm^{-1} for resveratrol) and the absorbance. Therefore a series of known concentrations of curcumin and/or resveratrol were injected into the HPLC resulting in peaks correlating to the concentrations. A calibration curve was developed, by plotting the area under the curve (AUC) from the peaks versus the known concentration of the standard (**Fig. 28 A** , Results section).

Using the calibration curve of standard, quantification of samples was executed. To quantify the amount of compounds in the liposomes and immunoliposomes, one volume of methanol was added to the liposome suspension, vortexed and centrifugated to ensure the fully liposome disgregation and avoiding column blockade.

The parameters are listed in **Table I**.

Mobile phase	Methanol : Isopropyil alcohol : Water: Acetic acid (27:20:48:5)
Column	Merck LiChrospher 100 RP-18, 5 μm, 250 x 4 mm
Flow rate	0.5 ml/min
Detection wavelenght	420 nm (excitation), 540 nm (emission)
Injection volume	20 μl

Mobile phase	10% Methanol in water; 90% Methanol in water
Column	Merck LiChrospher 100 RP-18, 5 μm, 250 x 4 mm
Flow rate	0.6 ml/min
Detection wavelenght	304 nm
Injection volume	20 μl

Table I HPLC parameters used for quantification of curcumin and resveratrol

6. Cellular uptake using flow cytometer analysis

6.1. Principle of FACS analysis

In a commun flow cytometer experiment, a cell suspension is injected through a nozzle whereby the cells are focused into the centre of a columnar flow by mechanical properties of the fluid (hydrodynamics). The cells in the single-cell stream, passes through a light source usually generated from a laser beam or a mercury lamp. Each cell then scatters the laser light, which is recorded by differente detectors that give information about the size (forward scatter light, FSC) and granularity of the cell (side scatter light, SSC). If the cell that passes through the laser beam has been labelled with a fluorescent reagent (fluorochrome) it emits fluorescence and can be recorded by another detector. Fluorescence may also be emitted by the cells naturally (autofluorescence). Scattered and fluorescent light is collected by the optics, transferred through specific filters to photodetectors (phomultiplier tubes, PMTs) and further digitalised and analysed by a computer. Fluorochromes absorb light of certain specific colour and then emit light of a different colour, usually at longer wavelength. These fluorochromes may be coupled to antibodies, which are directed to molecules expressed on the cell surface or to intracellular markers. Thus, the fluorescence of a cell is proportional to the amount of molecules to which the antibody has bound.

Cells can be scanned at a high rate and a large number of cells can be enumerated and characterized individually upon their specificity, frequency, function, state of activation, differentiation, viability etc, which may give some information of the phenotype and function of different cell subsets.

In our research, flow cytometry has been used to analyse cellular uptake and verify that HER2 receptor, expressed in breast cancer cells, was present on cell surface.

6.2 Uptake Studies

JIMT1 and MCF7 were plated into T25 flask and 24h later, were incubated at 37 °C with free curcumin, liposomated curcumin and curcumin encapsulated into immunoliposomes labeled with rhodamine-PE. After 3 hours of incubation, cells were washed with PBS, trypsinized and centrifugated for 5', the pellets were washed twice with PBS, resuspended in PBS and run on the ImageStream multispectral imaging flow cytometer (Amnis Corporation, Seattle, WA) using 488 nm laser excitation. It is a flow based imaging cytometer integrated with a digital camera detector, a scheme of its architecture is shown in **Fig. 24**.

Classifiers were set when needed to eliminate collection of debris based on low area in the bright-field imagery, clusters of cells based on high area in bright-field imagery, and camera saturating events based on the presence of peak intensities greater than 1022. A typical file contained imagery for 5000 or more cells. Each cell was imaged with side scatter, bright-field, a channel for curcumin stain and a channel for rhodamine stain (in the experiments with immunoliposomes).

Images of cells collected on the ImageStream were analyzed using ImageStream Data Exploration and Analysis Software (IDEAS). The quantitative measurement of liposome cell binding was calculated using features available in IDEAS.

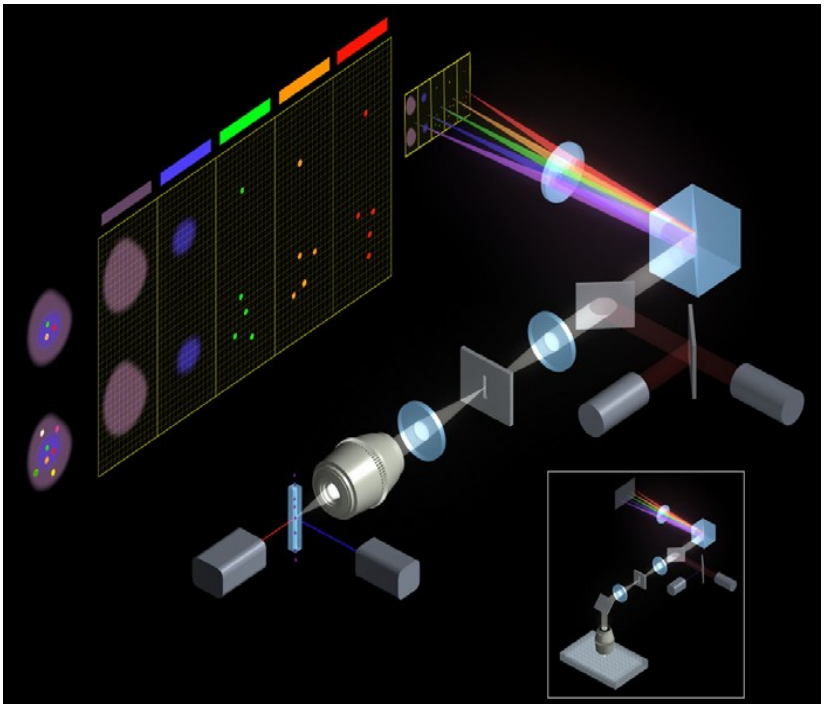


Figure 24: ImageStream Architecture

Cells are hydrodynamically focused within a flow cuvette and are illuminated from the side and from behind with lasers or other light sources. Fluorescence, side scatter, and transmitted light from cells is imaged by an objective lens and relayed to a spectral decomposition element, which divides the imagery into spectral bands located across the detector. Different spectral bands are used for different imaging modes or different colours of fluorescence imagery.

6.3 Analysis of HER2 expression

MCF7 and JIMT1 were trypsinized and washed repeatedly with PBS EDTA 0.5% to obtain

single cell suspensions. Then, one and a half million of cells of each cell line were fixed in 3.5% Formalin (Sigma–Aldrich Co., St. Louis, MO) during 5 min at 4 °C followed by 10 min at room temperature (RT). After three cycles of washing with PBS-EDTA 0.5%, cells were blocked for 30 min with PBS BSA 2% at RT. Once the blocking solution was removed, the cells were incubated overnight at 4 ºC with gently agitation with a rabbit anti-human HER2/neu antibody (sc-284, Santa Cruz Biotechnology, INC) at a concentration of 0.5 µg/10^5 cells. Cells were washed three times with PBS before proceeding with the secondary antibody staining. Anti-rabbit FITC-conjugated IgG (F-2765, Molecular Probes) was used as the secondary antibody at a dilution of 1:25 for 30 min at RT. As control, the secondary antibody alone was used after blockade to show nonspecific binding of the IgG to cells. After washing and recollecting cells in PBS, the FITC-stained cells were quantitated by flow cytometry in an Epics XL instrument (Beckman Coulter Co., Miami, Florida) by analyzing the intensity of the green fluorescence associated to cells.

Results

1. Expression of HER2 on breast cancer cell lines

1.1 Detection of HER2 by Western blot

One of the targets of the project was to compare the cytotoxicity of various phytochemicals against breast cancer cells either in their free form, incorporated into liposomes or incorporated into anti-HER2 immunoliposomes. Then, first task was to screen a variety of breast cancer cell lines in order to find two opposite models for low and high HER expression levels to test the efficacy of anti-HER2 immunoliposomes. After this, we found two cell lines with very significant differences in their HER expression levels. For this purpose western blot and flow cytometer analysis were used. We chose the trastuzumab-resistant JIMT1 cells [192, 193] and, as control line, MCF7 that was substantially HER2-negative [195].

Total protein lysates from the two cell cultures were made and then, these lysates were separated by SDS-PAGE and examined by Western blot.

HER2 was detectable in JIMT1 cells, whereas as expected, it was almost negligible in the lysates from MCF7 cells (**Fig. 25**). The figure 25 shows, at left, HER2 expression levels obtained by WB, while, at right, the quantification of the protein bands by densitometric analysis is shown (using the software Image J, web link: http://rsbweb.nih.gov/ij/).

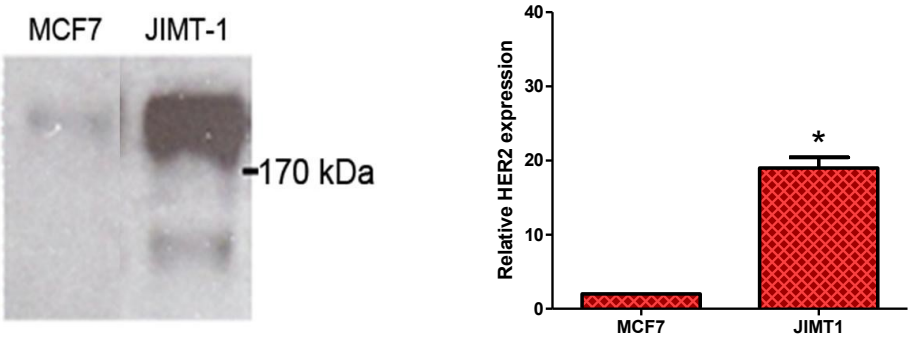


Figure 25 Western blot and densiometric analysis of HER2 expression

Whole lysates from MCF7 and JIMT1 cells were prepared and HER2 expression was quantified. Asterisk represents significant difference relative to MCF7 cells (*p <0.05).

1.2 Detection of membrane surface HER2 by Flow Cytometry

Total amount of the protein was detected and quantified by Western blot, but to verify if the HER2 protein detected in WB assays, expressed in breast cancer cells, was present on cell surface, flow cytometry was used. In this way, we assured that immunoliposomes coupled to trastuzumab would be able to recognize cell surface HER2 and cancer cells would be properly targeted.

JIMT1 and MCF7 cells were stained with an anti-HER2/neu antibody that binds HER2 on the C-terminal portion of the extracellular domain, as previously described in materials and methods section, paragraph 6.3. As shown in **Fig. 26** there was a modest binding of the antibody to MCF7 cells, whereas a more significant binding was evident in the case of JIMT1 cell line. This was in agreement to that predicted from western blot analysis.

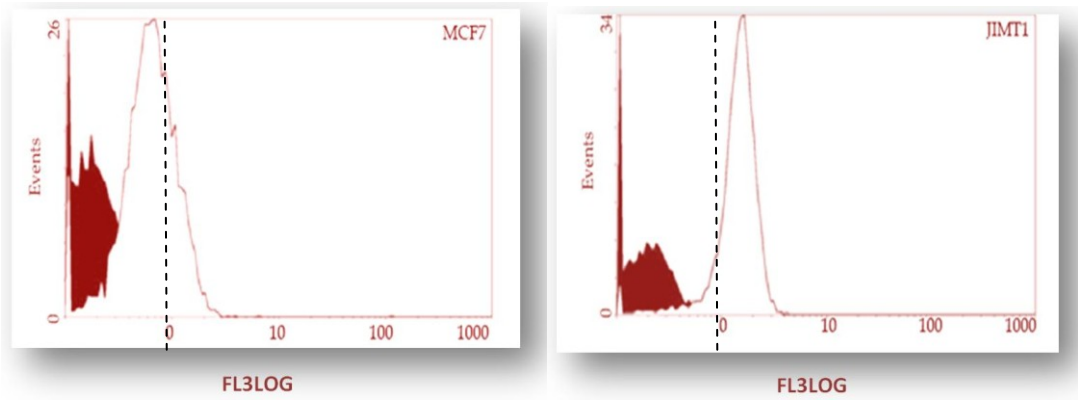


Figure 26 Flow cytometry analysis of HER2 expression

Flow cytometry analysis on cells stained with an anti-HER2/neu antibody. Filled peaks of each histogram represent control cells stained only with the secondary antibody (anti-rabbit-FITC).

2. Cytotoxic effects of free curcumin, free resveratrol and a combination of both on the viability of JIMT1 and MCF7

The efficacy of curcumin and resveratrol and their pharmacological safety make them potential compounds for breast cancer treatment, but the pharmacokinetic and bioavailability studies revealed their poor absorption and rapid elimination from the body. One of the strategies to improve their bioavailability is encapsulation or vehiculization into liposomes in order to increase blood circulation and reduce clearance. In first instance we studied the cytotoxic effects of these drugs in cellular models as free compounds, then incorporated into liposomes and finally encapsulated into liposomes coupled to trastuzumab (immunoliposomes).

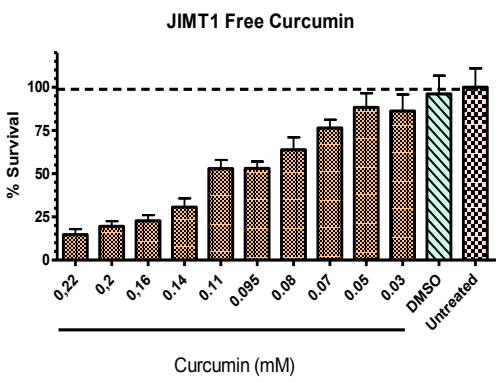
JIMT1 and MCF7 breast cancer cells at a confluence of 80%, were treated with different concentrations of curcumin, resveratrol and both compounds together depending of their cytotoxicity in the two cellular models in order to test their efficacy and /or synergy. These concentrations were ranging from 0.03 to 0.22 mM for curcumin, 0.22 to 0.87 mM for resveratrol and 0.058 to 0.566 mM for the two compounds together.

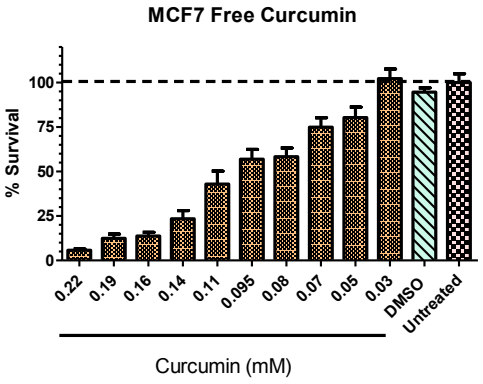
Calculation of drugs' concentration in case of the system based on the co-administration of curcumin and resveratrol was made calculating the number of moles of each compound and then adding the number of moles of both dividing for the volume. The molar ratio curcumin: resveratrol, for this system, was 1:1.6.

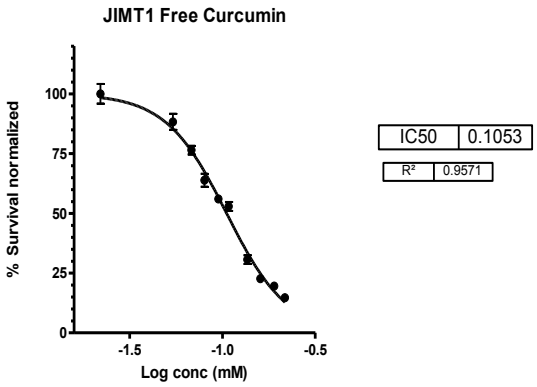
The treatments were performed for 72h, to assess a chronic effect and both cytotoxic and cytostatic effect was tested. Exposure to curcumin and resveratrol inhibited cell growth in the two cell lines tested in a concentration dependent manner (**Fig. 27**). Proliferation survival was assessed by MTT assay.

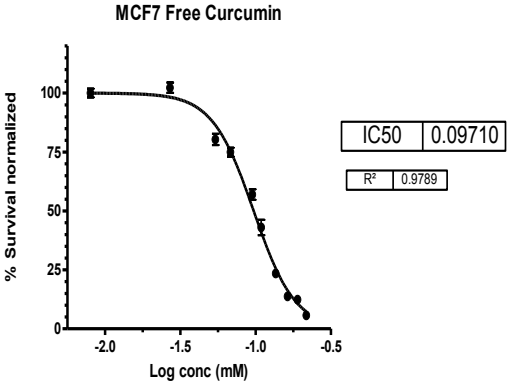
The IC_{50} values were determined from the survival plots and indicate the concentration causing the death of 50% of cells; the coefficient of determination (R^2) ranged from 0.9 and 0.98 in all cases, indicating a highly significant correlation between experimental and fitted data points.

A

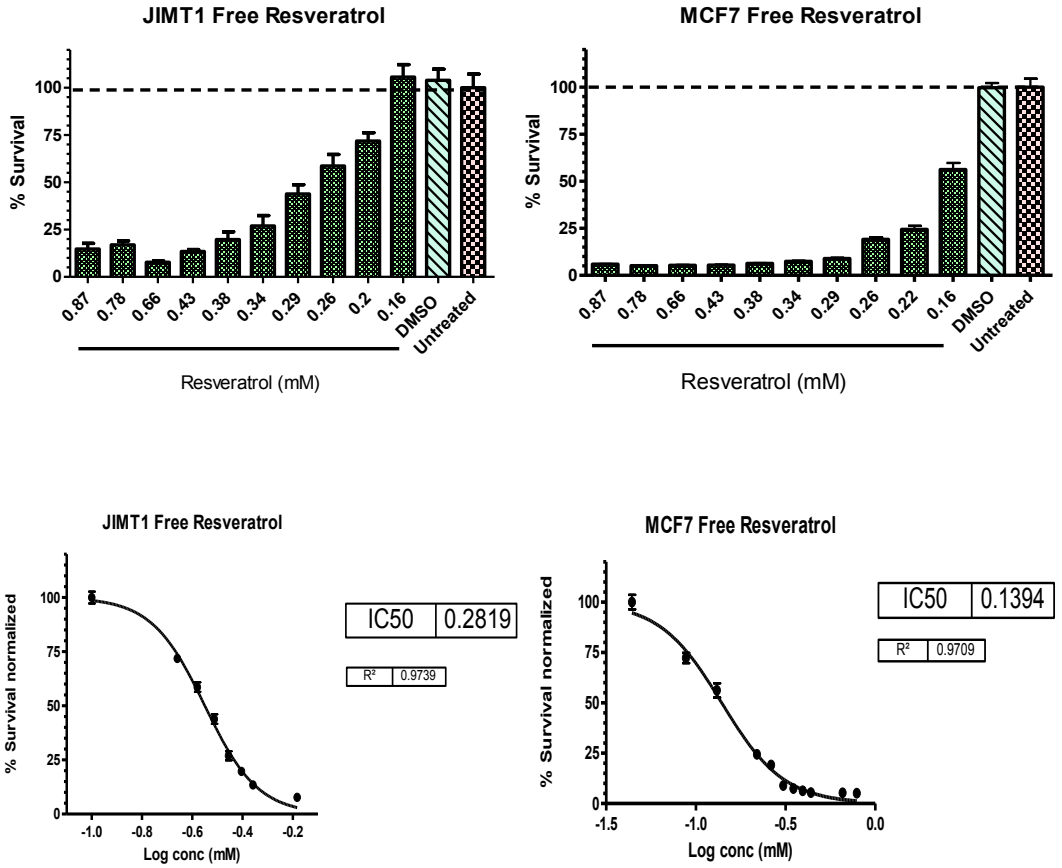

JIMT1 Free Curcumin
% Survival
100
75
50
25
0
0,22
0,2
0,16
0,14
0,11
0,095
0,08
0,07
0,05
0,03
DMSO
Untreated
Curcumin (mM)


MCF7 Free Curcumin
% Survival
100
75
50
25
0
0,22
0,19
0,16
0,14
0,11
0,095
0,08
0,07
0,05
0,03
DMSO
Untreated
Curcumin (mM)


JIMT1 Free Curcumin
% Survival normalized
100
75
50
25
0
-1.5
-1.0
-0.5
Log conc (mM)
IC50
0.1053
R²
0.9571


MCF7 Free Curcumin
% Survival normalized
100
75
50
25
0
-2.0
-1.5
-1.0
-0.5
Log conc (mM)
IC50
0.09710
R²
0.9789

B


JIMT1 Free Resveratrol
% Survival
100
75
50
25
0
0.87
0.78
0.66
0.43
0.38
0.34
0.29
0.26
0.2
0.16
DMSO
Untreated
Resveratrol (mM)
MCF7 Free Resveratrol
% Survival
100
75
50
25
0
0.87
0.78
0.66
0.43
0.38
0.34
0.29
0.26
0.22
0.16
DMSO
Untreated
Resveratrol (mM)
JIMT1 Free Resveratrol
% Survival normalized
100
75
50
25
0
-1.0
-0.8
-0.6
-0.4
-0.2
Log conc (mM)
IC50
0.2819
R²
0.9739
MCF7 Free Resveratrol
% Survival normalized
100
75
50
25
0
-1.5
-1.0
-0.5
0.0
Log conc (mM)
IC50
0.1394
R²
0.9709

C

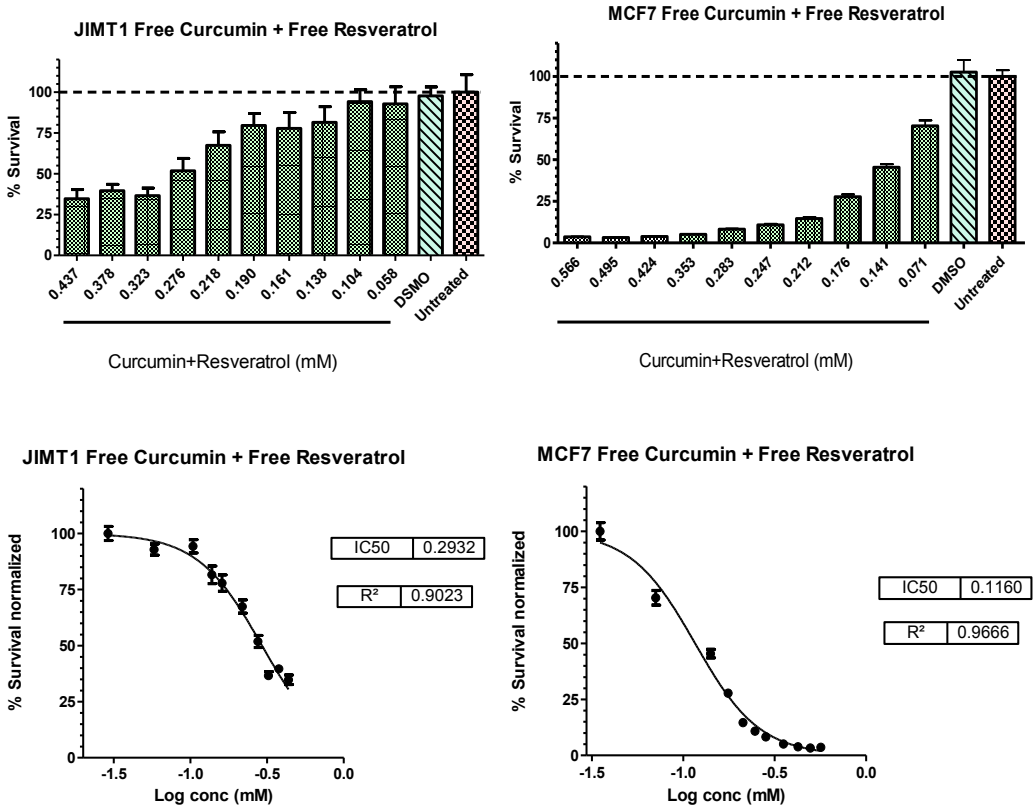


Figure 27 Effect of free curcumin (A), free resveratrol (B) and a combination of both (C) on JIMT1 and MCF7 proliferation after 72 hours of exposure.

Percentage of cell survival after 72h of treatment with different concentrations of compounds; untreated represents control cells treated only with culture medium; DMSO indicates cells treated with the higher DMSO concentration (0.1%) used to dissolve drugs. The data presented are mean of 3 data sets in which each condition was made in 8 replicates**.** The values of standard deviation (± SD) as presented as error bars. In case of curcumin with resveratrol combination (coadministered) (C) concentrations values represent the sum of the number of moles of each compounds dividing by volume, in which the molar ratio curcumin:resveratrol was 1:1.6.

The estimated IC_{50} values ranged between approximately 0.1 and 0.097 mM for free curcumin, 0.28 and 0.14 mM for free resveratrol and between 0.293 and 0.116 mM in the case of combination of both drugs as resumed in **Table II**. Results show a greater sensitivity of both cell lines to curcumin. It was also noticeable that resveratrol exhibited higher cytotoxic effect in MCF7 cells than that shown for JIMT1 cells, effect that was also observed in the combination of compounds.

Compounds	**IC_{50} JIMT1**	**IC_{50} MCF7**
Free Curcumin mM	0.1053 ± 0.0028	0.0971 ± 0.0026
Free Resveratrol mM	0.2819 ± 0.0056	0.1394 ± 0.0052
Free Curcumin+Resveratrol mM	0.2932 ± 0.0154	0.1160 ± 0.0056

Table II IC50 values of free curcumin, free resveratrol and free curcumin+free resveratrol on JIMT1 and MCF7 cell lines.

In case of curcumin and resveratrol coadministration, IC_{50} values represent the sum of the number of moles of each compounds dividing for the volume, in which the molar ratio curcumin:resveratrol is 1:1.6.

3. Quantification of drugs' content by HPLC analysis

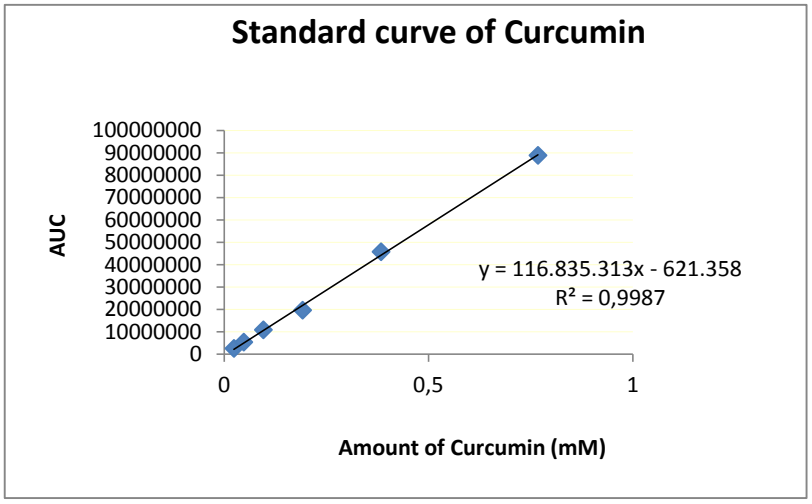
Determination of the concentration of curcumin and resveratrol in the experiments where these compounds were used in a free form, incorporated into liposomes or into immunoliposomes was performed by high performance liquid chromatography. In the systems based on liposomes, only curcumin and resveratrol incorporated into liposomes were determined.

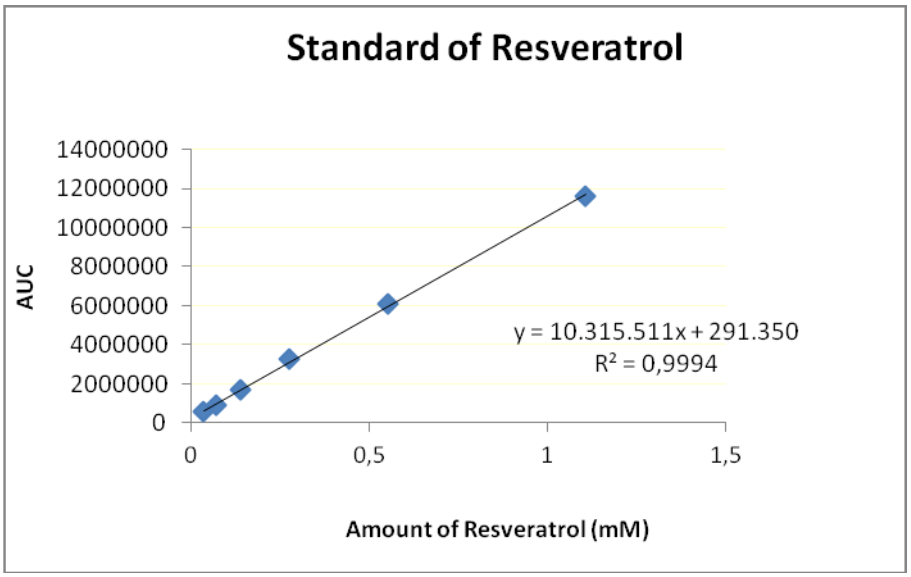
In first instance, external standards of curcumin and resveratrol with known concentrations were injected into HPLC in order to set their respective calibration curves (**Fig. 28 A**); then samples were analyzed and their quantification was performed.

In the case of curcumin the typical chromatogram shows three different peaks which represent the three main compounds of *Curcuma longa*. These are, in addition to curcumin, demethoxy-curcumin and bis-demethoxy-curcumin that have respectively one and two methoxylic groups less than curcumin. These compounds elute later than curcumin (**Fig. 28 B**).

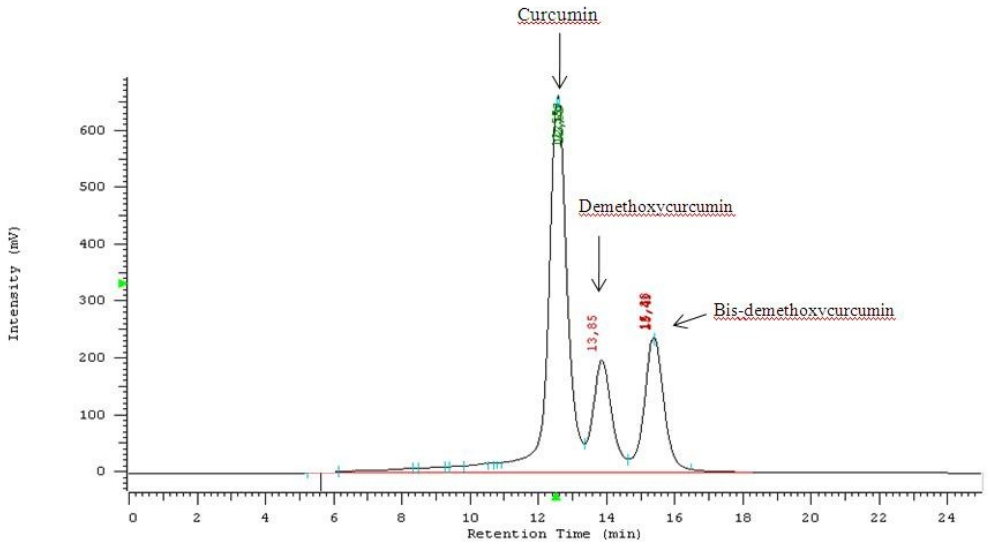
Trans-resveratrol showed a pure compound so it gave one single peak (**Fig. 28 C**).

A


Standard curve of Curcumin
AUC
100000000
90000000
80000000
70000000
60000000
50000000
40000000
30000000
20000000
10000000
0
0
0,5
1
y = 116.835.313x - 621.358
R² = 0,9987
Amount of Curcumin (mM)


Standard of Resveratrol
AUC
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
0
0,5
1
1,5
y = 10.315.511x + 291.350
R² = 0,9994
Amount of Resveratrol (mM)

B



C

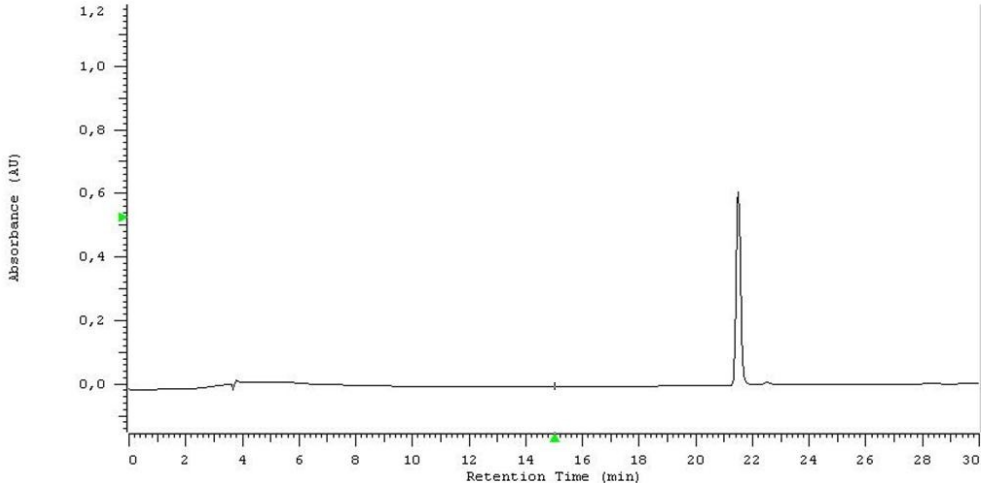


Figure 28 A) Standard curve of curcumin and resveratrol used for quantification of samples' concentrations; Representative chromatograms of (B) the three curcuminoid polyphenolic compounds and (C) *trans*-resveratrol.

4. Cytotoxic effect of curcumin and resveratrol incorporated into liposomes

The combination of curcumin with other agents to increase chemopreventive efficacy has been deeply studied previously [205-207]. However, the efficacy of the combination of curcumin and resveratrol in the same liposomal formulation against breast cancer cells has not been investigated to date.

Curcumin and resveratrol are supposed to be uncharged compounds at physiological pH. Moreover, these compounds exhibit a poor water solubility, which make them attractive candidates for inclusion in the hydrophobic core of a lipid system like that of liposomes and immunoliposomes. In spite of this, there are a series of parameters that must be considered and optimized to improve the encapsulation's efficiency.

To develop liposomes and immunoliposomes was firstly necessary optimize the systems with free compounds and this was namely elaborated in the preparations including two drugs. Different tests were performed with free compounds and the optimal molar ratio that can be dissolved in DMSO and later into culture media, in the coadministration system was found to be curcumin:resveratrol 1:1.6.

Next step was to perform liposomal preparations in which 1:1.6 molar ratio of free compounds was attempted to maintain, but results demonstrated that liposomes preferentially encapsulated resveratrol rather than curcumin, with a higher molar ratio for resveratrol, compared to free system, that was 1/3.6 (curcumin/resveratrol). This was probably due to the low solubility of curcumin in DMSO, which results in the formation of precipitates and therefore in a decreased ability of encapsulation.

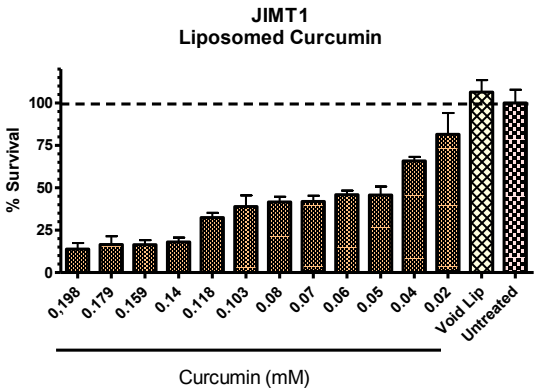
In first instance, we performed the cytotoxicity assays on the two breast cancer cell lines using the same bioactive compounds but encapsulated into liposomes. The aim of these experiments was to evaluate a potential IC_{50} decrease due to their incorporation into liposomes compared to the free compounds.

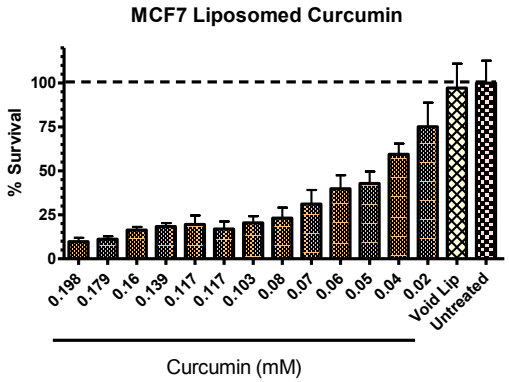
As shown in **Fig. 29** all liposomal formulations showed a decrease of IC_{50} values and consequently, an improvement of therapeutic index, i.e. achieving

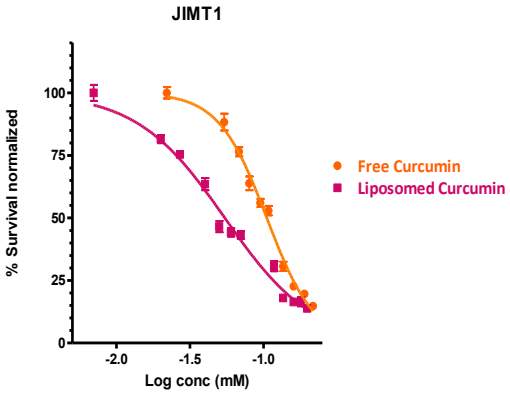
the same effect but using lower concentrations of drugs. Incorporation of curcumin into liposomes improved its IC_{50} value almost twice compared to free compounds both in MCF7 and JIMT1 cells. The result was even better for resveratrol since IC_{50} decreased around three times in both cell lines. This decrease was less evident in the case of the combination of the two compounds.

In addition, MCF7 cells were a little more sensitive to both curcumin and resveratrol than JIMT1 cells when these compounds were incorporated into liposomes.

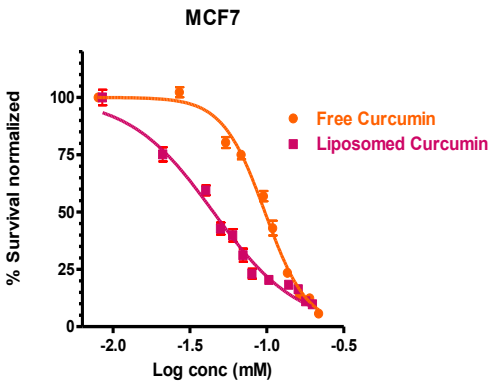
A





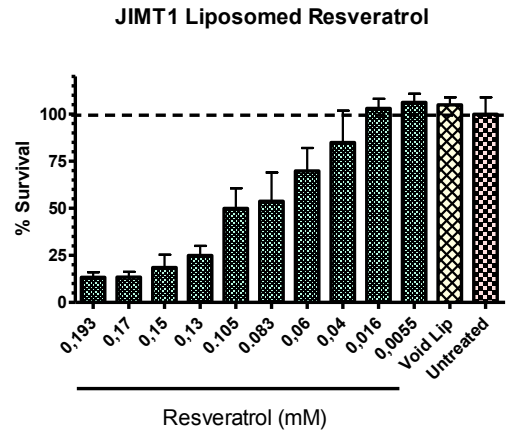


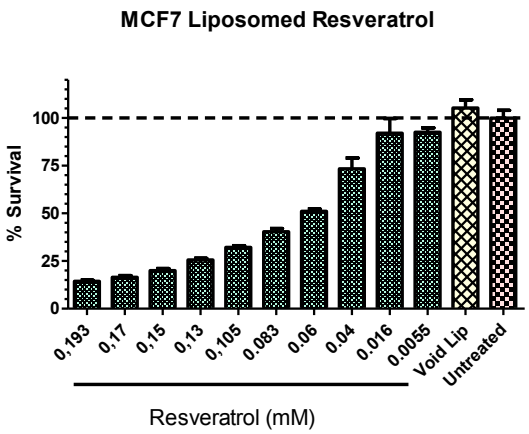
	Free Curcumin	Liposomed Curcumin
IC50	0.1053	0.05458

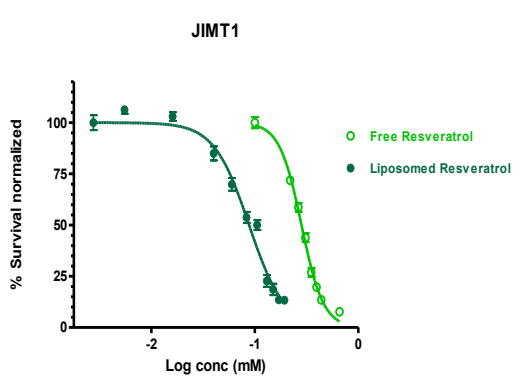


	Free Curcumin	Liposomed Curcumin
IC50	0.09710	0.04492

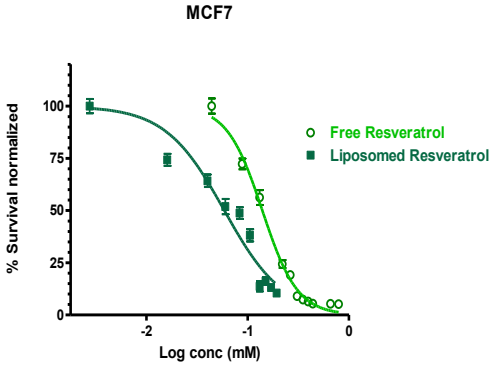
B





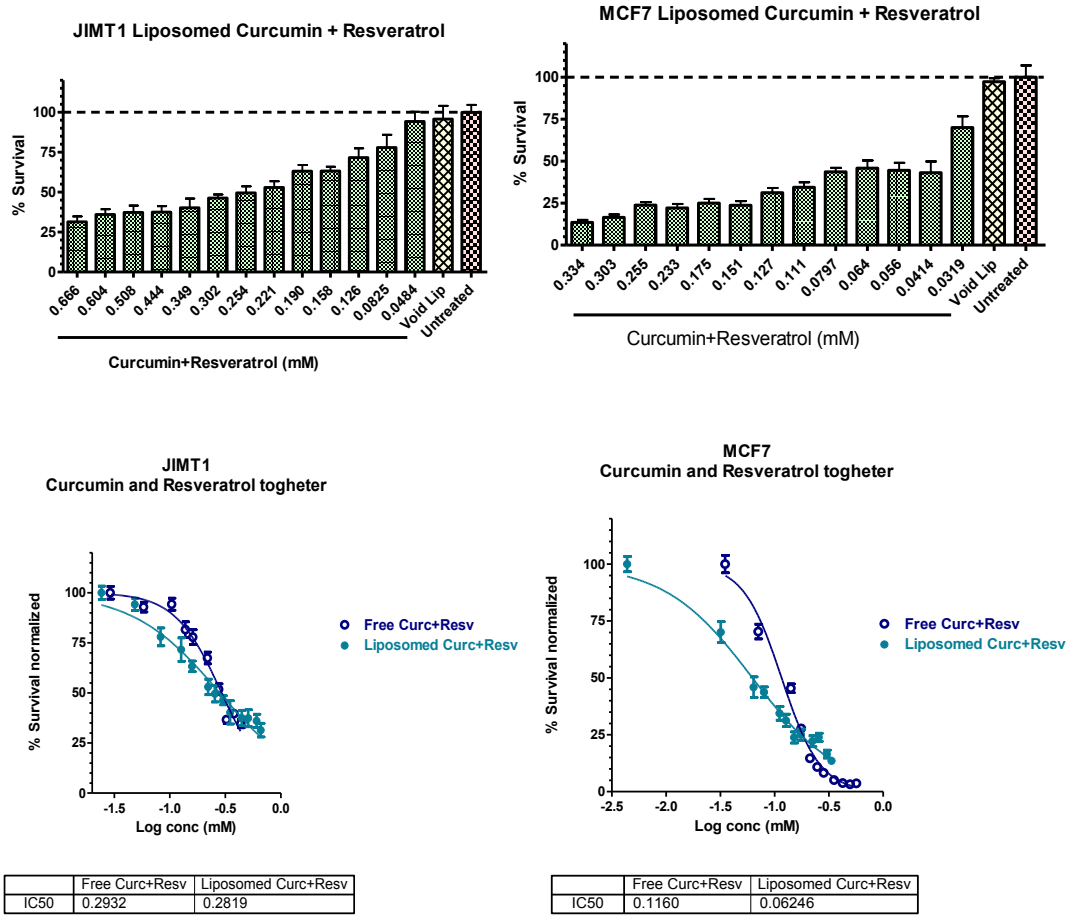


	Free Resveratrol	Liposomed Resveratrol
IC50	0.2819	0.08793



	Free Resveratrol	Liposomed Resveratrol
IC50	0.1394	0.05877

C



	Free Curc+Resv	Liposomed Curc+Resv
IC50	0.2932	0.2819

	Free Curc+Resv	Liposomed Curc+Resv
IC50	0.1160	0.06246

Figure 29 Comparative effect of free and liposomed preparations of (A) curcumin, (B) resveratrol and (C) curcumin+resveratrol on JIMT1 and MCF7.

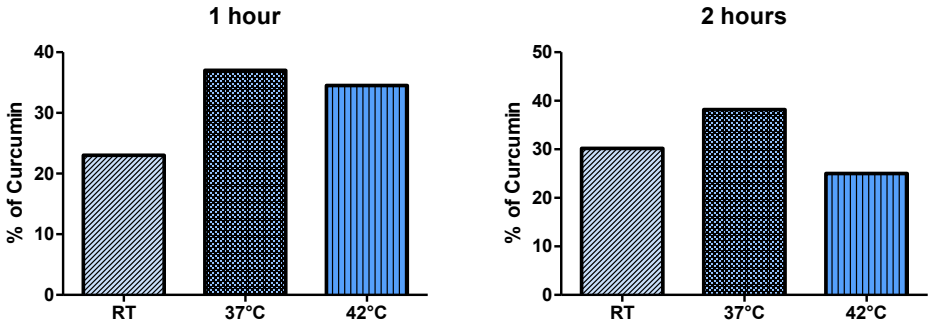
Cell survival after 72h of treatment with different drugs' concentrations; untreated represents negative control cells treated only with DMEM; void liposomes indicates cells treated with the higher concentration of void liposomes used (0.7 mM EYPC). In case of curcumin with resveratrol coadministration (C) concentrations values represent the sum of the number of moles of each compounds dividing by the volume, in which the molar ratio curcumin:resveratrol was 1:3.6.

5. Optimization of drugs' encapsulation into immunoliposomes

Optimizations of drugs' encapsulation became even harder in the immunoliposomes system both in case of a single drug encapsulation and with two drugs. Next, a series of tests are reported: the type of solvent to solubilize drugs, time of incubation with the compounds, solvent's percentage, incubation's temperature and time, were varied in order to find the optimum conditions for each system. As an example, the optimization of the incorporation of curcumin or resveratrol into the combined system (curcumin+resveratrol immunoliposomes) is reported. Then, based on the results obtained, optimal conditions were extrapolated for the other systems containing single drugs.

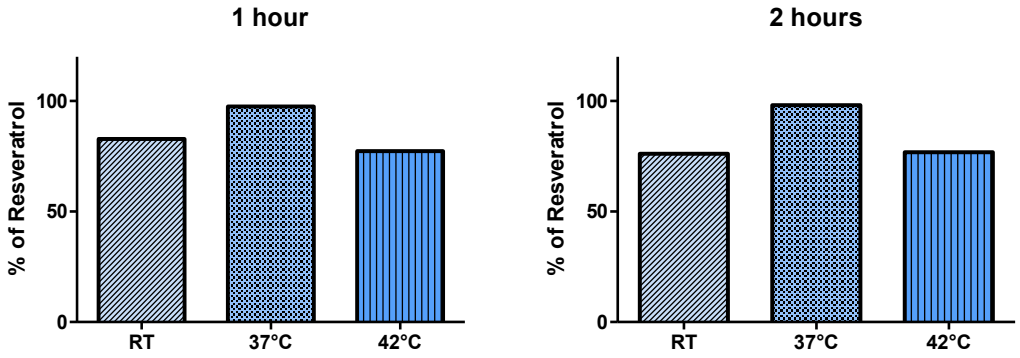
Immunoliposomes were incubated with different amounts of resveratrol and curcumin and were left to reach equilibrium in experiments at 1 h or 2 h at different temperatures: room temperature, 37 °C and 42 °C. The aim was evaluate what temperature favored the drugs' encapsulation when the compounds were added in the presence of low concentrations of organic solvents. Temperatures higher than 42 °C were not used to avoid denaturation of the antibody. Temperatures lower than room temperature were not utilized since encapsulation efficiency would be very low. Tests were performed in the presence of 10% DMSO. After 1 or 2 h the concentration of compounds incorporated into immunoliposomes after filtration was determined by HPLC. Results are shown in the following tables and figures indicating the percentage of the compound incorporated into the liposomes after the incubations (**Fig. 30 e 31, Table III e IV**).

Results showed that resveratrol, in all selected conditions, was encapsulated more easily than curcumin and this was in agreement to previous results obtained with liposomed system. The optimal condition resulted to be 37 °C. Based on the results, 37 ºC was chosen as optimal temperature for the following experiments. Incubations for 1 h yielded a little better results than 2 h.



Time of incubation	Temperature	% Curcumin
1 hour	RT	23%
1 hour	37 °C	37%
1 hour	42 °C	34.5%
2 hours	RT	30.2%
2 hours	37 °C	38.2%
2 hours	42 °C	25%

Figure 30 and Table III Percentage of Curcumin incorporated in Curcumin+Resveratrol immunoliposome preparations at three different temperatures for 1 and 2 h of incubation (RT: room temperature)



Time of incubation	Temperature	% Resveratrol
1 hour	RT	82.8%
1 hour	37 °C	97.6%
1 hour	42 °C	77.3%
2 hours	RT	76.2%
2 hours	37 °C	98.2%
2 hours	42 °C	76.8

Figure 31 and Table IV Percentage of Resveratrol incorporated into Curcumin+Resveratrol immunoliposome preparations at three different temperatures for 1 and 2 h of incubation (RT: room temperature)

To improve the percentage of curcumin encapsulation, two additional experiments were performed. The difference among them was based on the time at which compounds were added. Two type of experiments were performed:

- Option 1: Formation of immunoliposomes (as described in materials and methods section, paragraph 4.4), purification by size exclusion chromatography to remove unbound antibody (as described in material and methods section, paragraph 4.5), addition of compounds in organic solvent, and subsequent purification.

- Option 2: Formation of immunoliposomes (as described in materials and methods section, paragraph 4.4), addition of compounds in organic solvent, purification by size exclusion chromatography (as described in material and methods section, paragraph 4.5).

Option 1 resulted to be the best condition, so further tests were performed following this protocol.

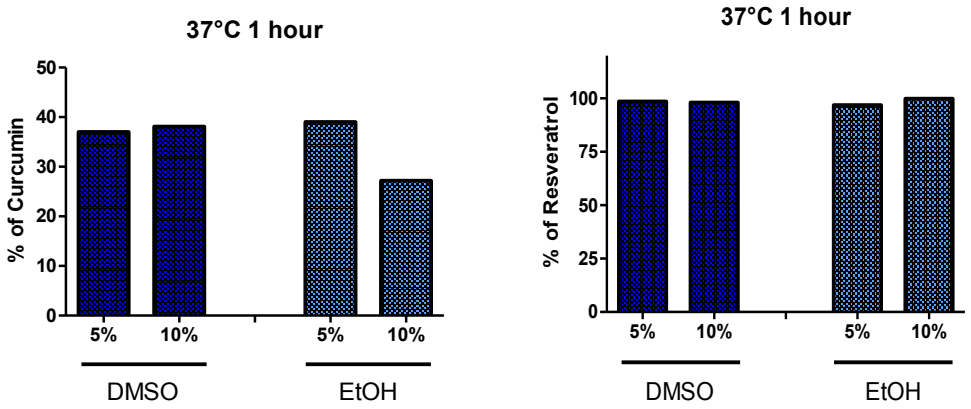
In another set of experiments, the type of solvent and its percentage was also varied. DMSO and EtOH were used because both drugs well soluble in these solvents. Moreover cytotoxicity assays on cell lines allow the use of these solvents at low concentration without compromising cell viability.

The results showed that drugs had a good incorporation both in DMSO and in EtOH, but curcumin seemed to prefer DMSO. A good incorporation efficiency was obtained both at 5% and 10% solvents whereas high concentrations of EtOH (10%) and longer incubations (2 h) yielded lower level of incorporation for curcumin. However, these factors didn't seem to affect resveratrol incorporation (**Fig. 32 and 33, Table V and VI**).

Based on the results obtained, shorter incubation (1 h), and 5% DMSO at 37 °C were selected as optimal condition of encapsulation, both in case of coadministration of the two compounds and in the incorporation of a single compound into the liposomes.

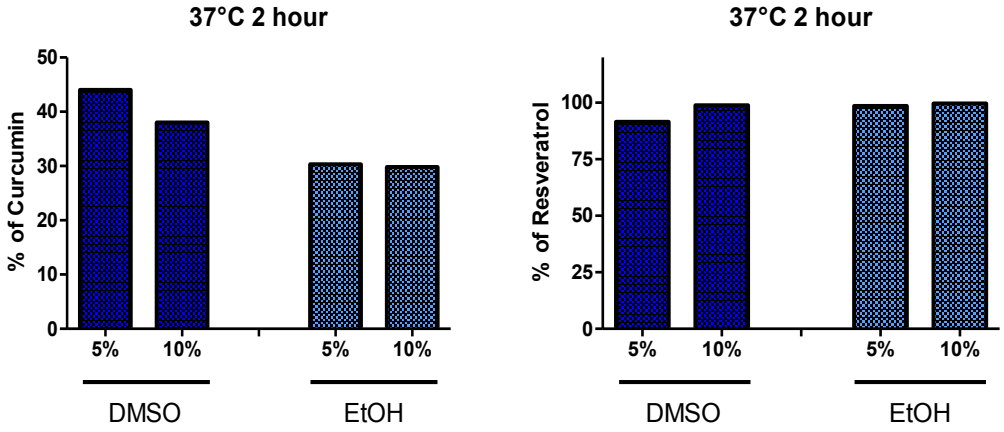
In the case of coadministration, the results show that the level of incorporation of compounds was difficult to control both in liposomal and in

immunoliposomal systems. This incorporation was limited by the affinity of the compounds for the phospholipid bilayers and their solubility into the low organic solvent concentration present in the incubations.Liposomes and immunoliposomes seemed to prefer encapsulating resveratrol rather than curcumin when when both were coadministered. This could be due to an incomplete solubilization of curcumin in DMSO and ETOH, which led to formation of precipitates limiting encapsulation's efficiency. Liposomes yielded a molar ratio curcumin:resveratrol of 1:3.6 and immunoliposomes yielded a molar ratio of 1:3.1.



Solvent	% Curcumin	% Resveratrol
5% DMSO	37%	98.6%
10% DMSO	38.1%	98%
5% EtOH	39%	96.8%
10% EtOH	27.2%	99.8%

Figure 32 and Table V Incorporation of curcumin and resveratrol into Curcumin+Resveratrol loaded immunoliposomes at 37°C for 1 hour.



Solvent	% Curcumin	% Resveratrol
5% DMSO	44%	91.5%
10% DMSO	38.%	98.8%
5% EtOH	30.3%	98.6%
10% EtOH	29.8%	99.7%

Figure 33 and Table VI Incorporation of curcumin and resveratrol into Curcumin+Resveratrol loaded immunoliposomes at 37°C for 2 hours.

6. Purification and characterization of liposomes and anti-HER2 immunoliposomes

Liposomes represent a very efficient delivery tool. They may act as a source from which the entrapped compound or compounds are slowly released over time. This release process is used to maintain therapeutic drug levels in the bloodstream or at the local administration site for prolonged periods of time. Despite advantages they offer, liposomes have a relevant limitation: they are not selective for the selected targets. For this reason we used immunoliposomes directed against HER2, which is overexpressed in a high percentage of breast cancers and play an important role in the pathogenesis of breast and other cancers.

The immunoliposome system was composed of different parts: (a) trastuzumab, the humanized anti-HER2 antibody; (b) sterically stabilized liposomes containing PEG; (c) the encapsulated anticancer agents (curcumin and/or resveratrol). Anti-HER2 immunoliposomes were constructed by conjugation of liposomes to anti-HER2 antibody (trastuzumab) and incorporating the anticancer compounds as described in methods section.

Then unbound antibody and, in a second time, no-encapsulated compounds were separated by size exclusion chromatography. **Fig. 34**, shows a chromatographic profile at 280 nm of a separation of immunoliposomes from unbound antibody. It can be clearly seen that immunoliposomes present a very intense sharp peak which elutes first, and the unbound antibody, with less intensity, elutes at longer retention time.

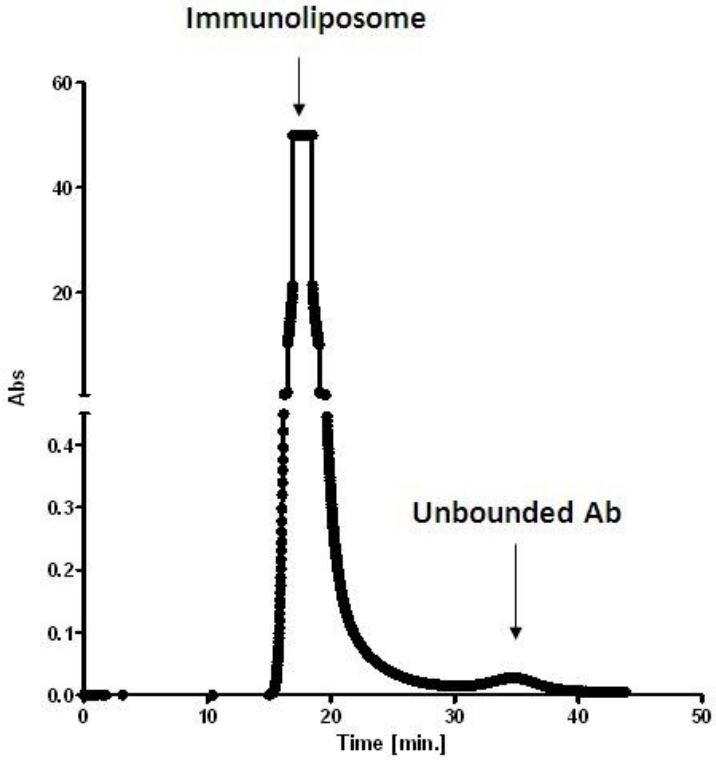


Figure 34 Chromatographic profile of the separation of immunoliposomes from the unbound material.

In order to check the unilamellar character of the liposomes and the absence of aggregation, all liposomes and immunoliposomes preparations were also characterized in size. Determination of size was performed using the technique of dynamic light scattering as described in Material and Methods section (paragraph 4.7) (**Fig. 35**). Figure 35 shows the average diameter of an typical immunoliposome preparation containing resveratrol. As expected, the diameter (Z) of preparation was around 100 nm with a polydispersity (P.D.I.) less of 0.2 indicating the reliability of the measurement.

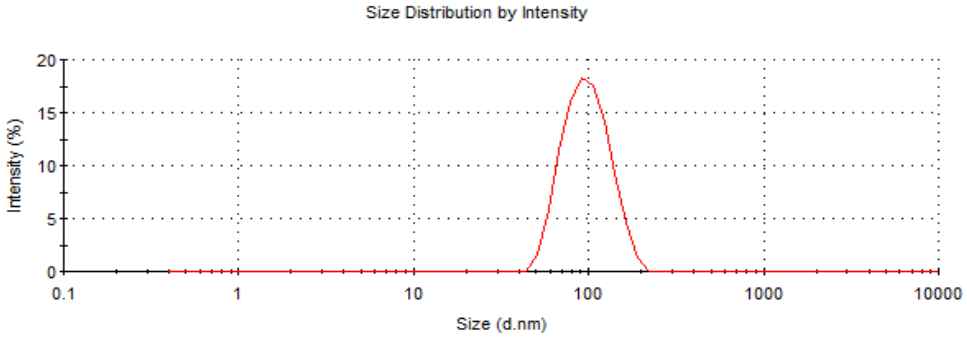


Figure 35 Example of size distribution vs intensity, obtained from the immunoliposome preparation containing resveratrol (AbLip+Resv) with Z= 100,5 d. nm and PDI=0.077

7. Comparative effect of the three delivery systems: free compounds, liposomes and immunoliposomes

Once immunoliposome systems were formed, we tested them on JIMT1 and MCF7 cell lines. JIMT1 is a HER2-overexpressing cell line, while MCF7 cell line was substantially HER2-negative as shown in Fig. 25; in this way, a positive and a negative controls for HER2 expression were available to test for selectivity of the immunoliposomes.

Cells were treated for 72 hours and also in this case, a dose-dependent response was evident (**Fig. 36**). From the graphs presented it's clear that the system based on immunoliposomes was much more effective than the system in which the compounds were simply dissolved in the solvent but also there was more efficacy compared to liposomes lacking antibody.

Furthermore, a different behavior pattern was observed when MCF7 and JIMT1 cells were compared. Whereas almost no big differences were observed in the survival plots of the treatments of MCF7 cells with liposomes or immunoliposomes carrying curcumin, significant differences were noticed between these two systems when JIMT1 cells were treated (*p <0.01 compared to **p <0.001 for JIMT1). IC50 value for JIMT1 dropped around four-fold when liposomes and immunoliposomes carrying curcumin were compared, and only a one a half-fold decrease was observed for MCF7 cells.

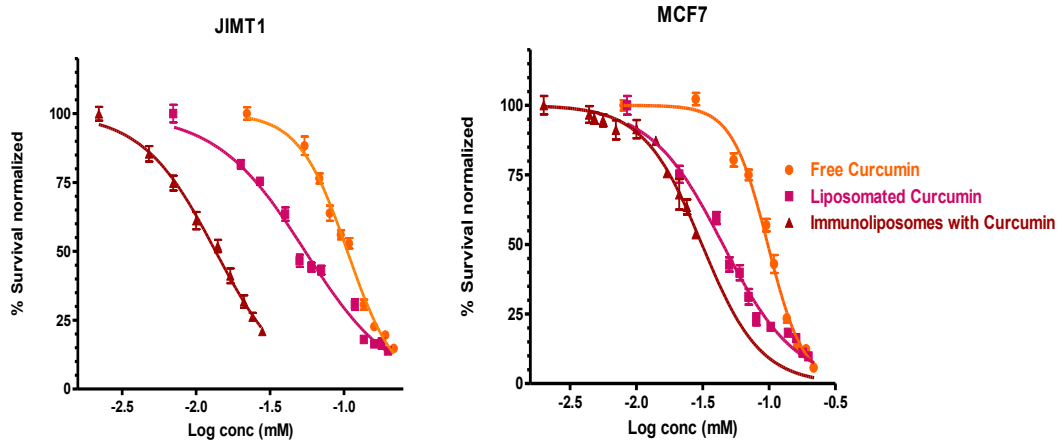
Regarding resveratrol (**Fig. 36 B**), liposomes also decreased IC_{50} values compared to free compounds, i.e. 3.2 fold in JIMT1 and 2.3 fold in MCF7 cells. No significant differences were observed between liposomes and immunoliposomes in MCF7 cells (*p >0.01). On the contrary, a significant decrease (1.5 fold) was observed between liposomes and immunoliposomes in JIMT1 cells (*p <0.01).

In case of curcumin and resveratrol' combination (molar ratio 1:3.1) (**Fig. 36 C**), a similar effect than that observed previously for curcumin alone seemed to occur. The combination of both compounds especially affected JIMT1 cells, in which IC_{50} values decreased almost 14-fold when drugs were

encapsulated into immunoliposomes (**p <0.01). No differences were observed between liposomes and immunoliposomes in the case of MCF7 cells for the combined system (*p >0.05).

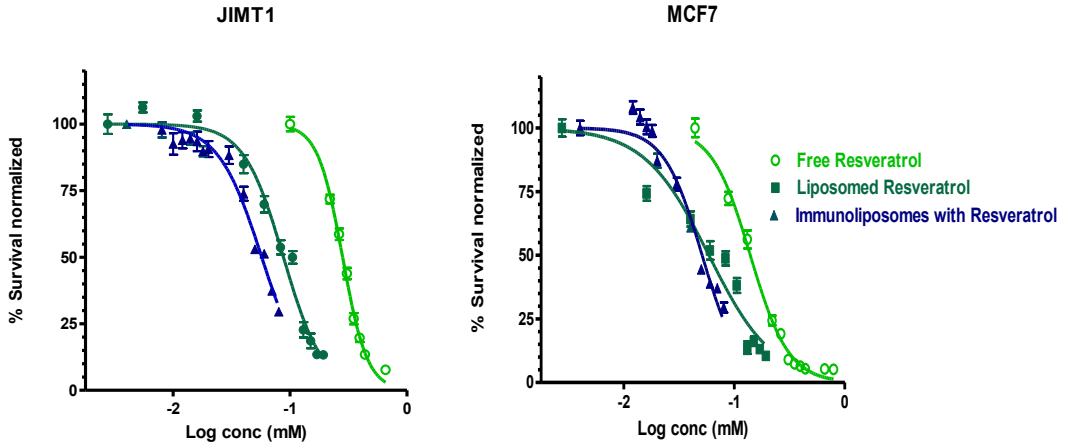
These results confirm that the presence of the antibody in the immunoliposome system provides with a higher degree of specificity in HER-2 overexpressing cell line such as JIMT1.

A



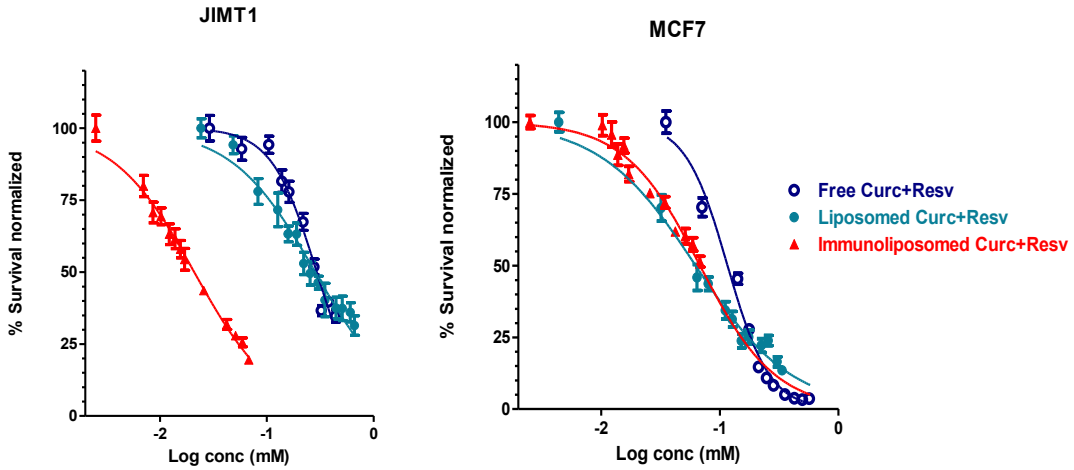
Compounds	IC_{50} JIMT1	IC_{50} MCF7
Free Curcumin mM	0.1053 ± 0.0028	0.0971 ± 0.0026
Liposomed Curcumin mM	0.0546 ± 0.00257	0.0449 ± 0.0024
Immunoliposomed Curcumin mM	0.0135 ± 0.00062	0.0312 ± 0.0030

B



Compounds	IC_{50} JIMT1	IC_{50} MCF7
Free Resveratrol mM	0.2819 ± 0.0056	0.1394 ± 0.0052
Liposomed Resveratrol mM	0.0879 ± 0.0047	0.0587 ± 0.0064
Immunoliposomed Resveratrol mM	0.0573 ± 0.0022	0.0511 ± 0.0023

C



Compounds	IC_{50} JIMT1	IC_{50} MCF7
Free Curc+Resv mM	0.2932 ± 0.015	0.1160 ± 0.0056
Liposomed Curc+Resv mM	0.2819 ± 0.024	0.0625 ± 0.0070
Immunoliposomed Curc+Resv mM	0.0209 ± 0.001	0.0679 ± 0.0007

Figure 36 Comparative effects of the three systems: free compounds, liposomed and immunoliposomed compounds containing (a) curcumin, (b) resveratrol and (c) curcumin+resveratrol

Percentage of cell survival after 72h of treatment in free, liposomed and immunoliposomed systems containing a) curcumin, b) resveratrol and c) a combination of both compounds. In case of the combination (coadministered) the concentrations values represent the sum of the number of moles of each compound dividing by volume, in which the molar ratio curcumin:resveratrol was 1:1.6 in the free system, 1:3.6 for liposomed system and 1:3.1 for immunoliposomed system.

8. Quantitative uptake and immunoliposomes' binding in JIMT1 and MCF7 cells using MIFC technology

Our cytotoxicity data indicated that, in most cases, the system based on immunoliposomes resulted in a significant lowering of the IC_{50} values in the cell population that overexpressed the target HER2. Therefore we decided to perform an assay that allows us to evaluate drug's uptake and to verify the possible increase of incorporation of immunoliposomes material to cells when drug was incorporated into these system compared to liposomes. For this purpose a novel image-based flow cytometric approach (multispectral imaging flow cytometry [MIFC]) was utilized, which has greater specificity and sensitivity than standard flow cytometry methods. This technology combines flow cytometry with microscopy and allows high-throughput quantitative and qualitative characterization of single cells by assessing a combination of morphology and immunofluorescence staining patterns.

JIMT1 and MCF7 breast cancer cell lines were incubated for 2 h with free curcumin (Fr-Curc), liposomed curcumin (Lip-Curc) and curcumin incorporated into immunoliposomes. Both, liposomes and immunoliposomes were labeled with a fluorescent phospholipid containing the probe rhodamine (Rhod-Ab-Lip-Curc). A rhodamine-labeled lipid was incorporated into the immunoliposomes in order to verify whether the incorporation of trastuzumab antibody to the liposome surface would increase the binding rate of immunoliposomes to cells overexpressing HER2/neu antigen or not. Curcumin was the compound selected in these experiments since bigger differences were previously observed in the compared efficacy between liposomes and immunoliposomes through cytotoxicity assays. Furthermore curcumin has strong auto-fluorescence so its uptake can also be easily measured. After the 2 h incubation of cells with the above-mentioned systems, curcumin and rhodamine fluorescences associated to cells were evaluated.

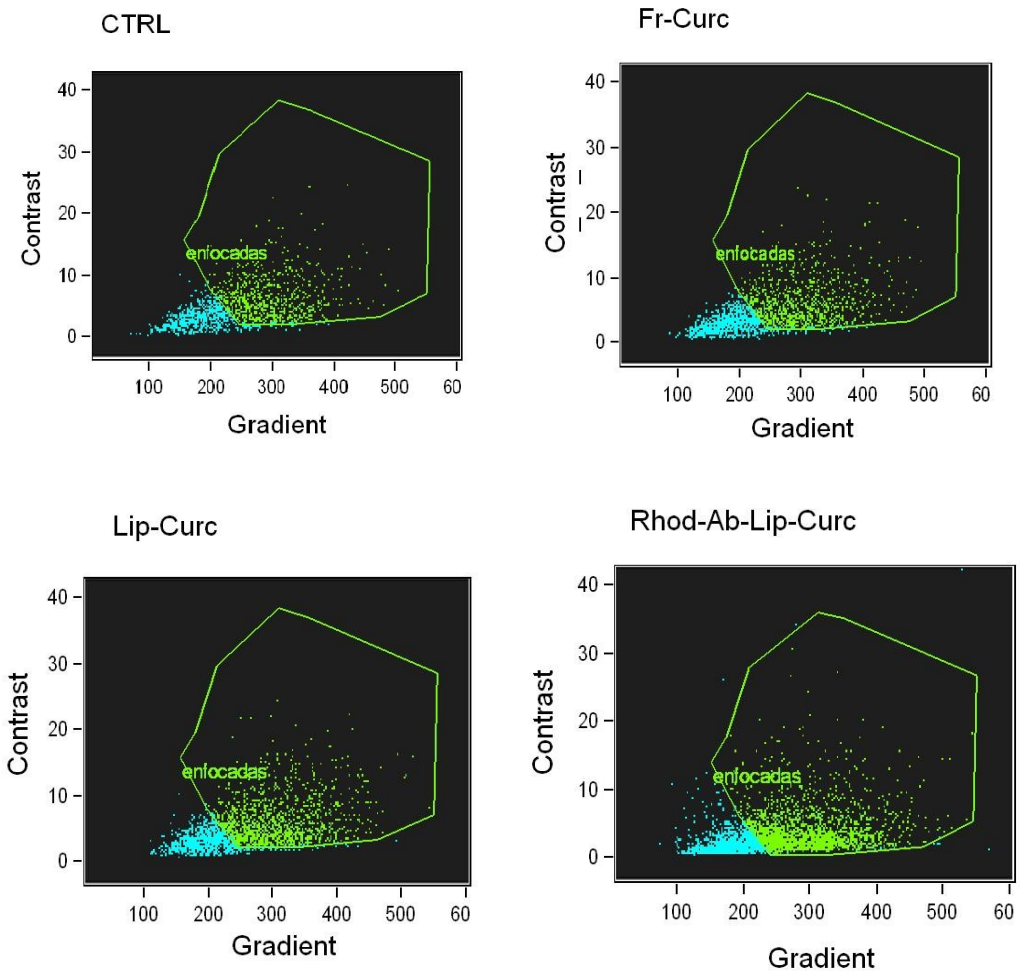
By using the ImageStream analysis software (IDEAS), curcumin uptake and liposome incorporation to cells were determined and compared between

JIMT1 cells that overexpressed HER2/neu antigen and MCF7 cells with a low HER2 expression level.

In **Fig. 37** the dots indicate MCF7 and JIMT1 cell populations. Green dots show the focused cells, while blue dots represent the cells that were excluded from the analysis because were considered cellular debris.

A

MCF7


CTRL
Fr-Curc
Lip-Curc
Rhod-Ab-Lip-Curc
Contrast
Gradient
enfocadas
0
10
20
30
40
100
200
300
400
500
60

B

JIMT1

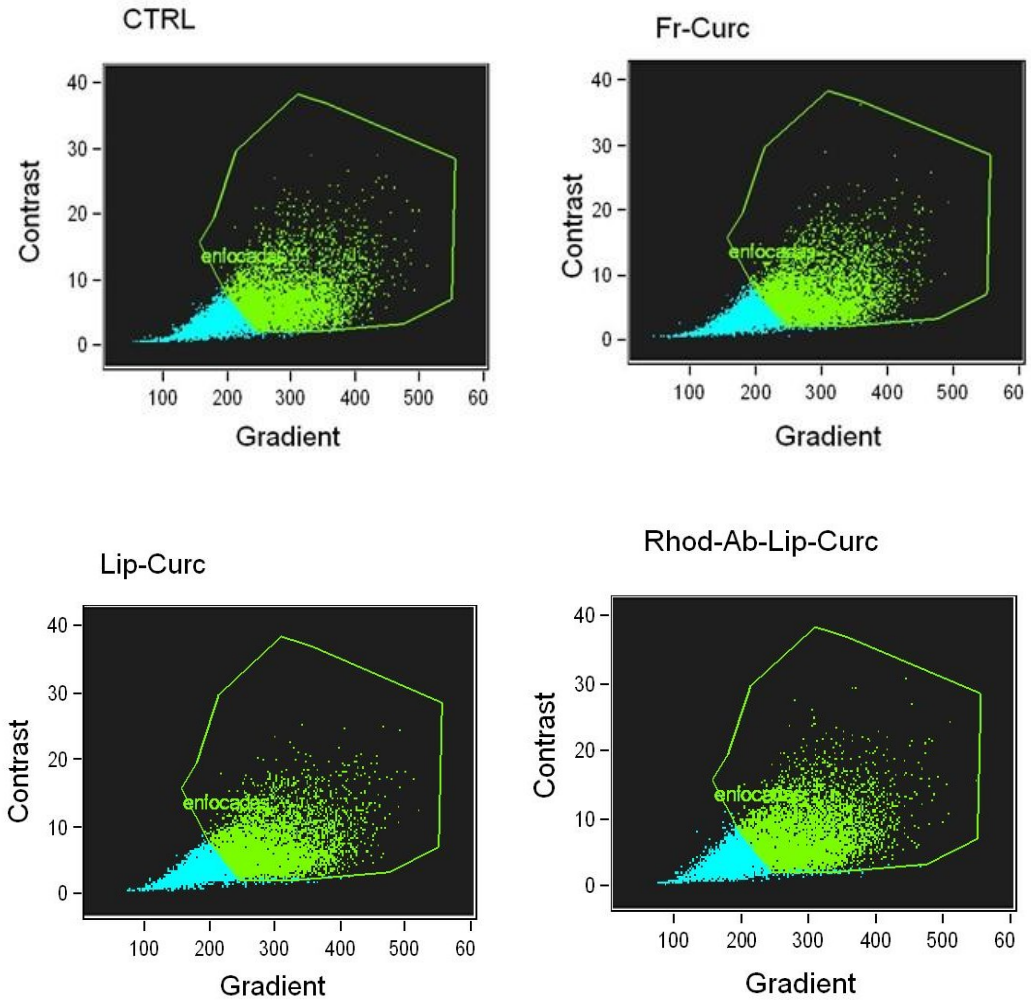


Figure 37 MCF7 and JIMT1 cellular population in the quantification assay of curcumin's uptake.

Plots of area vs gradient of MCF7 (A) and JIMT1 (B), in cells treated only with medium (CTRL), or after incubation with free curcumin (Fr-Curc), liposomed curcumin (Lip-Curc) and immunoliposomed curcumin (Rhod-Ab-Lip-Curc).

In **Fig. 38** only focused cells obtained from the previous plots (Fig. 37) were considered for further fluorescence's quantification. Peaks, represent the amount of curcumin incorporated into the cells; the control represent fluorescence background of untreated cells, indicated by blue lines and considered as negative labeled. Orange lines indicate cells with fluorescence intensity due to drug's uptake, the percentage of these positive labeled cells was represented in **Table VII**.

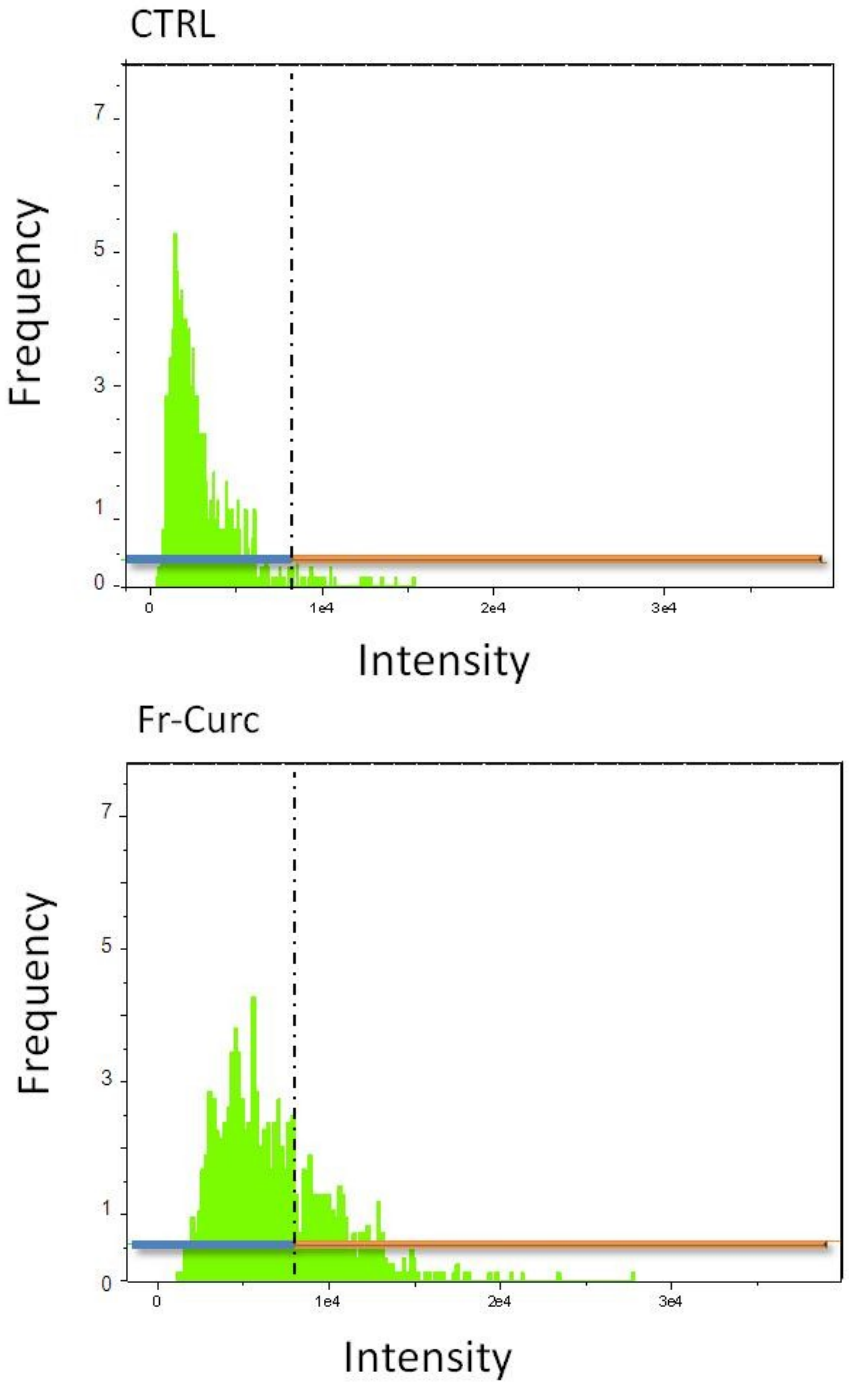
The intensity of the peaks varied depending on the type of treatment used. The intensity was greater for liposomed curcumin with respect to free curcumin in both cell lines. In the case of immunoliposomes containing curcumin, fluorescence intensity was higher compared to both liposomed curcumin and free curcumin. This behavior was consistent both in JIMT1 and MCF7 cell lines.

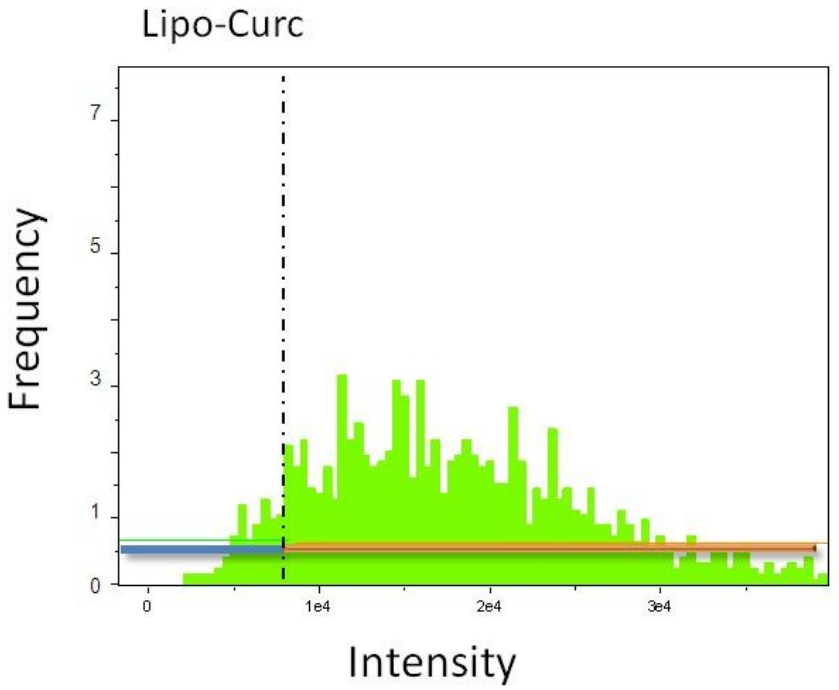
Nevertheless, in the case of JIMT1 cells, cell population with fluorescence due to curcumin-loaded immunoliposomes treatment was higher compared to curcumin-loaded liposomes lacking antibody. On the contrary, smaller differences were observed in the case of MCF7 cells comparing the same administration systems. Anyhow, the results also showed that curcumin-loaded liposomes are more effectively incorporated in JIMT1 cells than in MCF7 cells, indicating that passive transference or uptake of liposomes by JIMT1 is more effective. These results are in agreement to that one observed in the cytotoxicity assays and could explain the differences observed between the two cell lines.

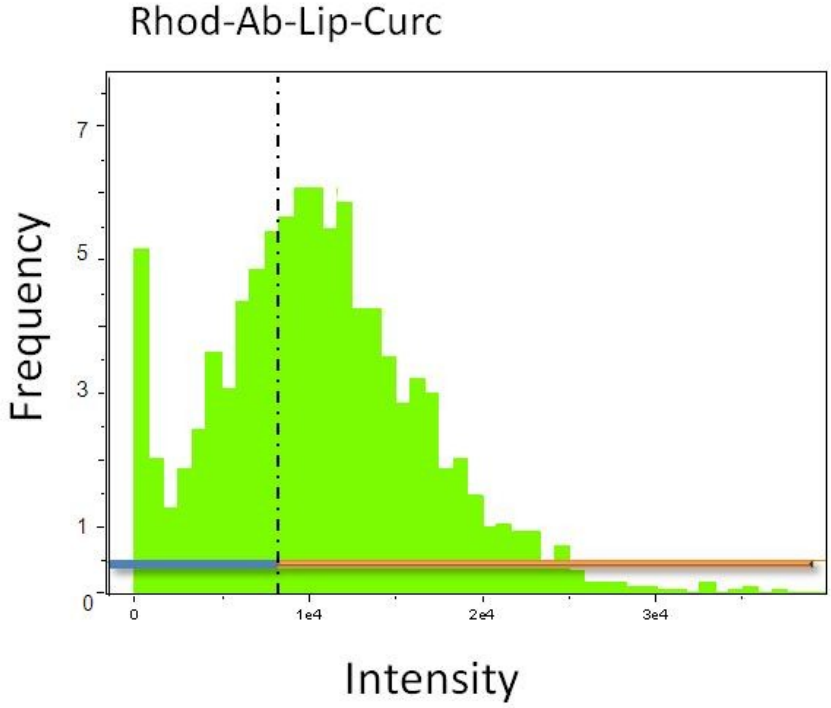
This brings us also to the conclusion that not only immunoliposomed preparations penetrate most effectively into cells, but their uptake is closely related to the presence of HER2 receptor on cells' surface.

A

MCF7




Lipo-Curc
Frequency
7
5
3
1
0
0
1e4
2e4
3e4
Intensity


Rhod-Ab-Lip-Curc
Frequency
7
5
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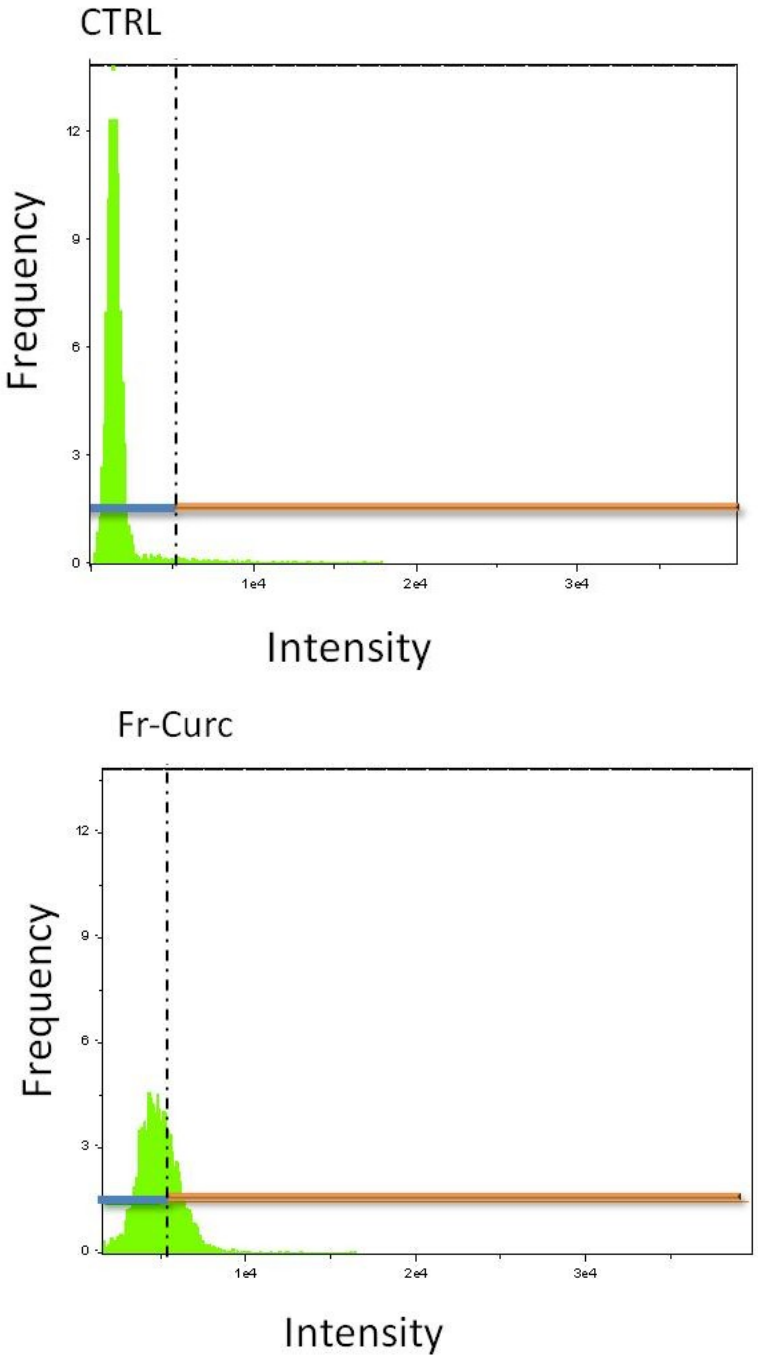
1
0
0
1e4
2e4
3e4
Intensity

B

JIMT1


CTRL
Frequency
12
9
6
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Intensity
Fr-Curc
Frequency
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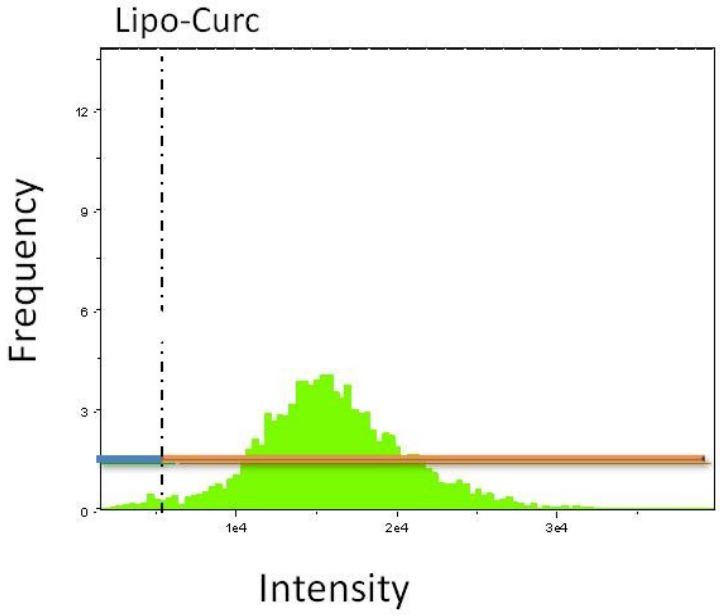
Intensity


Lipo-Curc
Frequency
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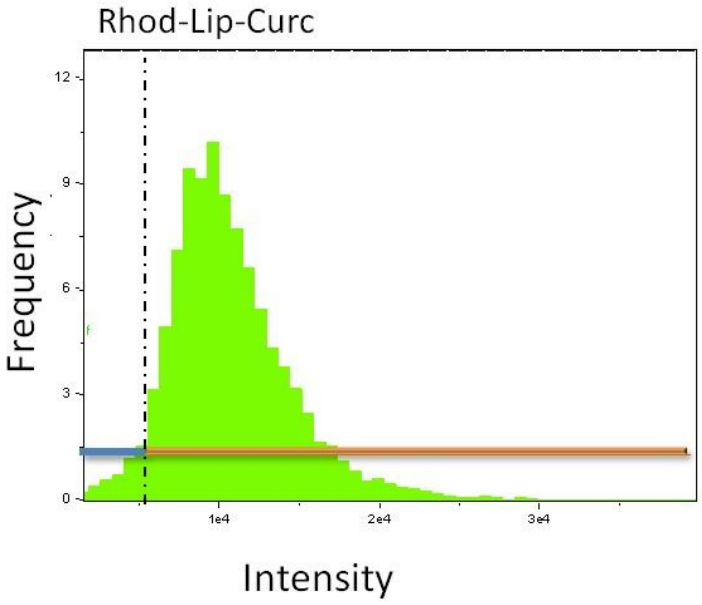
Intensity


Rhod-Lip-Curc
Frequency
12
9
6
3
0
1e4
2e4
3e4
Intensity

C

Type of treatment	% of labeled cells (in MCF7)	% of labeled cells (in JIMT1)
CTRL	1.01	1.64
Fr-Curc	26.9	11.8
Lip-Curc	69.5	51.8
Rhod-Ab-Lip-Curc	59	87.9

Figure 38 Quantification of curcumin uptake in MCF7 and JIMT1 cell lines treated with Fr-Curc, Lip-Curc and Rhod-Ab-Lip-Curc after 2 hours.

Figures show frequency vs. intensity plots of a) MCF7 and b) JIMT1 cell lines treated with medium (CTRL), free curcumin (Fr-Curc), liposomed curcumin (Lip-Curc) and immunoliposomed curcumin bearing rhodamine. Blue lines indicate the fluorescence background of untreated cells, orange lines indicate fluorescence intensity due to curcumin's uptake that was quantified and reported in table (c) as percentage of cells respect to total population.

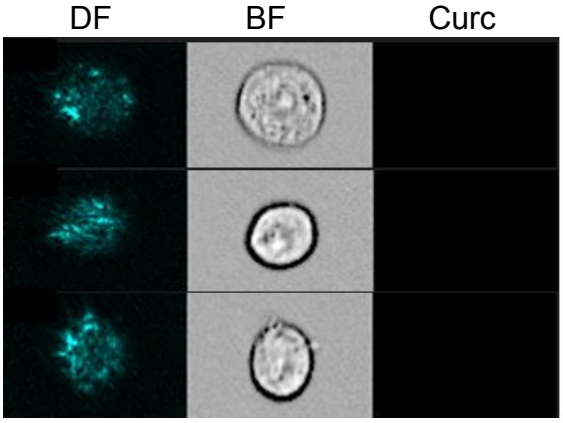
The **Fig. 39** represents a gallery of the most representative images of the assay. In this case cellular uptake is not represented as graph intensity vs. frequency, but as representative images of individual cells at different treatments. Cells were gated by graphing mask that includes darkfield (DF), bright-field (BF), curcumin fluorescence (Curc) and also rhodamine fluorescence (Rhod), in case of immunoliposomes. Pictures show a progressive fluorescence's increase, passing from free to liposomed form. The strongest signal due to curcumin was observed in JIMT1 cells treated with immunoliposomes, compared to MCF7 treated in the same way. The pictures also show the intracellular location of curcumin in the treated cells. Rhodamine, which reflects the presence of labeled lipids deriving from the liposomes, shows a signal significantly higher in JIMT1 compared to MCF7 cells, confirming a stronger level of binding of immunoliposomes to JIMT1 cells.

This result demonstrates both the efficacy and selectivity of the immunoliposomed system. Comparing the three different forms of delivery, results clear, in both cellular populations, an improved of curcumin uptake in the case of delivery with immunoliposome. Although in MCF7 cell line there was a light increase of the fluorescence in the immunoliposomed form compared to liposomed one, in case of JIMT1 this increase was more accentuated.

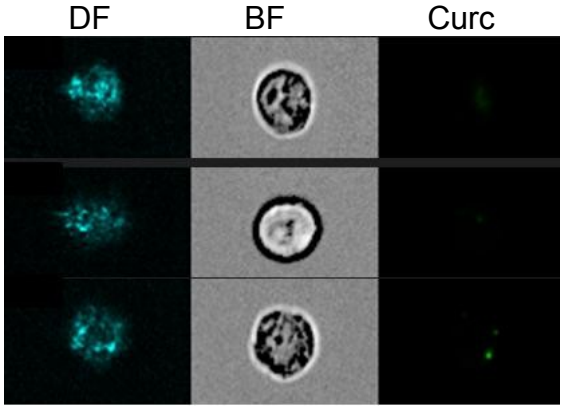
A

MCF7

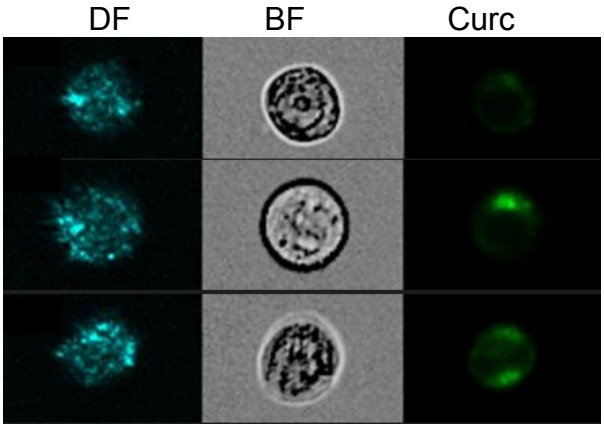
CTRL



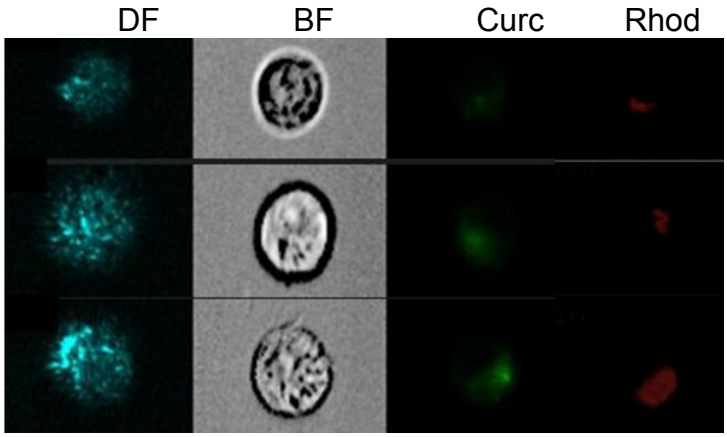
Free-Curc



Lip-Curc



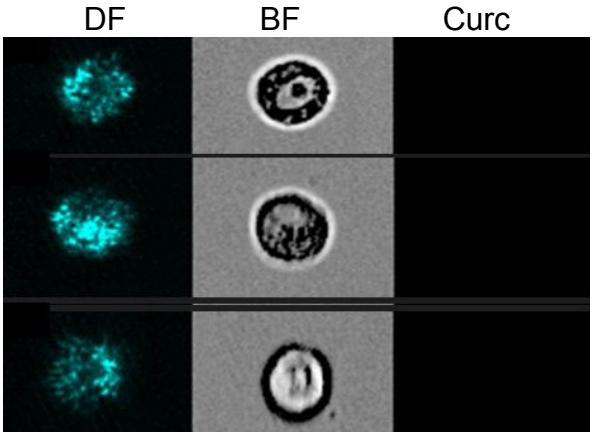
Ab-Lip-Rhod-Curc



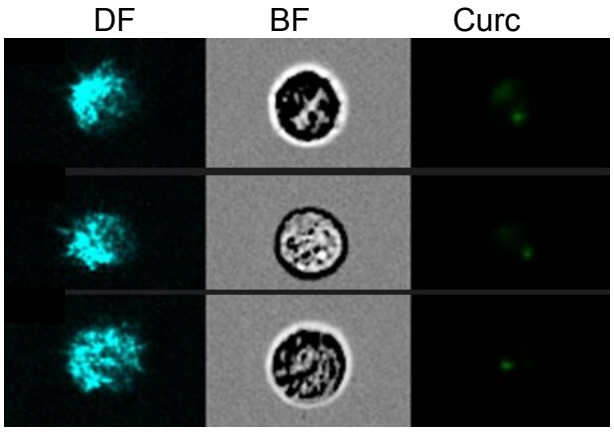
A

JIMT1

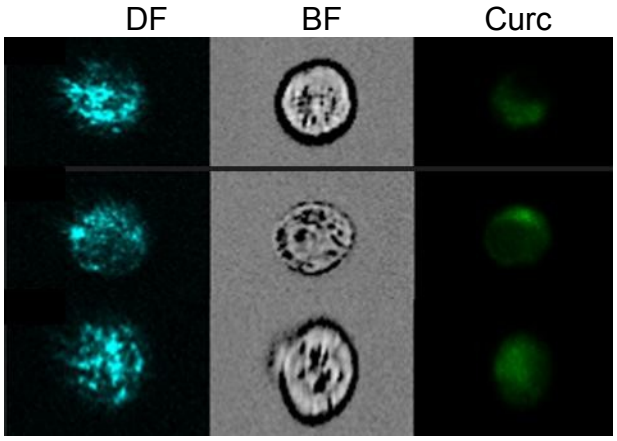
CTRL



Free-Curc



Lip-Curc



Rhod-Ab-Lip-Curc

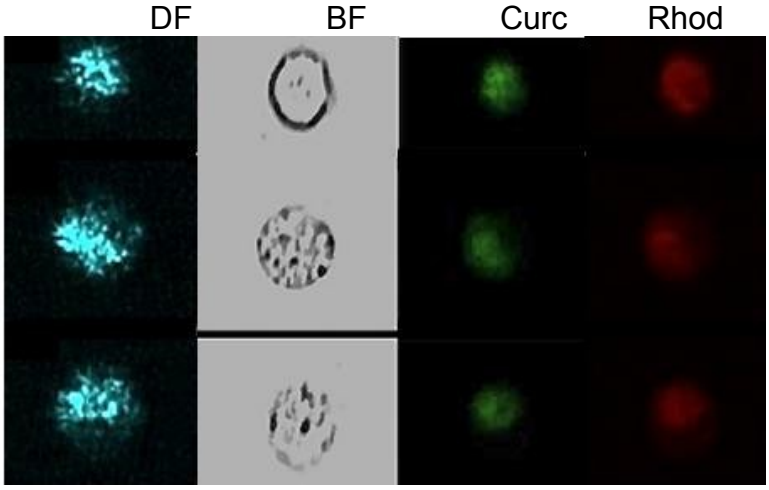


Figure 39 Representative images gallery of MCF7 and JIMT1 cells.
Representative compensated images of MCF7 **(**A) and JIMT1 (B) cells selected at random from files of 5000-9000 images. Channels represent (indicate) images of darkfield (DF), bright-field (BF), curcumin (Curc) and Rhodamine (Rhod) fluorescence.

Discussion

Curcumin and Resveratrol as chemopreventive agents

Among bioactive compounds, polyphenols represent the most intriguing and studied class of compounds that can be therapeutics for a large spectrum of the most common diseases, including cancer [208]. Curcumin and Resveratrol are two polyphenols that have gained much popularity and attention for their chemopreventive effects in cancer. The interest that allow us to choose these two drugs for our research, arise from their plurality of effects, pharmacological safe and effectiveness. Curcumin has broad biological and pharmacological properties including anti-inflammatory, antiviral, anti-infective, anti-proliferative and anti-oxidative effects [102,209]. Curcumin can suppress activation of NF-κB and the expression of various NF-κB regulated genes including COX-2, Bcl-2, TNF, MMP-9. It can also induce apoptosis through consecutive activation of caspase 8, cytochrome c release, caspase 9 and caspase 3 [103,210]. It has potent chemopreventive and anti-cancer activity on various human malignancies but is non-toxic towards normal cells [209]. RESV exhibits cancer chemopreventive activity in MCF-7 breast cell lines [132]. Many studies have shown that RESV suppresses growth of breast cancer cell lines inhibiting the activity and expression of several enzymes that play key functions in the regulation of the cell growth and apoptosis. The manner by which resveratrol inhibits cell growth probably involves a combination of induction of apoptosis and disruption of cell cycle control. The loss of cell cycle control and lack of normal apoptosis are two of the hallmarks of malignant cells. The effects of RESV on cell viability and proliferation appear to be concentration and cell-type dependent [211–213].

Cellular model

Cytotoxic effects of selected drugs were tested on two different breast cancer cell lines: MCF7 and JIMT1, for two main reasons:

- Clinical relevance: many breast cancers are estrogen-dependent, i.e., they depend on estrogen hormones for their development and growth; MCF7 cell line, shows a positive ER expression and suppose a model for these clinical cases. On the other hand, JIMT1 cell line, lacking expression of estrogen and progesterone receptors represents clinical cases hormone-independent.
- Different HER2-expression: HER2 represents the target of choice for the immunoliposomed systems. It is overexpressed in 20-30% of breast and ovarian cancers [44,45] and is pathologically associated with aggressive disease, increased risk of relapse and poor long-term survival [43, 50]. JIMT1 shows gene amplification and overexpression of HER2 [192-193], but at the same time, it is resistant to the conventional therapy used for HER2-overexpressing breast cancers based on trastuzumab and another HER2 inhibiting drugs. In contrast, MCF7 cell line presents a very low HER2 expression level.

Cytotoxic assays with free drugs

The first step of the research was to evaluate the cytotoxicity of selected drugs as free compounds. The treatments were performed for 72 hours in order to test both cytotoxic than cytostatic effects. The results obtained demonstrated that both cell lines are sensitive to curcumin and resveratrol in a dose-dependent manner, but with a different sensitivity. Free curcumin resulted to be the most effective, with similar activity in both cell lines (IC_{50} values were approximately 0.097 mM for MCF 7 and 0.1 mM for JIMT1).

MCF7 cells were more sensitive to resveratrol (IC_{50} 0.14 mM) compared to JIMT1 (IC_{50} 0.28 mM); the different response of these cell lines, could be related to mechanism of action of resveratrol. Several studies reported resveratrol as agonist of estrogen receptor, but many conflicting reports have

also appeared, and the effect of resveratrol on ERs is controversial [214-217]. Nevertheless, this could explain the greater sensitivity of the hormone-dependent MCF7 cell line, compared to JIMT1 lacking of estrogen receptor.

The free curcumin-resveratrol combination (molar ratio 1:1.6) was intended to improve the efficacy of the therapeutic potential of two drugs that present a wide spectrum of action and may have complementary cellular targets. Both compounds are hydrophobic, and then any kind of phospholipid vesicles may suppose an appropriate form of delivery for these compounds. As far as we are concern, no studies on the combination of these two compounds on breast cancer cells are available in the literature. The combination of the two compounds didn't seem to improve the therapeutic effect of the single compounds in the case of JIMT1 cells probably due to the resistance of these cells to resveratrol. In contrast, the combination improved the IC_{50} value compared to single compounds in the treatment of MCF7 cells. Nevertheless a simple additive but not synergistic effect of the two compounds was observed.

Optimization of drugs' encapsulation into liposomes and immunoliposomes

Once the effects of free compounds were analysed in cellular models, the incorporation of these compounds in phospholipid vesicles (liposomes) was attempted.

First, the compounds were incorporated into liposomes of optimized composition but lacking antibody in order to assess a potential increase of their therapeutic efficacy. Second, the compounds, resveratrol, curcumin and both together, were loaded onto Immunoliposomes (ILs) bearing the anti-HER2 antibody covalently attached to their surface (IL-Curc, IL-Resv and IL-Curc+Resv). The aim was to decrease the previously obtained IC_{50} values and to develop a selective therapy for breast cancer cells overexpressing HER2.

The process of encapsulation of single drugs into liposomes was quite

simple. However, the incorporation of both compounds into liposomes and their incorporation into IL-systems (immunoliposomes), either combined or as single compounds, required a series of trials to optimized the amount of bioactive compounds incorporated.

No previous studies on the incorporation of curcumin and resveratrol in liposomes or immunoliposomes are reported in the literature.

In 2009, Narayanan et al., demonstrated that the combination of liposomes containing curcumin and liposomes containing resveratrol deriving from different preparations reduced prostate cancer incidence in PTEN knockout mice [218]. Their cytotoxic effects were studied for the single formulations and then co-administrated (lipo-curc and lipo-resv) together using half concentration of each compared to the single drugs preparations. In our studies curc and resv were encapsulated together into the same liposomed and immunoliposomed preparations.

We have chosen the coencapsulation of both drugs, rather than administering as a mixture of individual encapsulated compounds due to several reasons:

1) Each liposome preparation could affect the pharmacokinetic profile of the other one, thereby altering drug delivery to tumor cells.
2) Increase in the total dose of lipid administrated when the two drugs are separated, with the subsequent increase of cytotoxic effects.
3) When the two compounds are in different liposomes, two different liposomes need to reach the same target cell to exert their pharmacological effect. In the case of the combinated liposomes, this occurs as a single event and then increases the pharmacological feasibility for *in vivo* treatments.

Regarding the last issue, the probability of resistance to a single chemotherapeutic agent in vivo is high when compounds are in separated vehicle, since a visible tumor contains $\geq 10^9$ cancer cells with large heterogeneity. Therefore, it is very important to expose the cancer cells simultaneously to more than one agent.

In order to overcome these obstacles, an effective procedure to co-

encapsulate the two drugs was developed.

The first goal of the preparation of liposomes containing both drugs was to maintain the same molar ratio curc:resv (1:1.6) as in the free system. Nevertheless, when the combined compounds were incorporated in the same preparation, it was quite difficult to maintain the same molar ratio due to the limited water solubility of the compounds when added to the liposome preparation.

Tests performed in liposomes, allowed to obtain a molar ratio curc:resv of 1:3.6; this means that liposomes preferentially encapsulate resv, when both compounds are present in the same formulation.

These optimizations became even harder for the IL-systems, both for the incorporation of single drugs and also for the combined system. A series of tests were performed varying single conditions at a time (temperature, time of incubation, solvent), and evaluating which was the most promising one.

Optimal conditions were achieved using the most complicated system, i.e. immunoliposomes containing both drugs (IL-curc+resv), then conditions were extrapolated and used for the other IL systems containing single compound.

Regarding the time at which compounds needed to be added, the best option was to add the compounds in a low concentration of organic solvent once the immunoliposomes were formed and separated for the unbound antibody and the non-encapsulated material. Then the immunoliposomes were chromatographied again for further purification (option 1).

In previously reported liposomal drug combinations, one of the drugs was passively loaded into the liposomes, whereas, the other one was remotely loaded [219-221]. In remote loading, the drugs to be encapsulated are introduced once the liposomes were formed. The use of remote loading for both drugs, as in our studies, supports similar physical stability. Moreover, remote loading enables to load more drug into the liposomes and to control better the drug release compared to passive loading [222].

The results obtained in the case of combined incorporation of curc and resv for ILs systems show that molar ratio curcumin:resveratrol was 3:3.1. A very similar result than that obtained for liposomes' system, but different

compared to free drugs. Liposomes and ILs seemed to prefer the incorporation of resv rather than curc when the two compounds are co-encapsulated. The different chemical structures of the compounds (curcumin more hydrophobic than resv) might explain that curcumin doesn't complete solubilise in the solvent and precipitate when it comes to only 5% organic solvent in the media where liposomes are present, reducing in this way encapsulation's efficiency. This hypothesis is supported by the evidence that the same behaviour occurred both in liposomes and in ILs preparation with a very similar final molar ratio. On the other hand, in the free system, where the drugs were only dissolved in DMSO, the molar ratio curcumin:resveratrol 1:1.6 still shows a preference to resv, but lower compared to liposomed and immunoliposomed preprations.

Cytotoxic assays with liposomed drugs

Once the liposomed preparations were optimized, we tested their effects on cellular cytotoxicity, compared to free drugs. Results showed an improvement of the efficacy of liposomed systems in all cases, with a halving of IC_{50} values in all conditions, except in the case of the treatment of JIMT1 cells with the resveratrol system, which IC_{50} value was decreased more than three fold. When the two compounds were combined in the same preparation and MCF7 cells were treated, IC_{50} global value decreased almost to the half but the higher contribution was due to their curcumin content. In contrast, when the combined liposomed system was used to treat JIMT1, no improvement of the global IC_{50} value was observed compared to the compounds in their free form. Anyhow the contribution of curcumin in the liposomed system seemed to be more effective (0.117 in the free form vs. 0.070 in the combined one). Therefore, curcumin seems to enhance their cytotoxic activity in the liposomed system when resveratrol is present.

Cytotoxic assays using Immunoliposomes and comparative effects of the three delivery systems

Effectiveness of immunoliposomes *in vitro* was reflected in the significant reduction of IC_{50} values required for cytotoxic activities against the two different cancer cell lines. JIMT1 and MCF7 were sensitive to free drugs, especially to free curcumin with IC_{50} values ranging from 0.1 to 0.097 mM for free curcumin, 0.28 to 0.14 mM for resveratrol and 0.29 to 0.12 mM for the combination of both. The sensitivities of these cells toward liposomal preparations were significantly different. These features provided an argument for us to evaluate the effectiveness of anti-HER2 immunoliposomes systems respect both free and liposomed compounds. Cellular responses to the treatments based on anti-HER2 immunoliposomes differed between the two cell lines in agreement with their HER2 expression.

As shown in Fig.36 in JIMT1 cells an average from 14 to 5-fold reduction was achieved in comparison to IC50 values of JIMT1 cells treated with free drugs, while in MCF7 the average of reduction was weaker, from 3 to 1.7-fold. These results suggest all three immunoliposomed systems are highly effective in killing cells, probably due to their high selectivity because the cytotoxic effect occurs mainly and most markedly in the targeted cell lines overexpressing HER2.

Surprisingly, when the combined immunoliposomed system was used to treat JIMT1 cells, IC_{50} value decreased 13 fold compared to liposomes, i.e. from 0.281 to 0.021. Based on the comparison of the IC_{50} values between the combined systems (liposomed and immunoliposomed) and the molar ratio of the compounds, curcumin improved its performance around 12 times (0.070/0.006) and resveratrol 14 times (0.210/0.015). This phenomenon was not so dramatic when the IC_{50} values of liposomed and immunoliposomed single compounds were compared. Therefore, in this particular case a possible synergistic effect of both compounds incorporated into immunoliposomes could be assumed.

Briefly, the data obtained on breast cancer human cell lines, indicate that the combination of drugs with liposomes, and even more with immunoliposomes,

significantly enhances the cytotoxicity of the active compounds. This finding, although expected, is not granted, as in the literature JIMT1 is reported to be a cell line resistant to conventional therapy based on the use of the antibody trastuzumab [193].

The striking sensitivity of JIMT1 cell line towards immunoliposomes, compared to MCF7 cell line, may be due to the higher level of HER2 expression on JIMT1 cellular surface.

In addition, previous studies, conducted in the laboratory where this research project was developed, showed that there were no visible effects on cell viability of JIMT1 or MCF7 cells when incubated with the free antibody even at high concentrations (100 μg/mL) for 4 days of treatment [195].

Cellular uptake

The results obtained in the cytotoxicity assays have fully demonstrated the selectivity of immunoliposomed systems and a high grade of efficacy especially in the case of the curcumin system.

Immunoliposomes' specificity to HER2-overexpressing cells was also demonstrated using the novel technique ImageStream, multispectral imaging cytometer which combines the sample handling and quantitative power of flow cytometry with high-resolution brightfield, darkfied, and fluorescence image gallery. Each cell is imaged in flow, allowing for acquisition rates of over 100 cells/second. The IDEAS™ post-acquisition analysis software is able to quantify over 200 parameters for each cell.

System based on curcumin was selected among all, because it showed significant differences among the free, liposomed and immunoliposomed systems in the cytotoxicity assays and also because curcumin shows autofluorescence, so its uptake can be easily measured without staining with probes.

MIFC experiments show that the incorporation of curcumin into the two breast cancer cell models is more effective when this compound is administered in a liposomed form, and even more effective when the vehicle

are immunoliposomes. In addition, these increase is even more dramatic for JIMT1 cells compared to MCF7 cells, what is in agreement to their higher level of expression of the HER receptor. The amount of fluorescence in cells treated with liposomed curcumin is between 2.6 and 4.4 fold higher, in MCF7 and JIMT1 cells respectively, than that for the treatment with free curcumin. When immunoliposomed curcumin was used, no improvement was observed compared to liposomed curcumin for MCF7 cells. In contrast, a 1.7 fold fluorescence increase was observed for JIMT-1 cells. These results are in correlation to the higher level of HER2expression in JIMT1 cells compared to that of MCF7 cells. These results are also in agreement with the fluorescence images of isolated cells, which show the intracellular location of curcumin in the treated cells.

The ability of anti-HER2 immunoliposomes to deliver curcumin in an intracellular manner is significantly more important than even for both free curcumin than liposomed curcumin in JIMT1 and provides for a greater degree of specificity in delivery to HER2-overexpressing cancer cells, instead this result was significantly lower in MCF7 in which liposomed form resulted more efficacy compared to Immunoliposomes formulations.

In summary, the results obtained in MIFC experiments are in agreement to those of the cytotoxicity assays and prove that anti-HER2 immunoliposomes loaded with curcumin are more effective to decrease cell viability that liposomed curcumin or free curcumin in human breast cancer cell lines. The observed cytotoxic effect is in correlation to the HER2 expression of the cell line and also with the cellular uptake of curcumin..

Conclusions

Conclusions

1. JIMT1 breast cancer cells are less resistant to free curcumin than resveratrol, an agonist of estrogen receptor, which might be related to the lower estrogen receptor expression of JIMT1 cells.

2. The incorporation of curcumin or resveratrol into liposomes of specific composition improves their cytotoxic effect on human breast cancer cell models through the decrease of their IC50 values at an average of 2-3 fold.

3. The incorporation of relatively hydrophobic compounds into immunoliposomal delivery systems can be achieved by the incubation of formed immunoliposomes with the compounds in 5% DMSO for 1 h at 37ºC, without affecting immunoliposomes viability.

4. The incorporation of curcumin or resveratrol into immunoliposomes improves its cytotoxicity on breast cancer cells through the decrease of their IC50 values at an average of 1.5-4 fold decrease compared to liposomes, and an average of 3-8 fold decrease compared to free compounds. The effect was especially intense for the treatment of JIMT1 cells with immunoliposomed curcumin, in correlation to their higher HER2 expression level.

5. The cytotoxicity of immunoliposomes containing curcumin and resveratrol together on JIMT1 cells was dramatically enhanced compared to liposomes containing both compounds, i. e. 12-14 fold decrease. A possible synergistic effect of both compounds incorporated into immunoliposomes is proposed, which deserves further attention.

6. The treatment of breast cancer cells with immunoliposomed curcumin incorporates higher amounts of curcumin and lipid deriving from the delivery system in targeted cells, than that observed for liposomed or free curcumin. In addition, this effect was more intense in JIMT1 cells than in MCF7 cells, in correlation to their higher HER2 expression level. These results were also in agreement with the higher cytotoxic activity of the immunoliposomed curcumin. Therefore, the presence of the antibody in the immunoliposomes provide with a high degree for specificity in HER2-overexpressing cell lines.

7. Anti-HER2 immunoliposomes loaded with bioactive chemopreventive compounds may suppose a potent and selective cytotoxic therapeutic delivery system against HER2-overexpressing breast cancer cells, and may increase drug concentration at the tumor sites. Anyhow, whether the proposed immunoliposomal formulations might offer therapeutic advantages or not for the treatment of breast cancer *in vivo*, at least in animal models, remains to be elucidated in future studies.

List of Abbreviations

List of Abbreviations

ADCC antibody-dependent cellular cytotoxicity
AIF apoptosis inducing factor
AUC area under curve
BC breast cancer
BRCA1 and **BRCA2** Breast Cancer genes 1 and 2
Chol cholesterol
COX cyclooxygenase
Curc Curcumin
D200M 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (PEG 2000]
DDS Drug delivery system
DMEM Dulbecco's modified Eagle medium
DMSO Dimethylsulfoxide
DNA deoxyribonucleic acid
ECD extracellular domain
ECM extracellular matrix
EDTA Ethylenediaminetetraacetic acid
EndoG endonuclease G
EPR enhanced permeation and retention
ER estrogen receptor
ETOH Ethanol
EYPC egg yolk phosphatidylcholine
FBS fetal bovine serum
FDA Food and Drug Administration
HER2 human epidermal growth factor receptor 2
HPLC High performance liquid chromatography
IGF-1R insulin-like growth factor-1 receptor
IC_{50} inhibitory concentration 50%
IL Interleukin
MAPK mitogen-activated protein kinases
MLVs multilamellar vesicles
LUVs unilamellar vesicles

MMPs Matrix Metallo proteinases

MPS mononuclear phagocyte system

MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)

NF-κβ nuclear factor-kappa β

NK natural killer

PBS phosphate buffered saline

PDI polydispersity index

PEG Polyethylene glycol

PI3K Phosphatidylinositol 3' kinase

PR progesterone receptor

RB (Retinoblastoma gene)

RES reticuloendothelial

Rhod-PE Lissamine rhodamine B 1,2-dihexa-decanoyl-sn-glycero-3-phosphoethanolamine

ROS reactive oxygen species

RT room temperature

RTKs receptor tyrosine kinases

SUVs small unilamellar vesicles

TBS Tris-buffered saline

TDLU terminal ductal-lobular unit

TGF-α Tumor Growth Factor alpha

THIS Histidine buffer

TP53 Tumour Protein_53

VEGF Vascular endothelial growth factor

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