The effects of the nanosphere carrier for the combined carmustine-busulfan trastuzumab in human breast cancer tissue culture

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Abstract
Cancer of the breast is from the highest cancer type’s incidence. Cancer in general represents a high therapeutic challenge. Considerable adverse effects and cytotoxicity of highly potent drugs for healthy tissue require the development of novel drug delivery systems to improve pharmacokinetics and result in selective distribution of the loaded agent. Targeted therapy is a novel maneuver to achieve proper selectivity index. And as the main goal of nanocarriers is to target specific sites and improve the circulation time of the drug which is entrapped, encapsulate or conjugate in the carrier system so we chose nanoliposome as a drug carrier. Liposomes improved a potent drug targeting successfully in the last decade, but nanoliposomes offer more surface area and they have more solubility, improve controlled release, enhance bioavailability, and permit precision targeting of the material that is encapsulated to a greater extent. The Aims and objectives of the study is the formulation of HER2 Ab directed nanosphere carrier for a combined Carmustine-busulfan and trastuzumab (LCBT), then Assessing antineoplastic efficacy of (LCBT) in lung carcinoma cell line. A dose-dependent cellular growth inhibition on all three cell lines (P-value < 0.001) was seen. The concentration of 100 µg/ml of LCBT show highly significant inhibitory effect (P-value < 0.001) on cancer cell line in comparison to other concentration and other positive control drugs that forming the combination separately (P-value <0.001). The means of the half-maximal inhibitory concentration (IC50) values for LCBT combination show highly significant difference (P-value < 0.001) from other positive control groups. At the same time, the interaction indexes IAI for LCBT and for LT and LCB separately show synergistic effect that was less than one. The results of the present study also revealed a reduced expression percentages of proliferation marker Ki67 in MCF-7 breast cancer cell line that was treated with IC50 of LCBT and positive control (LT, LCB, T and CB) after 48 hour from drug exposure in comparison with the negative control group (P-value <0.001). Also, there was a significant decrease in expression percentages in CB containing positive control groups in comparison to T containing positive control (P-value <0.01). From the above results, we conclude that the LCBT has a prominent anticancer effect in MCF-7 breast cancer cell line as it significantly decreased the growth percentage and show apoptotic effects.

Keywords: Carmustine, busulfan, trastuzumab, nanoliposomes, MCF-7 cell line, cytotoxicity.


Introduction
Cancer is considered as a complex illness characterized by abnormal growth and extent of abnormal cells [1]. Breast cancer is defined as an overgrowth of the healthy cells within the milk-producing glands, such as ducts and lobules. Breast cancer classified according to the specific cell receptors status mainly progesterone receptor, estrogen receptor, and the human epidermal growth receptor (HER2) [2]. All treatment modalities have many side-effects includes loss of appetite nausea and vomiting, diarrhea, decrease in blood cell counts, allergic reaction, bleeding, joint pain, change in taste, tingling or numbness in the fingers or toes, hair loss, and weight gain [3-4]. Nanomedicines are nanoscale implements (1–1000 nm-sized) used in the prevention, treatment, and diagnosis of diseases [5]. Nanocarriers or drug delivery system has very significance includes elongated circulation half-lives, better pharmacokinetics and compact side effects [6]. Drugs loaded into nanoparticles by entrapment, adsorption, or chemical conjugation, their Pharmacokinetics and therapeutic index can be improved as compared to the free drugs. Advantages of nanoparticles drug delivery, principally at the systemic level, that include improve drug solubility in serum and provide sustained, controlled, combined and targeting drug delivery also it can cross the tissues and organs’ biological membranes in compare to larger-sized particles [7].
Nanoliposomes have the same structural, chemical and thermodynamic properties, as Liposomes but in compared to Liposomes, they offer additional surface area, they also have the tendency to surge the solubility, improve controlled release, improve bioavailability, and permit exact accuracy in targeting of the encapsulated material [8]. Carmustine is a highly lipid-soluble nitrosourea compound. It is a bifunctional alkylating agent, that alkylates both DNA and RNA, so can cross-link DNA, and by carbamoylation, it can inhibit several enzymes. It is a cell-cycle phase nonspecific. [9]. Busulfan is one of the alkylating agents that have a bifunctional action. Following systemic absorption carbonium ions are formed quickly, resulting in DNA alkylation, which results in breaks of the DNA molecule with double strands cross-linking, causing interference of DNA replication and RNA transcription. It has cell cycle phase-nonspecific action [10]. Trastuzumab is a monoclonal IgG antibody human type that is targets HER2 selectively, it inhibits the HER2 over expressed tumor cell growth that includes breast, lung, gastric, prostate, and ovarian cancer cells. Mechanisms involved are activating antibody-dependent cell-mediated cytotoxicity, decreasing VEGF production, inhibiting intracellular signaling pathways and G0/G1 cell cycle cytotoxicity [11]. The cancer cell lines availability is an important basis for cancer research. These cell lines offer an experimental model for therapeutic studies. The in vitro and in vivo techniques were used to investigate the inhibitory effect of many agents on these cell lines [12]. The amount of pKi67 present at any time throughout the cell cycle is regulated by an equilibrium between the degradation and the synthesis, as the half-life of Ki67 protein is 1-1.5 h [13]. It is only noticed in dividing cells (mitosis) in all active phases of the cell cycle (G1-, S-, G2- and M-phase) and not in inactive cells (G0 phase) [14]. Through mitosis, it submits to phosphorylation and dephosphorylation by protease degradation. In telophase and anaphase (end of mitosis phases), Ki67 levels sharply decline, the levels are peak during the beginning of mitosis, and it is of little amounts in the G1 and S phases [15]. The Ki67 is a proliferation marker to measure the growth segment of cells in human tumors. Its expression is strongly associated with cell proliferation and is broadly used in routine pathology; pKi67 is well described at the molecular level and widely used as a prognostic and predictive marker in cancer as well as in the diagnosis and treatment of a wide range of malignancies [16]. Moreover, photolytic pathways can regulate Ki67p expression the key regulatory complex is cycling B/CDK2 [17].

Materials and methods
This study was accomplished in the postgraduate Cancer Research Lab, at the College of Medicine/ University of Babylon, during the period from November 2018 to March 2019.

Cell lines
Human breast cell cancer cell line MCF-7 this line was obtained from the University of Dublin in Ireland as a gift. Cultivated and maintained in RPMI 1640 media supplemented with 10% FBS, 100 U/ml penicillin and 100mg/ml streptomycin, cultured as a monolayer in a 37°C incubator and 5% CO2 [18].

Drugs stocks Preparation
Liposomes of CBT, CB, and T were prepared by modified TFH technique and sonication which is a simple method for reducing the size of Liposomes to produce nanoliposomes [19]. Equal concentration from each drug and a half from liposome were used in the prepared mixtures. The two-fold serial dilutions from each of the LCBT, LCB, LT, CB, T and Lip was made at concentrations of (100, 50, 25, 12.5, 6.2, and 3.1) µg/ml. In vitro cytotoxicity assessment with crystal violet assay the crystal violet assay was performed according to the protocol of Feoktistova and his colleagues [20].

Pre-drug exposure cellular handling
When the cells of MCF-7 line reached 70-80% confluence growth, they were trypsinized, counted, and returned in RPMI 1640 cultivation medium and seeded in sterile flat bottom 96-wells plates. 200µl of cell suspension /well and incubated for 24 hours at 37°C and 5% CO2 incubator.

Cellular drugs exposure
Next day, the medium was aspirated from the plates and then they were treated with the new fresh sterile serum-free RPMI medium which was previously prepared and supplemented with scalar concentrations each of the tested agent’s that ranged from 100 to 3.1µg/ml, and a three control wells were incubated with only culturing medium without any drug or additives. The plates after that were incubated in 37°C for 48 hour.

Staining with crystal violet dye
After target treatments incubation time had been finished, the wells were washed with PBS, then 50µl of 0.5% crystal violet stain solution was poured to each well then the plates were incubated in 25°C for 20 min. The plates were then gently washed with tap water and dried. After that, 200µl of methanol was administered to each well then the plates were incubated at room temp for 20 min with gentle rocking, finally the optical density of each well was read with ELIZA plate reader [21]. The cellular growth inhibition was calculated according to the following equation Growth Inhibition % = 100-mean of the optical density of experimental wells/ mean of the optical density of control wells [22].

IC50 determination
The excel sheet was used to outline the dose-response curve to the different tested concentrations of the tested
drugs and their cellular GI%. It was achieved for the agents LCBT, LT, LCB, T, CB, and Lip on MCF-7 cell line. The values of IC50 were extrapolated and then calculated from the dose-response curves of tested agents [23].

**The calculation of the Interaction index (IAI) of drug combinations**

The IAI can be determined as follow:

\[
IAI = \frac{d_1 + d_2}{D_1 + D_2}
\]

D1 and D2 are the concentration required to make response y for the drugs 1 and 2 when they were used alone; d1 and d2 were the concentration in the combination that yields the equal response when the result =1 addition interaction present, more than 1 antagonism interaction and when it is less than 1 that mean synergistic interaction present [24].

**The Ki-67 immunohistochemical staining**

Staining procedure was performed according to the Dako/Denmark manufacturer instructions and the percentage was calculated as Dowsett, et al. (2011) stated [25].

**Statistical analysis**

Statistical analysis was done by using SPSS version 23. Analysis of variance ANOVA test was used to compare the different groups mean. P-value <0.05 was considered statistically significant. Variables were presented as mean ± SD with a 95% confidence interval. The half inhibitory concentration (IC50) of tested groups was calculated by Graph Pad Prism6.

**Results**

The cytotoxic effect of LCBT on MCF-7 cells were evaluated *in vitro* by C.V staining, a wide range of concentrations represented by (3.1, 6.2, 12.5, 25, 50, and 100 µg/ml) were used for LCBT, and for the positive control groups (LT, LCB, T, CB, and Lip). While the negative control group was treated with RPMI-1640 medium. After 48 hours incubation, the absorbance was measured for each well by ELISA reader and the growth inhibition (GI) percentages were determined. A dose-dependent cellular growth inhibition (P-value < 0.001) was seen. The concentration of 100 µg/ml of LCBT show highly significant inhibitory effect (P-value < 0.001) in comparison to other concentration and other positive control drugs that forming the combination separately with highly significant inhibition in comparison to the other positive treated groups (77.81±0.99 vs 51.95±1.67, 68.32±3.13, 47.01±2.05, 61.72±1.04, 21.38±1.04 and 9.74±0.67) for LT, LCB, T, CB, Lip, and with the negative control groups respectively (P-value <0.001). The lower concentrations of 6.2 and 3.1 µg/ml show no significance differences from negative control groups (p-value > 0.05) as shown in figure 1. The half-maximal inhibitory concentration (IC50) values for LCBT were highly significantly lower from other positive treated groups (P-value < 0.001). They were 51.8±1 µg/ml vs 87.5±0.9, 59.8±1, 100.8±2.6, 63.7±2.8 and 318±3.7 µg/ml for LCBT, LT, LCB, T, CB, and Lip respectively as shown in figure 2.

**Figure 1:** Percentage of growth inhibition produced by different concentrations of different tested groups on the growth of MCF-7 cells after 48h of exposure as evidenced by crystal violet represented by mean± standard deviation p-values <0.001.
Figure 2: Comparison between the mean of half-maximal inhibitory concentration (IC_{50}) values in different tested treatment groups on MCF-7 cell line represented by mean± standard deviation *= P value<0.05

The interaction indexes for LCBT were calculated and they show synergistic effect in MCF-7 cancer cell line that was less than 1, it was 0.37. Also, the IAI for LT and LCB was (0.21 and 0.16) respectively in MCF-7 line which is less than 1 for all as shown in table 1 below.

Table 1: Interaction Index (IAI) for tested compounds

<table>
<thead>
<tr>
<th>Drug/Compound</th>
<th>breast MFC-7 IC_{50} (μg/mL)</th>
<th>IAI in MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCBT</td>
<td>51.8</td>
<td>0.37</td>
</tr>
<tr>
<td>LT</td>
<td>87.5</td>
<td>0.21</td>
</tr>
<tr>
<td>LCB</td>
<td>59.8</td>
<td>0.16</td>
</tr>
<tr>
<td>T</td>
<td>100.8</td>
<td></td>
</tr>
<tr>
<td>CB</td>
<td>63.7</td>
<td></td>
</tr>
<tr>
<td>lip</td>
<td>318</td>
<td></td>
</tr>
</tbody>
</table>

(When IAI <1 that mean synergism effect, and if IAI =1 so additive effect occur, while when IAI >1 this mean an antagonism effect)

3.1.3. The IC50 of LCBT effects on proliferation of cancer cell lines:

The results of the present study revealed a reduced expression percentages of Ki-67 protein proliferation marker in MCF-7 cancer cell line that was treated with LCBT and positive control LT, LCB, T and CB in IC 50 of each of them separately after 24 hour from drug exposure in comparison with the negative control group (30.5±1.5, 53.5±5, 33±2.6, 50±2, 33.6±3.2 vs 75±4.3) respectively (P-value <0.001). The expression percentages of Ki-67P show highly significance difference within MCF-7 cells treated groups in which there was highly significant reduction in LCBT treated group from other positive control groups, with a significant decrease in proliferation percentage in CB containing positive control in comparison to T containing positive control (33±2.6 for LCB and 33.6±3.2 for CB vs 53.5±5 for LT and 50±2 for T (P-value <0.01). The liposome only positive control groups showed no significant difference from untreated cell (negative control) groups (66.5±3 vs 75±4.3) as shown in figure (3) and images figure (4).
Figure 3: Comparison between means of proliferation percentages in MCF-7 breast cell lines resulted from treatment with IC50 of different tested drugs in compare to the control untreated group, a: significant difference from normal control group. b: significant difference from other treated groups. *refers to p-values <0.05, non refers to p-values <0.01 and <0.001.
Discussion

Though high progress happened in cancer therapies, and many treatment modalities are evaluated, mortality rates still significantly high, the control of cancer growth and metastatic progression remain the main challenges met most researchers [26]. Trastuzumab directed nanosphere were hypothesized to achieve good kinetic profiles for the carried anticancer drugs within the nanosphere. The rationale behind carmustine and busulfan selection in nanoliposome formulation is that the carmustine is one of the alkylating agents and represent the same target mechanism of all alkylating group. However, it has high lipophilicity or hydrophobicity of about 90%, while busulfan share the same mechanism of action of alkylating agent with carmustine, but it has high hydrophobicity thus we can make a combination between carmustine and busulfan more or less amphipathic that can fit the emulsification effect of nanoliposome. However, trastuzumab causes down-regulation of the tumor cell activity so that it can reduce the potency of any cell cycle-specific chemotherapeutic agents [27]. Thus, we chose alkylating agents as they are cell-cycle nonspecific chemotherapeutic agents. The antineoplastic effects of the
prepared agent LCBT as well as LT, LCB and the pure drugs without nanocarriers trastuzumab and the
carmustine busulfan combination on MCF-7, was performed by crystal violet cytotoxicity assay because this
procedure has been reliably applied in proliferation and cytotoxicity studies to screen the new drug products
potential on cell growth [28]. In this study trastuzumab alone or loaded in nanoliposome (LT) inhibit the growth
of MCF-7 cell line which conflict with the Bunn et al. (2011) in which trastuzumab alone show no effect on
growth rate and cell cycle in cell lines not expressing her2 [29]. MCF-7 was one of the used lines in Collins et al.
(2012) study in which they suggest that trastuzumab can bind cells of low HER-2 level or negative and
initiate antibody-dependent cell-mediated cytotoxicity [30]. Another study showed an additive interaction
between trastuzumab and docetaxel or paclitaxel in HER2-weak to moderate or higher expressing cells, that
may suggest that trastuzumab has a role in inhibiting her2 negative tumors by other unknown mechanism [31].
Current study also shows a synergistic effect between T and other anticancer drugs loaded in nanosphere LCBT.
At same time Bunn et al. (2011) study also found that a synergistic interaction seen between trastuzumab and
other anticancer drugs occur in both lung and breast cancers having Her-2/neu expression, even in low level, in
spite of that Her-2/neu over expression in NSCLC are generally lower than that in breast cancer as Bunn et al.,
in 2011 state. The trastuzumab anticancer effect can come from an increase in p27Kip1 that can inhibit cycling-
dependent kinase and consequently arrest the cell cycle [29]. Carmustine busulfan combination whether as pure
agents or loaded in the nanoliposome shows dose-dependent cytotoxicity effect on both cancer cell lines and on
the normal cell line which indicates less selectivity toward carcinomas in comparison to the loaded drugs in
nanosphere LCBT and trastuzumab that show high selectivity. This result was due to the pathways mechanisms
of carmustine action as one of nitrosourea compounds by the generation of isocyanides beside [-N≡N–CH2–
CH2–Cl]+OH that as a result of its reactivity, can further breakdown to produce DNA alkylation and strand-
breaking, and various other metabolites, in addition to the release of reactive nitric oxide (NO) which is another
mechanism for its action [32]. Zhirong Zhong, et al. (2012) study carmustine and adenosivirus as co-encapsulates
in Liposomes forming a co-delivery system in which they were significantly suppressed B16 and LLC tumors
growth in mice and lengthen their survival [33]. Liu et al., in 2016 discovered that busulfan alone is dose and
time-dependent inhibitory medicine to prostate cancer cell LAPC4 growth and proliferation by enhancing its
apoptosis which resemble our results [34]. Nanoliposome (Lip) effect was not significantly differing from
negative control except in high dose (100 µg/ml) which shows low inhibitory effects. This result was adjusted in
preparation of encapsulated drugs LCBT, LT, and LCB to be started with 50 µg/ml in the higher stock
concentration to get rid of the liposomal cytotoxicity, this observed result can be from chloroform and diethyl
ether used in the preparation of nanoliposome [34]. When the entrance of nanoliposomes into the cell occurs
by any mechanism of fusion and endocytosis of lipid exchange and adsorption, this can release this organic
solvent (chloroform and diethyl ether) even if it was in small amount this can cause cytotoxicity whether after
cytotoxicity or when it stays outside the cell it can interact by a lipid exchange with membrane proteins [35].
The interaction index for LCBT show, synergistic effect that were less than one, this effect could be from the
combination of different mechanism of action of 3 drugs on both cell lines in addition to the targeted effect of
nanoliposome to overcome the drug resistance [36]. Hence the combination of cytotoxic drugs loaded in
nanoliposome may be able to overcome this difficulty, as that nanoliposome entrance to the cell and directly
provide drug distribution and targeting could enhance the pharmacological activity of the encapsulated drugs.
This nano vehicles can release active drugs inside acidic organelles, like endosome and lysosome by which they
can bypass P-glycoprotein which consider as an efflux pump of drugs through direct drug internalization by
adsorption and endocytosis, thus can overcoming this multidrug resistance cancer cells [37]. The results of the
present study revealed a reduced percentages expression of Ki-67 protein proliferation marker in MCF-7 cancer
The lower Ki67 level seen in LCBT treatment in MCF-7 cell line that was treated with LCBT and positive control LT, LCB, T, CB in IC 50 of each of them separately after 48 hour from drug exposure in comparison with the negative control group (P-value <0.001). The expression percentages of Ki-67P was significantly lower in CB containing positive control in comparison to T containing positive control (P-value <0.01). The liposome only positive control groups showed no significant difference from untreated cell (negative control) groups P-value >0.05. As we mentioned before that B. Qi J. et al., (2011) showed that combination Carmustine and all-trans retinoic acid can down regulate mRNA expression of cycling E and cycling-dependent kinase 2 (CDK2) [38]. At same time Vijayalakshmi et al. (2016) studied the Carmustine-serenity combination found that they can down regulate proliferative proteins c-Myc and cycling-D, by which it can inhibit Kif67 protein synthesis [39]. The lower Ki67 level seen in LCBT treatment in MCF-7 could be from the synergistic effect of combined drugs used beside the targeted effect of nanoliposome vehicle used that can facilitate the drug entry in higher concentration into the tumor cells and overcome their resistance. However, there is no previous study to compare with it.

Conclusion and recommendations: LCBT has a prominent antineoplastic effect in the MCF-7 cancer cell line as it significantly decreased the cellular viability and show ant proliferative effect. At same time a synergistic interaction between drugs loaded in nanoliposomes were seen. However, further studies are required to assess the LCBT efficacy and safety profile and to determine the pharmacokinetics in laboratory animals.
References


