Studies on the anticancer potential of High Molecular Weight bovine lactoferrin in targeting ovarian carcinoma

By

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ABSTRACT

Cancer is one of the greatest public concerns and remains the world’s leading cause of deaths. In the female population, ovarian cancer (OC) is a leading cause of death worldwide. Despite conventional therapies which have been developed in recent years, such as surgery, radiotherapy, chemotherapy and laser therapy, new strategies need to be used to treat OC due to the limitation and side effect of conventional procedures. However recently, many natural herbal and dairy products such as lactoferrin (Lf) and curcumin, have been studied in the treatment of different cancer types. In the present study a natural therapeutic bovine lactoferrin (bLf) with emerging anti-cancer effects was chosen to treat OC. Lactoferrin (Lf) is a ~78-80 kDa multifunctional iron-binding glycoprotein found at high concentrations in bovine, human colostrum and in milk. Lf is a natural protein that plays an important role in host defence against microbial infections, cancer and inflammation. Since bLf has a potential anti-carcinogenic effect, its role in treating OC remains unexplored. Therefore, this current study was aimed to investigate therapeutic efficacy of bLf in targeting OC. The study hypothesis was that bLf could be successfully purified from Australian bovine colostrum and could be effectively employed as an anti-carcinogenic bioactive protein to treat different types of cancers including OC.

Lf used in this study was purified from Australian bovine colostrum using cation-exchange chromatography and was importantly identified as a high molecular weight (250 KDa) protein (HMW) using SDS-PAGE and confirmed by Western blotting. This novel trimeric form of bLf protein was further compared for its anti-cancer effects using iron saturated and unsaturated forms of commercial monomeric bLf. The physico-chemical characterisation was carried out using differential scanning calorimetry (DSC) and Fourier Transform Infrared Spectroscopy (FTIR). By employing human OVCAR-3 cell line derived from a Homo sapiens (female) ovary adenocarcinoma, cell bioassays like lactate dehydrogenase (LDH) release assay, MTT assay and CyQuant cell proliferation assay were carried out to determine the anti-cancer effects of HMW-bLf. The gene expression analyses of the cellular internalisation receptors and apoptosis mediator and protein expression were studied through quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR). Flow cytometric analysis was further carried out to study the
protein expression of different apoptosis-related proteins such as survivin, Bcl-2, Bcl-xL, BAD and Bax caspase-7, -8, -9 and cell receptors such as low density lipoprotein receptor-related protein (LRP-1), transferrin receptor and (TfR) molecules.

Interestingly, HMW-bLf exhibited high thermal stability and was found to be more stable against a digestive enzymes’ cocktail (Omnizyme). Also, HMW-bLf had a similar chemical structure as that of ~78kDa Apo-bLf (Iron free bLf) and native monomeric bLf (NM-bLf) in the FTIR spectrum. Furthermore, HMW-bLf demonstrated highly significant cytotoxicity (p ≤ 0.01) with the highest concentration dose of about 3200 µg/ml. Correspondingly, cell proliferation significantly decreased (p ≤ 0.01) in OVCAR-3. Further, Annexin-V staining revealed that HMW-bLf at its highest concentration induced cell death in OVCAR-3 cells via apoptosis with 56% of cells showing Annexin-V positivity (p ≤ 0.01). HMW-bLf at high (3200µg/ml) doses increased the gene expressions of apoptotic factors like Fas and Fas-L, Bax, caspase-3, -8 and -9 and TRAIL. It induced a significant (p ≤ 0.01) down-regulation of gene and protein expressions of anti-apoptotic molecules like survivin, Bcl-2 and Bcl-xL when analysed through qRT-PCR and protein expression assays using flow cytometry, respectively. The significant (p ≤ 0.01) up regulation of mRNA expression of LRP-1, Lf receptors (LfR) and TfR in OVCAR-3 cell line supported their role in facilitating receptor mediated endocytosis of HMW-bLf. Furthermore, immunofluorescence using confocal microscopy showed high binding and internalisation activity of HMW-bLf at 2 and 4h in OVCAR-3 cells cytoplasm and nuclei.

To confirm these findings, other cancer cell lines MDA-MB-231 derived from Homo sapiens (female) breast carcinoma and SW 480 derived from Homo sapiens (male) colorectal adenocarcinoma were also used. Similar cell bioassays mentioned above like LDH, MTT, CyQuant, propidium iodide (PI staining assay) and immunofluorescence staining followed by confocal microscopy (to determine the levels and localization of HMW-bLf in these cancer cell lines), were carried out on these two cell lines. HMW-bLf significantly (p ≤ 0.01) induced cancer cell cytotoxicity and also reduced cell proliferation significantly in both these cell lines at its highest concentration of 3200 µg/ml. Moreover, HMW-bLf induced the caspase-3 activity in both cell lines, when analysed spectroscopically. Collectively, the findings of this study are novel and have shown promising results for the anti-cancer efficacy of
HMW-bLf in the *in vitro* bioassays. Hence, future studies that employ other ovarian cancer cell lines, normal cells and *in vivo* ovarian cancer mice models would unravel its real potentials as a safe anti-cancer therapeutic or chemopreventive biomacromolecule.
Title Page Figure: Internalization of high molecular weight bovine lactoferrin (HMW-bLf) in the cytoplasm and nucleus of OVCAR-3 (ovarian cancer cells) after 4h treatment.

OVCAR-3 cells treated with HMW-bLf 1600 μg/ml for 4 h. Confocal images shows the internalization of HMW-bLf (green) inside the cytoplasm and nucleus. Nuclear DNA is stained with DAPI (blue).
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List of Abbreviations

AIF ........................................... Apoptosis inducing factor
APS .......................................... Ammonium persulfate
Apo-bLf ..................................... Apo (Iron free) bovine lactoferrin
Apaf-1 ....................................... Apoptotic protease activating factor-1
ATCC ......................................... American Type Culture Collection
bLf ............................................. Bovine lactoferrin
Bad ........................................... Bcl-2 associated agonist of cell death
Bak ........................................... Bcl-2 antagonist/killer 1
Bax ........................................... Bcl-2 associated X protein
Bcl-2 ......................................... B-cell lymphoma-2
BID ........................................... BH3-interacting domain death agonist
BSA ........................................... Bovine serum albumin
EGFR ....................................... Epidermal growth factor receptor
DAPI ......................................... 4’, 6-diamidino-2-phenylindole
DIABLO .................................... direct inhibitor of apoptosis-binding protein with low pl
DISC ......................................... Death inducing signalling complex
DTT ........................................... Dithiothreitol
dNTP  Deoxyribonucleotide triphosphate
FACS  Fluorescence-activated cell sorting
Fas   Tumour necrosis factor receptor superfamily member 6
Fas-L Fas ligand (TNF superfamily member)
FADD Fas-associated death domain protein
FITC Fluorescein isothiocyanate
Fe-bLf Iron saturate bovine lactoferrin
Fe-NTA Ferric nitrilotriacetate
HMW-bLf High molecular weight bovine lactoferrin
IAP Inhibitor of apoptosis protein
IL Interleukin
IP Intra-peritoneal
NM-bLf Native monomeric bovine lactoferrin
HCl Hydrochloric acid
JNK c-Jun N-terminal Kinase
kDa Kilodalton
LDH Lactate dehydrogenase
LfR Lactoferrin receptor
Milli-Q Millipore ultra-purified water
MWCO Molecular weight cut off
ml Millilitre
NaOH Sodium hydroxide
NK Natural killer cells
NM-bLf Native monomeric bovine lactoferrin
OC Ovarian cancer
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>EOC</td>
<td>Epithelial ovarian cancer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>pH</td>
<td>Potential of hydrogen</td>
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<tr>
<td>Pgp</td>
<td>Permeability glycoprotein</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspase</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-immunoprecipitation assay</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/borate/EDTA buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffer saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffer saline Tween- 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF related apoptosis inducing ligand</td>
</tr>
<tr>
<td>TVS</td>
<td>Trans-vaginal ultrasound scans</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>µg</td>
<td>microgram/s</td>
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<tr>
<td>µl</td>
<td>microliter</td>
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<td>µM</td>
<td>micromolar</td>
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<td>mins</td>
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Chapter 1: Literature Review

1.1 Introduction

Cancer is defined as an uncontrolled division and growth of cancerous cells which lead to a formation of benign or malignant tumours [1]. Cancer can affect all age groups without exception. The majority of cancers occur mainly in the elderly (estimated 77%), while other malignancy shows an increase in young people and children [2]. Cancer is a leading cause of death worldwide and accounts for 7.6 million deaths (around 13% of all deaths) in 2008. The main types of cancer are: Lung cancer (1.4 million deaths) stomach cancer (740 000 deaths) liver cancer (700 000 deaths) colorectal (610 000 deaths) and breast cancer (460 000 deaths). The World Health Organization (WHO) also estimated that by the year 2030, cancer will kill 12 million people per year [3].

Furthermore, cancer prevails in all world nations and is diagnosed in all ethnic races. However, the cancer incidence and cancer types notified vary from race to race. In developing countries, survival rates are very low due to the late diagnosis of cancer at its advanced stages and limited access to verify the suitable treatment. Malignant cancers when diagnosed at an early stage are highly curable, while cancer at an advanced stage tends to be otherwise. Hence the successful treatment of cancer lies in its early detection [4]. Cancer is still leading cause of death around world due to limited treatments choices including surgery, radiotherapy, chemotherapy and laser therapy. Chemotherapy option has been used broadly for cancer treatment but it has several side effects such as anaemia, nephrotoxicity, immune system depression, fatal infection, nausea, vomiting, hair loss and fatigue [5].

To reduce these unfavourable side effects, newly developed techniques that are relatively more specific to tumours cells that cause fewer side effects are currently undergoing clinical trails [6]. These techniques include immunotherapy molecular based targeted therapies, gene therapy and the use of nanoparticles for targeted therapy and diagnosis. Currently, anticancer therapies available in the medical field include chemotherapeutic agents such as doxorubicin, paclitaxel, actinomycin and
mitomycin destroy both tumour and normal cells and causes severe cytotoxicity effects as well [7].

However, the above mentioned approaches and the drugs available are insufficient to cure cancer patients, hence the recent research that focuses on naturally sourced and derived proteins such as lactoferrin (Lf) [8, 9]. Historically, Lf was first isolated from bovine milk but this glycoprotein is also an important component of innate immune system and found in most mammalian milks and exocrine secretions [10]. Lf possess various biological functions and several physiological roles have been attributed to Lf, namely regulation of iron homeostasis, host defence against infection and inflammation, regulation of cellular growth, differentiation and protection against cancer without causing any side effects [11, 12]. As mentioned previously, there are several kinds of cancer, in which ovarian cancer (OC) is one of them. OC has the highest mortality rates among gynaecological malignancies [13]. Although the efficacy of OC treatment has improved, the survival rate is still low with 5 year survival rate of only 50%. Therefore, the demand for new treatment strategies is high [14].

1.2 Ovarian carcinoma

OC is caused by the rapid growth and division of cells within one or both ovaries, which are the reproductive glands in which the ova or eggs and the female sex hormones are produced. The ovaries contain cells that, under normal circumstances, proliferate to maintain tissue health. When there is uncontrolled proliferation of cells, they divide too frequently and too rapidly and hence a cellular mass or tumour is formed. OC is the sixth most common cancer among women worldwide, with an estimated 200,000 new cases and 140,000 deaths due to this neoplasia each year [15]. In Western countries, OC leads to the highest mortality rate among all kinds of cancer which affects the female reproductive system [16]. In Australia, ovarian cancer ranks 9th and is the most frequent cancer diagnosed [17]. There are three major kinds of ovarian cancer: (i) epithelial, (ii) sex cord stromal and (iii) germ cells tumours [18]. The majority of ovarian tumours are epithelial in type and most of these are believed to originate from the growth of ovarian surface epithelium (OSE) [19]. While the granulose form consists of 5%, germ cells tumours are with the lowest
percentage of incidence 1% [20]. Importantly, epithelial ovarian carcinoma (EOC) has become an extremely fatal disease in recent years because it has the highest mortality rate of all gynaecological malignancies [21]. Since the diagnosis of ovarian cancer is only possible at an advanced stages, the survival rate of patients who are affected by EOC is very low [22]. Many factors decide the kind of treatment that will be useful, but the most important factor is if the patient has received any chemotherapy in the past [23].

1.2.1 Symptoms of ovarian cancer

Ovarian cancer poses a very important clinical challenge to gynaecologists, medical oncologists and radiotherapists. Females with ovarian cancer at an early stage do not have clear symptoms and their symptoms can be vague. In addition, the range of symptoms associated with OC is wide and nonspecific [24]. Furthermore, OC is often referred to as the ‘silent killer’ because of its late presentation and difficulty in diagnosis [25]. Hence, OC is relatively asymptomatic until later stages. Unfortunately, most patients with OC are not diagnosed until the disease is in its advanced stages [26]. This results in metastasis of the cancer to the upper abdomen and other vital organs. Only 25% of all OC can be diagnosed in its early stage and more than two thirds of women with OC are diagnosed at an advanced stage of the disease [27]. There are numerous physiological symptoms that may be observed in patients who are in the advanced stages such as nausea, vomiting, anaemia, fatigue and pain. Other emotional features such as loss of appetite and depression can be some additional unspecific symptoms [28]. Symptoms may be related to the presence of a pelvic mass, urinary frequency, pain and constipation [29]. A recent study demonstrated the major symptoms of OC are bloating, pelvic or abdominal pain, difficulty in eating or feeling full quickly, and an increased frequency in needing to urinate at both early and advanced stage [30].

1.2.2 Ovarian cancer etiology and risk factors

Many factors have been recognized that increase the probability of epithelial OC incidence among women’s such as family history, age and early menarche. There are four major factors that can increase the probability of OC incidence among
females. These are genetic, hormonal, reproductive history, and lifestyle [20]. The majority of ovarian carcinoma incidence is not related to a specific cause [31]. The most considerable risk factor is family history which indicates a female who have inherited mutation of both breast cancer gene (BRCA1 or BRCA2), or have a site specific family history [32]. Hereditary OC represents valuable reason to increase the possibility of OC by 25%-50%. The probability of ovarian tumour increases by 4-5% for women who have a single first degree relevant member diagnosed with ovarian malignancy [33]. The percentage further increases to 7% if two or more family members have a case history of OC [34]. Hormonal factors which include early menarche, late menopause and infertility consist of factors which give rise to the risk of ovarian carcinoma due to frequent ovulation time [35]. Reproductive factors such as pregnancy, breast feeding and oral intake of contraceptive pills can reduce OC incidence by interrupting and suppressing the ovulation process [33].

1.2.3 Existing and emerging hypotheses

Although the etiology of OC is unknown, there have been many recent hypotheses suggesting an explanation of the main causes and the etiology of EOC [36].

**Incessant ovulation hypothesis:** Incessant ovulation theory suggests that due to the process of ovulation, repeated trauma is caused to the layer of epithelial cells leading to an increased exposure of the ovary surface and ovarian cells to the genetic abnormalities and or other risk factors. Moreover, every ovulatory cycle causes wound at the ovarian surface, therefore cells undergo repair and growth frequently which may increase the probability of genetic mutation [37].

**Pituitary gonadotropin hypothesis:** Pituitary gonadotropin hypothesis states that an excessive level of gonadotropin leads to an increased production of estrogen, in addition to an increase in the levels of both the luteinizing hormone (LH) and follicle-stimulating hormone (FSH), then stimulate the epithelial cells to form an inclusion cyst. The epithelial cells are exposed to further proliferation leading to an eventual malignant transformation [38].

**Androgen/Progestosterone hypothesis:** This theory suggests that the androgen hormone, which is elevated during the stages of menopause and obesity, leads to an
increased risk of developing OC while the progesterone hormone works as a protective agent [39].

**Inflammation hypothesis:** Inflammation has been suggested to be a main factor leading to EOC. Damaged ovarian surface epithelia with ovulation induce inflammation, promotes reconstruction and mutation susceptibility [40]. Numerous inflammatory mediators such as cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, TNFα), which are produced by leukocytes, have been reported as one of the causes for EOC [41].

1.2.4 Ovarian cancer staging

OC has been classified according to the spread of the tumour from the beginning of diagnosis. The classification of cancer stages depends on the surgical and pathological findings. Since 1985, the International Federation of Gynaecology and Obstetrics (FIGO) have made minor changes to previous classifications. The stages have now been classified to four stages those with limited diseases are stage I, stage II, while those with advanced diseases are stages III and IV (Table 1.1) [42].
<table>
<thead>
<tr>
<th>Stages</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Tumour confined to the ovaries</td>
</tr>
<tr>
<td>IA</td>
<td>Growth limited to one ovary, external surface is clear, no ascitis, capsule intact.</td>
</tr>
<tr>
<td>IB</td>
<td>Cancer exists in both the ovaries and there are no adjacent organs affected.</td>
</tr>
<tr>
<td>IC</td>
<td>Neoplasm on the surface of one or two ovaries, rupture of ovarian capsule with positive peritoneal washings.</td>
</tr>
<tr>
<td>Stage II</td>
<td>Growth involving one or both ovaries and has spread to other sites in the pelvic area.</td>
</tr>
<tr>
<td>IIA</td>
<td>Metastases of cancer to adjacent organs such as the fallopian tube and uterus.</td>
</tr>
<tr>
<td>IIB</td>
<td>Metastases to other pelvic tissues.</td>
</tr>
<tr>
<td>IIC</td>
<td>IIA or IIB with serosal involvement or capsule rapture or ascites or peritoneal malignant(3).</td>
</tr>
<tr>
<td>Stage III</td>
<td>Tumour spread to abdominal cavity or to lymph nodes located in the abdomen.</td>
</tr>
<tr>
<td>IIIA</td>
<td>Node negative, microscopic extra-pelvic disease.</td>
</tr>
<tr>
<td>IIIB</td>
<td>Node negative, abdominal disease &lt; 2 cm.</td>
</tr>
<tr>
<td>IIIC</td>
<td>Retropertitoneal or inguinal lymph nodes, abdominal disease &lt; 2 cm or both.</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Metastases to organs beyond and outside the abdomen.</td>
</tr>
</tbody>
</table>

**Table 1.1** Major stages of ovarian cancer. Data adapted from [42].
1.2.5 Diagnosis and biomarkers

As reviewed earlier in Section 1.2.1, the prognosis and symptoms of OC patients are still unclear and vague. Because of this, when incidence of ovarian cancer is present, diagnosis is often delayed while other suspected disease are investigated. Symptoms such as prolonged pelvic pain, incontinence and abdominal bloating [43] are representative of other kind of diseases too. According to the International Federation of Gynaecology and Obstetrics (FIGO) more than 70% of the OC patients are diagnosed with advanced stage (stage III or IV), which is after the cancer has spread to the adjacent organs [44]. In fact the detection and diagnosis of ovarian malignancy in early stage is tedious, and at present there is no valuable screening mechanism for OC [45]. There are many biomarkers with high sensitivity to clinically diagnosing ovarian cancer, but few of these markers tend to have high sensitivity for preclinical disease. These tumour biomarkers are occasionally developed from the tumour itself (tumour derived) or developed as a reaction of the body tissue to the tumour (tumour associated). The major features of these valuable tumour markers for targeting should be of high sensitivity and high specificity [46]. Despite the current research work to create and discover a valuable biomarker to enhance the therapeutic efficiency and detection of OC in its early period, there have been a number of challenges. The first of these is the molecular heterogeneity of different kinds of tumours. The second challenge is the absence of a sensitive biomarker specific for ovarian cancer, and the third challenge is the low amount of biomarker at an early stage because the size of lesion is too small [47]. To date, diagnosis of EOC in its early stage is still not possible due to insufficient strategies. Screening modalities used right now is a combination between transvaginal screens (TVS) and investigation of the tumour biomarkers using proteomics and genomics approaches [48]. A number of selected biomarkers are reviewed in the following sections which are used in clinical investigation.

1.2.5.1 Proteomic and genomic approaches

The main goals for establishing this technology are to define and investigate biomarker expression against cancer diseases. The work is still ongoing from both
scientists and researcher to design a novel approach to address OC in an early stage [44]. Proteomics is a powerful technology to discover the biological process of many diseases. Briefly, proteomics is defined as a broad-scale study to address the protein structure, function and level of expression [49]. Genomic, proteomic and transcriptional profiling methods on DNA, RNA, and protein levels in tumours, blood, and urine are also used to identify new tumour markers with acceptable sensitivity and specificity for clinical use [50]. The research discusses the proteomics elements in the laboratory from different aspects. These are (i) recognition structure of the proteome; (ii) investigate the difference in expression level of proteomics among healthy and cancer affected tissue at the specific stage (iii) in vivo survey analysis to gain knowledge about how proteins interact with other kind of proteins that have been expressed due to cancer, and (iv) to identify the fate of these proteins are expressed in cancer and their post translational modification [51].

1.2.5.2 Transvaginal sonagraph (TVS)

TVS is one of the best imaging techniques used to detect OC. It’s an excellent tool to investigate the morphological changes of ovaries due to any pathological disorder condition including OC. TVS exhibits accurate and notable results compared to other imaging techniques such as Magnetic Resonance Imaging (MRI), Computed Tomography (CT) and ultra sound imaging [45]. Various studies suggest that TVS has been considered as a potential candidate for screening ovarian malignancy in its primary stages [52]. In a study using TVS conducted from 1987 until 2005, a quarter of a million women were involved in this procedure each year. The result demonstrates that the survey test had specificity of 98.7% and a sensitivity of 85.0%. The data from randomised trials shows that the regular TVS screening can improve the probability of detecting ovarian cancer at an early stages as compared to the unscreened population [53]. However, a limitation of TVS is that it is not cost effective when employed for population screening. Researchers suggest that a combination of TVS and Cancer Antigen (CA125) would be much more cost effective to investigate ovarian malignancy [54].
1.2.5.3 Tumour biomarkers

In general, tumour markers are cancer specific biochemical substances that have been detected in the serum or urine of patients with some kind of cancer. Biomarker research is a field that represents the design of significant tools to discover and understand the mechanism of disease, especially tumours. Scientists utilize this approach to create a new medicine and explain at what level this medicine is valuable [55]. These biomarkers may be produced via a particular organ which is affected by tumour (tumour-derived) or in other ways, such as the response from the body to the tumour (tumour associated). Biomarkers have different significant uses in cancer such as diagnosis, categorising cancer stages, prognosis monitoring and response to chemotherapy [56]. The ideal tumour marker should have a high sensitivity (SE) and a high specificity (SP). In an ovarian tumour, various biomarkers currently used are distinguished by a high sensitivity for the purpose of clinical diagnosis, but very few are relatively useful for early detection of OC. The glycoprotein CA125 has been pronounced as a valuable biomarker to observe the treatment response and diagnosis.

1.2.5.3.1 Cancer antigen 125 (CA125)

Currently, among the various kinds of biomarkers that have been used in the diagnosis of EOC, most attention has been given to CA125 [57]. CA125 has been described as a high molecular weight mucin with a 200 kDa molecular weight glycoprotein which is specifically recognized by a monoclonal antibody OC125 [58]. Moreover the concentration of CA125 increases in certain cases such as tissue damage, vascular invasion and inflammation related to cancer diseases. The concentration also rises above 90% in OC patients who are in advanced stage and an elevated level of CA125 is recorded in 40% of patients in an advanced intra-abdominal malignancy [59]. The specific value of CA125 is not sufficient to be approved for valuable screening [44]. CA125 has also been reported in elevated levels during pregnancy and menstruation which is considered as an unpathological state. Also, CA125 is present in high levels in other pathological conditions such as pelvic and abdomen inflammatory disease, breast cancer, lung, colon and liver
cancer [60]. For these conditions, this glycoprotein is less useful for general screening and could be more useful for investigating persistent or recurring tumours [61]. Hence, due to the nonspecific action of CA125 to the OC, it cannot be branded as a well characterized diagnostic tool for detection of this condition.

1.2.5.3.2 Human epididymis protein 4 (HE4)

HE4 is a novel biomarker whose expression is noticed in high expression in epithelial ovarian cancer cells and in minimum expression in normal OC [62]. Currently, HE4 appears to be a promising biomarker for early detection of ovarian cancer with higher sensitivity than that of the CA125 biomarker [63]. HE4 is a low molecular weight glycoprotein which consists of whey acid protein with a 4-disulfide and which has been obtained from human epididymis epithelial cells. HE4 is highly expressed in ovarian malignancy and can be evaluated by the ELISA technique with similar sensitivity as that of CA125. In addition, the current study had shown high accuracy of HE4 expression for the detection of ovarian malignancy at an early stage [64]. Also, HE4 is detected in urine sample with the same sensitivity. HE4 represents a promising biomarker to monitor ovarian carcinoma [65]. In 2009, the Food and Drug Administration (FDA, USA), approved the use of HE4 as an approved biomarker for the condition of monitoring women who are affected by EOC [66]. In a recent study conducted by Hollestram et al in 2010, the detection of HE4 from urine samples reported a high accuracy of 94%, with 86.6% in women with early stage (I/II), and 89.0% at an advanced stage (III/IV) [63]. Recently, the combination of HE4 and CA125 was found to be helpful in prediction of malignancy rather than being used individually [67].

1.2.5.3.3 Mesothelin

Mesothelin is another hopeful biomarker for early detection of OC [68]. Mesothelin is defined as antigen bound-protein with 40KDa found in normal peritoneal mesothelial cells [69]. Moreover, various studies have proved that mesothelin is highly expressed in certain cancer type particularly mesotheliomas, pancreatic, ovarian and lung cancer [70]. Over expression of mesothelin has been recorded in approximately 70% of women affected by ovarian carcinoma [71]. The physiological role of mesothelin is
still unknown, but recent works suggest that, mesothelin conjugate with CA125 may exhibit important factor in peritoneal metastasis of ovarian carcinoma [72]. The limited mesothelin expression in normal tissues and high expression in many cancers makes it an attractive candidate for cancer therapy. Due to this, mesothelin as a biomarker represents a promising element to investigate ovarian carcinoma in its early stage [73]. A study conducted in 2007 by Badgwell et al showed that, expression of mesothelin via urine sample had high accuracy than serum sample which were collected from OC patients at early and late stage compared with healthy controls [74]. In patients who are in stage I and II of ovarian carcinoma, around 42% of the patients had elevated mesothelin level in urine assay compared to 12% in serum assay. Similarly 75% of patients with advanced disease had elevated mesothelin in urine compared to 48% in serum. This suggests the potential role of combining of mesothelin and CA125 to increase the sensitivity percentage of diagnosing OC with 98% specificity, compared with CA-125 (78.8%) or mesothelin (59.6%) when used alone [75].

**1.2.5.3.4. Haptoglobin**

Haptoglobin is a glycoprotein mainly produced by liver. Many studies reported more expression of haptoglobin in ovarian carcinoma via ascetic fluid and serum [76]. Further studies were conducted to prove that the level of haptoglobin increases during infection, inflammation and neoplastic disease including lung cancer, lymphoma and breast cancer. Moreover, this glycoprotein is present in significant amount during the early stages of OC [77]. A previous study involving a total of 136 women, among which 10 were healthy, 66 were diagnosed with malignant ovarian carcinoma and 60 diagnosed with a benign ovarian tumour showed significant amount of haptoglobin expression in stage I and II compared with normal healthy control [78]. Haptoglobin has been recognized as a candidate biomarker with 90% specificity, and 64% sensitivity when used alone to detect ovarian malignancy with diagnostic specificity and a sensitivity of 95% and 91% respectively compared with CA125 [79]. This may indicate its possible importance in diagnosing the early stage of ovarian carcinoma. Further studies need to be carried out to verify and validate haptoglobin as a successful biomarker with high sensitivity and specificity.
1.2.5.2.5 Osteopontin (OPN)

Osteopontin is a ~35 kDa glycophosphoprotein whose expression is increased in white blood cells during inflammation. This biomarker was established by using the cDNA microarray method, and subsequently OPN was found to be elevated in females with OC compared to normal control groups, and significantly correlated with tumour response to surgery, chemotherapy and disease recurrence [80]. OPN was first reported to be a candidate biomarker in 2002 [81]. A study demonstrated that OPN was elevated in case of cardiovascular disorder and some cancers [82]. OPN was detected in breast cancer and prostate cancer patients and this was shown to be a valuable biomarker to investigate OC at an early stage, thus complimenting CA125 [83]. In a comparative study carried out by Kim et al in 2002, it was concluded that the level of OPN in serum of OC patients was over expressed and estimated to be 487 ng/ml, while in benign ovarian cancer patients the amount estimated was 254 ng/ml as compared with the normal healthy control who were involved in this study [80].

1.2.5.3.6 Vascular endothelial growth factor (VEGF)

The vascular endothelial growth factor (VEGF) is one of the most significant factors stimulating angiogenesis and has been proven to play a vital role in pathogenesis of ovarian malignancy. VEGF is a 45 kDa glycoprotein and defined as the prominent pro-angiogenic molecule, involved in tumour growth and the development of metastases [84]. It also has a potent effect by increasing the vascular permeability and acts as a strong mitogen for vascular endothelium [85]. These two factors are sufficient to induce angiogenesis. The level of VEGF is found to be higher in women diagnosed with OC at an early stage (I&II) compared to normal controls or patients with a benign disease. In vivo and in vitro studies were carried out to demonstrate the increased level of VEGF in different stages of ovarian malignancy. Over expression of VEGF was detected in ascites, cyst fluid and serum of OC patients and the levels of VEGF in serum were 10-fold higher in OC patients who are in advanced stages compared to healthy controls [86, 87]. In some studies there have been correlations between VEGF, clinical outcome and prognosis of OC patients.
Thus, VEGF can be used as a prognostic biomarker [88]. However, the clinical significance and implication of VEGF expression in OC is not completely understood, and further studies need to be conducted to identify the role of VEGF in OC prognosis.

1.2.6 Treatment options for ovarian cancer

A successful treatment for ovarian cancer is still to be developed. There are some methods for treatment, but none has shown success in eliminating the cancer or blocking the recurrence of tumour. Despite various treatment options such as surgery and chemotherapy drugs, OC still remains a fatal disease among females worldwide. In addition, 55-75% of OC responders relapse during 1-2 years from the end of the first line chemotherapy dose [89]. Early-stage EOC (stages I and II), in which the disease is limited to the ovary or the ovaries and adjacent pelvic structures, is mostly curable. Primary surgical cytoreduction followed by platinum-based chemotherapy is the standard treatment for patients with advanced EOC. In earlier stages of cancer, the patient is usually able to respond to the treatments. However, 90% of patients in stage I, and 80% in stage II relapsed to these type of treatments.

1.2.6.1 Surgery

Standard first line treatment of OC is surgical cytoreduction followed by platinum-based chemotherapy. Surgery is the primary choice in which the lesion is completely removed from the ovary and affected adjacent organs are also removed [90]. Well differentiated ovarian tumour patients at first the stage have excellent prognosis when the lesions are surgically removed [91]. Moreover, surgery assists gynaecologists to decide and restrict the tumour stage [92]. Although, systemic treatment including surgery and chemotherapy provide minor improvement to overall survival rate of ovarian cancer patients. Unfortunately, many women with OC do not undergo optimal cytoreduction, with one of the main reasons being, as shown in numerous studies, that to achieve an optimal cytoreduction rate greater than 50% often requires the incorporation of a variety of extensive upper abdominal surgical procedures, not always performed by general gynaecologists [93, 94]. Therefore, the identification of a more effective treatment for ovarian cancer would have a
substantial public health benefit. For patients in the advanced stage, optimal treatment after surgery has yet to be completely defined [93].

1.2.6.2 Chemotherapy

Chemotherapy has been suggested for all OC stages [95]. Females who are affected by EOC with a probability of metastasis of the condition to adjacent organs are usually treated using chemotherapy. Chemotherapy for EOC is administrated post-surgery (adjuvant).

1.2.6.2.1 First line chemotherapy.

Platinum-based chemotherapy drugs have been established since the 1980s for the treatment of OC [96]. Moreover, platinum agents like cisplatin and carboplatin represent the favourable choice of treatment for ovarian cancer patients, who are in advanced stages. Platinum-based drugs were approved as first line chemotherapy after meta-analyses [42]. Survival rate benefits of 5% at two to five years have been recorded for ovarian cancer patients who are treated by platinum-based therapy [97], but in most of the patients ovarian cancer recurrence notified. However, the second dose of chemotherapy for patients who have had a recurrence undergo unfavourable side effects such as cumulative peripheral neurocytotoxicity [98]. The International Collaborative Ovarian Neoplasm (ICON) trial compared the effect of carboplatin combined with cyclophosphamide, Adriamycin and cisplatin (CAP) and concluded that the administration showed no significant difference in survival rates between the drug combination group (CAP) and carboplatin as a monotherapy. Furthermore, the combination group (CAP) was found to be more toxic. In another study conducted by the (ICON) group in 2002, it showed that carboplatin; CAP, carboplatin and paclitaxel did not improve the survival rate among women who were affected by EOC [91]. Only carboplatin and paclitaxel are considered for treating this condition [99]. Recently, sever neurotoxicity and arthralgia was exhibited by patients who were treated with paclitaxel. For that, alternative toxins such as (Taxotere) have recently been used as first line chemotherapy in advanced OC [100]. Another randomised study showed similar efficacy and decrease in neurotoxicity when compared between docetaxel-carboplatin and paclitaxel-carboplatin [101]. At relapse, platinum
free intervals play an important role in deciding whether patients would be able to respond to retreatment by a second dose of platinum based chemotherapy. If this period consists of more than six months the same drug or combination between carboplatin and cisplatin can be used [102].

1.2.6.2.2 Second line-therapy.

Women with recurrent OC following an initial incomplete response, and those who do not respond well to initial chemotherapy, are candidates for further chemotherapy, often called second-line chemotherapy [103]. Data shows that, more than 50% of women suffering from OC will achieve complete remission in response to taxane/platinum therapy; while the majority of patients will relapse within three years [104]. In this instance, second lines of therapy choices are necessary at this stage and represent an important option [96]. If the relapse period is less than 6 months, there are still other opportunities to provide the patients with a second dose from the same first line therapy because the sensitivity of this drug still has a function within this short period [105]. In contrast, if the OC patients do not respond to the first-line therapy with paclitaxel and a platinum agent or the neoplasm recurrent within 6 months, other options can be considered including non- platinum agents [105]. Second line agents are described to the patients according to their response to first line chemotherapy. Patients can be characterized as having recurrent, intermediate or refractory disease (Table 1.2). Moreover, for patients susceptible to recurrent disease, the probability for response to the second line chemotherapy increases [96]. Non-platinum agent such as tamoxifen, docetaxel, topotecan, liposomal doxorubicin, oxaliplatin, etoposide, gemcitabine and alternatamine all have moderate activity in sensitive recurrent diseases [106].
Table 1.2 Second line treatment options for ovarian cancer

<table>
<thead>
<tr>
<th>Diseases category</th>
<th>Duration of treatment (months)</th>
<th>Treatment choices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recurrent (sensitive)</td>
<td>&lt; 12</td>
<td>Retreatment by platinum + paclitaxel single agent carobplatlin or paclitaxel second line agent.</td>
</tr>
<tr>
<td>Intermediate</td>
<td>4-12</td>
<td>Retreatment with platinum ± paclitaxel.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Second line agent.</td>
</tr>
<tr>
<td>Refractory</td>
<td>&lt; 4</td>
<td>Investigational treatment /clinical trials second line agent.</td>
</tr>
</tbody>
</table>

Table adopted from [96, 107]

1.2.6.2.3 Disadvantages of first and second line chemotherapies

Many clinical studies involving human model to discover the side effects of chemotherapy and unfavourable symptoms have proven that severe nausea and renal failure could mostly occure to patients who received the platinum-based drugs [108]. On the other hand, there are numerous side effects when patients are treated by platinum-based agents, as those groups have a metal chemical structure which can lead to unfavourable symptoms such as abdominal pain, nausea, vomiting, diarrhoea, fever, neuromuscular cytotoxicity, hemorrhagic cystitis, alopecia, reversible leukopenia and thrombocytopenia [109]. Furthermore, nephrotoxicity and gastrointestinal toxicities are common during a therapeutic course by a platinum compound. In addition, neuropathies due to the side effect of chemotherapy can
interfere with key aspects of quality of life, including physical and social aspects [108, 110].

1.2.6.3 Current clinical trial therapies for OC

The current standard treatment for ovarian cancer therapy is surgery followed by chemotherapy as the preferred first step in the management of advanced ovarian cancer. Unfortunately, these protocols often do not allow for cure at initial diagnosis, and many patients will often face recurrence of the tumour and eventually die from the disease. Targeted therapy has subsequently becoming the option of both research and clinical trials in an effort to overcome resistant disease and achieve improvement in patient outcomes. In addition to the development of new drugs, the improvement of drug administration is also a focus of these investigations [111].

1.2.6.3.1 Neoadjuvant chemotherapy

Many research studies have examined the role of neoadjuvant chemotherapy (NACT), mainly in patients whose cancers are not amenable to initial surgical resection [112]. Principally NACT regimens were suggested to cancer patients prior to the surgery option [113]. This approach may enhance the resectability of the tumour but it does not seem to improve the survival rate of OC patients [114]. Recently, two major trials have been carried out in advanced stage of OC; the Chemotherapy OR Upfront Surgery trials (CHORUS) and the EORTC 55971 trial. Previous report on EORTC trails showed the rate of optimal debulking of ovarian tumour cells is elevated and side effects decreased [115]. The trial is currently accruing patients with stage IIIIC or IV EOC [116]. To date, no randomized trial has been completed to assess whether NACT yields equivalent or superior outcomes to initial cytoreductive surgery followed by chemotherapy in advanced stage of OC.

1.2.6.3.2 Hormone therapy for ovarian cancer (HT)

Another therapy option for symptom management in patients who are refractory to chemotherapy or who cannot tolerate chemotherapy is hormonal therapy (HT). HT is the use of hormones or drugs that block hormones to fight cancer [37]. A hormonal agent blocks the activity of or reduces the synthesis of endogenous oestrogen, which
is a growth factor for many ovarian cancers [117]. Hormone therapy is rarely used to treat epithelial ovarian cancer. It is more often used to treat ovarian stromal tumours. HT has been used according to two observations. The first is that majority of OC happen after menopause in females due to excessive circulating gonadotropins, the second is the level of patients’ response to hormonal and anti-hormonal agents in control of other cancers such as uterine and breast cancer [118]. The efficacy of HT in OC is not well defined due to few uncompleted randomised clinical trials.

1.2.6.3.3 Intraperitoneal administration of drugs

Intraperitoneal (IP) chemotherapy is a hopeful treatment option for OC and considered an interesting method of administration by enhancing the diffusion of chemotherapy [119]. The peritoneal cavity represents the main location of tumour cells developed from OC. Delivering drugs directly into effected organs became attractive approach in OC because higher drug dose concentration could be achieved locally [120]. IP chemotherapy has the theoretical advantage of that in acting directly on the tumour cells. Administration of platinum chemotherapy agents via the peritoneum cavity may lead to increased survival rates of female OC patients as showed in the earlier study [121]. However, despite the benefits of using chemotherapeutics drugs as an anti-cancer therapy, the data achieved with IP are still controversial in terms of complete and lasting responses [122]. A recent IP comparative study involved carboplatin and paclitaxel administrated via an intravenous route which showed limited increase in OC survival rates and was further accompanied with severe toxicity [123].

1.2.6.4 Novel targeted therapies

The increased understanding of tumour biology provides a number of potential therapeutic targets. These include the use of growth factor inhibitors, anti-angiogenic therapy, and immunotherapy and gene therapy.
1.2.6.4.1 Growth factor inhibitor studies

In OC, there are many growth factors and their receptors that have been the focus of research but the epidermal growth factor receptor (EGFR) is the most extensively studied. Tyrosine kinase inhibitors (TKI) such as Erlotinib and Cefitinib act on EGFR by blocking signal transduction. These drugs have achieved good results in lung cancer while the result was unacceptable in case of OC [124]. An ongoing research study conducted by the Gynaecologic Oncology Group (GOG) on Lapatinib, a similar TKI had a similar effect on both the receptors EGFR and Her2, particular in advanced stages of OC. This seems to offer a more promising therapeutic strategy [125].

1.2.6.4.2 Anti-angiogenic therapy trials

Recent subset analysis of International Collaborative Ovarian Neoplasm (ICON 7) data indicates that the addition of Bevacizumab seems to impact both progression-free survival (PFS) and overall survival (OS) endpoints to the greatest degree in the advanced stage residual disease population with much less effect in early stage or no residual disease patients [126]. Bevacizumab plays an important role of suppressing angiogenesis due to inactivation of all isoforms of VEGF-A, thereby leading to a reduction in the recurrent tumour among OC patients [127]. Research is still ongoing into the difference between Bevacizumab plus standard treatments and standard chemotherapy agents (carboplatin and paclitaxel) [128].

1.2.6.4.3 Immunotherapy approach

CA-125 is a high molecular weight glycoprotein antigen over expressed at a high level in the patient’s serum unt in 90% of OC patients during advanced stages. Moreover, the concentration of CA125 increases in some cases causing tissue damage, vascular invasion and inflammation related to cancer diseases [58]. Recent clinical trial phase I and II studies revealed that survival rates were considerably enhanced in patients who achieved an immunological response with a murine monoclonal anti-idiotypic antibody [129]. Another hopeful antibody-directed therapy is the use of the monoclonal antibody HMFG1, which is known as a repeated epitope
of CA125, and attached to yttrium-90. One study demonstrated that at 10 years prolonged survival rate was noticed in 78% of OC patients who used this approach in a subgroup population who were in complete remission after surgery and standard chemotherapy drugs [130]. An alternative approach is to deliver the tumour antigen via dendritic cells (DC) where the obtained results were promising [131]. Fusion between DC/ovarian malignancy cells has been generated and demonstrated the inducement of antitumor cytotoxic T-lymphocyte activity in vitro [132].

1.2.6.4.4 Gene Therapy

The overexpression of oncogenes and diminished expression of tumour suppressors are attractive molecular targets for a more causal anti-cancer therapy than the widely used chemotherapeutic agents. Gene therapy in OC has yet to reach its full potential [133]. The mutation in tumour suppressor gene P53 represents 50% of OC incidences [134]. Recently, gene therapy in OC by using adenoviruses as vectors to carry Wild-type p53 into mutated tumour cells have been studied [135]. A wide randomised phase I/II trial showed that a combination of IP p53 gene therapy and standard chemotherapy did not induce any improvement in OC patients [136]. An alternative option uses an attenuated virus that can mutate p53 leading to termination of tumour cells. An alternative study conducted in phase I trial involved the delivery of adenovirus ONYX-015 via IP route [137]. Another gene derived from herps virus named Thymidine Kinase (TK) was used with gangciclovir to destroy OC tumour cells. The idea of this approach depends on causing cancer cell death by delivery of an enzyme-encoding gene to target tumour cells leading to stimulation in the activity of gangciclovir [138].
1.3 Colon cancer
Colon cancer is also known as bowel cancer. In spite of respectable efforts to enhance and improve early diagnosis and treatment, colorectal cancer is reported as the third most common cancer related deaths in the world [139]. Colorectal cancer kills more than 1 million people annually around the world and can affect both male and female. Approximately 50% of patients diagnosed with colorectal cancer eventually die from the disease [140]. Like other malignancy diseases, metastasis is the biggest factor in making colorectal cancer mostly incurable. To date, this malignancy is still uncommon among the age group under 40 [141]. According to the stage of colon cancer, a suitable treatment is given. Surgery represents the primary option for different cancer stages. Chemotherapy agents like Platinum-based compounds such as carboplatin and oxaliplatin are used as an adjuvant therapy in colon cancer [142]. Unfortunately, the therapeutic efficacy of these drugs is known in regards to drug resistance and toxic side effects and depressed immune system. Moreover, chemotherapy agent used for a long period of time leads to drug resistance [143].

1.3.1 Symptoms of colorectal cancer
Adenocarcinoma of the colon grows slowly and is asymptomatic for many years. In some cases bleeding occurs due to blood loss from tumour site. Malignancy in the proximal part of colon usually grows large and show symptoms more than the carcinoma in distal part of colon (rectum) [144]. Although colon cancer in stages 0 and 1 are largely symptomatic they are hard to be distinguished [145]. These symptoms include diarrhoea or constipation, abdominal pain, bloody faeces, abdominal float and feeling incomplete evacuation of bowel and anaemia due to bleeding from tumour location [146].

1.3.2 Stages of colon cancer
In general colon cancer is the growth of malignant tumour in the colon. These abnormal tumour cells divide quickly and lose their functions leading to form mass of tissue called tumour cells. The symptoms of colon cancer play vital role to classify
these malignancy diseases to five different stages (Table1.3) describing the metastasis of tumour into colon tissue layer (Figure 1.1) [147].

**Table 1.3 Colon cancer stages**

<table>
<thead>
<tr>
<th>Stages</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tumour cells restricted in the innermost surface layer lining the colon.</td>
</tr>
<tr>
<td>Stage I</td>
<td>Malignant cells in the inner wall of the colon (submucosal).</td>
</tr>
<tr>
<td>Stage II</td>
<td>Tumour cell spread into adjacent tissue through the wall of colon.</td>
</tr>
<tr>
<td>Stage III</td>
<td>Cancer involved with lymph node, but does not affect adjacent organs.</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Cancer cells spreads to adjacent organs such as lungs and liver.</td>
</tr>
</tbody>
</table>

Table adapted from [148].
Figure 1.1 Different stages of colorectal carcinoma start from stage 0 and advance to stage IV which shows the metastasis of tumour into tissue layer of the colon and spread to adjacent lymph node then affect the other organs [149].

1.3.3 Risk factors of colon cancer
There are various factors which can lead to a rise in incidence rate of colon cancer among people. These involve the presence of defined and non-defined genetic syndromes, a history of inflammatory bowel diseases, age, dietary factors, alcohol and tobacco. Genetic and environmental factors are considered as the main causes contributing to colorectal cancer [150].

1.3.3.1 Genetic factor
The incidence rate of colon cancer is associated with several genetic syndromes such as family adenomatous polyposis coli (APC), which incorporate the familial adenomatous polyposis (FAP), are known as a mass of extensive polyp’s formation in the colon, with the time these polyps progress to malignant mass. This syndrome is related to the inactivation or reduced function of a tumour suppression gene. Other kind of genetic disorders leading to colon cancer is hereditary non-polyposis colorectal carcinoma (HNPPCC), this genetic disorder located on one or more DNA mismatch repair gene [151]. However, both genetic disorders involving FAP and HNPPCC are related to understanding of genomic instability in colon cancer [152].

1.3.3.2 Environmental factors
Environmental factors play an important role in increasing the colon cancer incidence rate. In developed countries, it is estimated that around 50% of colon cancer incidence are due to western type diet [153]. Other considerable risk factors can also lead to colon carcinoma such as food containing higher amount of fat (obesity), drinking too much alcohol and tobacco.

1.3.4 Treatment options
The selection of suitable treatment options depends mainly on the location of the tumour cells in the colon and on the various stages of the disease [154]. However,
surgery remains the first choice for various stages of colorectal cancer. In case of stages I, II of the disease, surgery is curative for 80-90% of the patients. The probability of survival rate decreases with advanced stages (stage III, IV) of the diseases to 5 year survival rate [155]. While chemotherapy and radiotherapy mainly are used as adjuvant options for colorectal patients according to their individual states, Benson *et al* (2004) demonstrated that the chemotherapy option for stage II of colon cancer did not increase the survival rate significantly [156]. While in stage III, chemotherapy 5-Fluorouracil based drugs agent has been considered as a favourable choice with survival rate of 10% [157]. In final stage of colon cancer (stage IV), chemotherapy provides survival rate expected less than one year with severe cytotoxicity [158].

1.4 Breast cancer

Breast cancer is defined as the formation of a malignant tumour that has originated from cells in the breast. Breast cancer is accounted as the most diagnosed cancer in women and the second frequent disease leading to death among cancer in women [159]. More than 1 million new incidences among females estimated every year, breast cancer represent 10% of all new cancer patients and 23% among all sort of cancer in female group [160]. In United states 1 in 8 women are likely to be diagnosed with breast cancer [161]. In the last two decades, the mortality rate of breast cancer has significantly reduced due to enhancement of early diagnosis and advancement in therapies [162]. Many factors such as stage of malignancy, age and kind of treatments which has been used, can determine the probability of survival after diagnosis. Moreover, many studies indicate that the incidence and mortality rate due to breast cancer increases with women's age. Females who are above 45 years of age are at high risk and this malignancy is very limited among age group under 25 years [163]. According to the diagnostic methods such as imaging studies or pathologic diagnosis, treatment decisions can be selected. Biopsy sample which are taken by qualified pathologist to investigate and describe histological features of tissue or cells can provide physician's a successful insight of the condition leading to appropriate diagnosis and an efficient treatment [164].
1.4.1 Risk factors of breast cancer

Generally, it is not possible to determine the cause of breast cancer in any individual woman. However, studies on large number of women have shown that there are some characteristics that are more common among groups of women who developed breast cancer compared to groups of women who did not [165]. There are several risk factors that can lead to the increase in probability of breast cancer incidence among female population. These risk factors are distinguished into following group as explained in (Table 1.4) [166, 167].

**Table 1.4 Risk factors of breast cancer**

<table>
<thead>
<tr>
<th>Category</th>
<th>Risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Rising incidence in women from age 45 to age 75.</td>
</tr>
<tr>
<td>Family history</td>
<td>First-degree relatives had previous history with breast cancer. Mutations of BRCA1 and BRCA2.</td>
</tr>
<tr>
<td>Race</td>
<td>More incidence in women from region of African and Latina.</td>
</tr>
<tr>
<td>Breast tissue density</td>
<td>Denser breast mass increase risk of disease.</td>
</tr>
<tr>
<td>Reproductive/hormonal</td>
<td>Pregnancy after 35 year of age and Late menopause.</td>
</tr>
<tr>
<td></td>
<td>Age at menarche under 12.</td>
</tr>
<tr>
<td></td>
<td>Recent oral contraceptive use.</td>
</tr>
<tr>
<td></td>
<td>Never breastfeed.</td>
</tr>
<tr>
<td>Previous incidence of malignancy</td>
<td>Endometrium, ovarian, colon cancer.</td>
</tr>
<tr>
<td>Live style</td>
<td>Obesity, alcohol, low physical activity.</td>
</tr>
</tbody>
</table>

Table adapted from [167]
1.4.2 Staging of breast cancer

Staging is an important issue for all types of cancers and it enables clinicians to group the patients [168]. According to the size of tumour, number of lymph node invaded by tumour cells and distant organ metastasis, breast cancer stages is divided to five clinical stages. Stages 0, I and II are accounted as early stages and stages III and IV considered as advanced stages [169].

Stage 0: When the tumour lesion are located in ductal system or in lobular part. Estimated 5 year survival rate is 92%.

Stage I: The diameter of tumour size is less than 2cm and metastasis has not involved the lymph node.

Stage II: At this stage metastasis reaches to axillary lymph node without distant metastases and tumour size reaches < 5 cm. The 5 year survival rate is 75%.

Stage III: This stage is distinguished from other stages by many abnormality features. This stage includes several categories: Invasive carcinoma 5 cm or less in diameter with four or more metastatised axillary nodes; invasive carcinoma with larger than 5 cm tumour with nodal involvement; invasive carcinoma with 10 or more involved axillary nodes; invasive carcinoma with metastases in the ipsilateral internal mammary lymph nodes; or invasive carcinoma that manifests in the skin (oedema, ulceration, or satellite skin nodules), chest wall fixation, or clinical inflammatory carcinoma. The 5 year survival rate of this stage is 46%.

Stage IV: Metastasis spread in all breast tissue with 5 year survival rate of 13%.

1.4.3 Therapeutic approaches of breast cancer

Treatment decisions are made by the patient and the physician after consideration of the optimal treatment available for the stage and biological characteristics of the cancer, the patient’s age and preferences, and the risks and benefits associated with each treatment protocol [170]. However, treatment of breast cancer mainly depends on two modes; local and systemic. The local approach includes surgery and
radiotherapy, while the systemic mode includes chemotherapy and hormonal therapeutic agent [171].

1.4.3.1 Surgery

The main purpose of using surgical approach is to remove the malignant tissue and the local lymph node which is affected by tumour as well [172]. Recently, surgeons are using modified radical mastectomy and breast conserving surgery with axillary dissection [173]. In contrast, surgical techniques available are inefficient in majority of locally advanced breast cancer due to failure in removing all the malignant cells with probability of systemic spread of disease [174].

1.4.3.2 Radiotherapy

Radiotherapy is usually prescribed after surgical approach. The main purpose of this kind of treatment is to enhance the local control by killing any tumour cells remaining in breast tissue. Although in case of women who are in early stage of breast cancer, radiotherapy is usually used an option before surgery to restrict the tumour size [175].

1.4.3.3 Chemotherapy

Chemotherapy considered as systemic approach to cure malignant cells, and may also be used to control and arrest metastatic or recurrent breast cancers. Chemotherapy therapeutic agents are usually given in combination to increase their efficacy. Also, many factors can assist to decide which sort of chemotherapy is more appropriate for breast cancer patients such as age, general health condition, and tumour characteristics [176]. The most common chemotherapeutic drugs used as a combination to treat breast cancer are doxorubicin and cyclophosphamide (AC), doxorubicin and cyclophosphamide followed by docetaxel (ACT), cyclophosphamide, methotrexate, and fluorouracil (CMF) and cyclophosphamide, doxorubicin, and fluorouracil (CAF) [177]. In case of patients with advanced stage of breast malignancy, other choices of therapeutic regimen that have been used are gemcitabine, vinorelbine and capecitabine [178]. In contrast, chemotherapy regimen is mostly accompanied with many undesirable side effects which influence on
psychological and physiological states followed by symptoms such as impaired bone marrow, diarrhoea, fatigue, hair loss and neurotoxicity [179]. Furthermore, due to the drug resistance not all of the breast cancer patients respond well to existing chemotherapeutic agents [180].

1.5 Drug resistance

Drug resistance is one of the most common problems in treating cancer patients today and represents a crucial factor in reducing the efficacy of cancer chemotherapy. The possible general pathways of mechanism of drug resistance are categorized in (Table 1.5). Some of these mechanisms play a role against specific drugs where as others result in the development of non-specific drug resistance [181].

Table 1.5 Main mechanism of drug resistance

<table>
<thead>
<tr>
<th>I. Cellular and Biochemical Mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Reduce drug accumulation</td>
</tr>
<tr>
<td>Decrease drug influx</td>
</tr>
<tr>
<td>Increase drug efflux</td>
</tr>
<tr>
<td>Altered intracellular trafficking of drug</td>
</tr>
<tr>
<td>B. Decreased drug activation</td>
</tr>
<tr>
<td>Increased inactivation of drug/toxic intermediate</td>
</tr>
<tr>
<td>C. Increased repair of drug induced damage</td>
</tr>
<tr>
<td>D. Drug targets altered</td>
</tr>
<tr>
<td>E. Altered cofactor or metabolite levels</td>
</tr>
<tr>
<td>F. Decreased apoptosis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Mechanisms Relevant in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Pharmacologic and anatomic drug barriers</td>
</tr>
<tr>
<td>Host-drug interactions</td>
</tr>
<tr>
<td>Increased drug interactions by normal tissues</td>
</tr>
<tr>
<td>Relative increase in normal tissue drug sensitivity (toxicity)</td>
</tr>
<tr>
<td>B. Host-tumour interactions</td>
</tr>
</tbody>
</table>

Table adapted from[181]
1.5.1 Multi drug resistance of cancer cells

Multi drug resistance (MDR) seems to pose a threat to most of the chemotherapeutic agents [182]. In malignant cells, adenosin triphosphate purptis (ATP) is responsible for MDR because ATP-dependent cycle is located on plasma membrane of cancer cells and acts to decrease the drug concentration on the cells surface due to secretion of ATP binding cassette (ABC) [183]. ABC is classified as a third group of resistance pathway. Furthermore, besides its role in MDR, this kind of protein shows expression in normal health tissue to prevent accumulation of toxic substances. Currently, there are 48 ABC genes known in humans. P-glycoprotein (Pgp) was the first protein to be announced whose over expression was studied in this family encoded by the ABCB1 gene [184], while the second discovered MDR protein was MRP1, and the third is related to Pgp and MRPs, and the breast cancer resistance protein (BRCP). Pgp and BRCP are classified in the same subfamily and share around 20% of amino acid homology with each other. While MRPs relevant to the same ABC category share around 50% of amino acid, Pgp acts by decreasing drug accumulation inside the cells by using ATPs, thus limiting the therapeutic efficacy between anticancer therapeutic doses and affected malignant organs [185]. Hen et al (2009) reported that level of Pgp increases with disease prognosis. Paclitaxel (taxol) exhibits significant preclinical and clinical anti-carcinogenic functions against various cancers. Resistance of ovarian tumour cells to paclitaxel is closely related to Pgp expression [186]. Furthermore, in a transfection study conducted by Dong et al in 2001, they demonstrated that Pgp raises the sensitivity of tumour cells to an apoptotic effect of taxol and decline sensitivity to G2M to arrest its effect [187]. Recently, several researches have been performed on the mechanism of drug resistance in cancer cells which can be fruitful in enhancing the mode of drug delivery inside cancer cells.

1.5.2 Role of permeability glycoprotein (P-gp/MDR-1) in drug resistance

P-gp seems to play a significant role in such cells by acting as an energy-dependent efflux pump to remove a number of natural product drugs from the cell before they have a chance to exert their cytotoxic effects [188]. P-gp/MDR1 is expressed in a variety of human solid tumours and hematological malignancies [189]. The molecular
weight of Pgp is 190 kDa, and is composed of 128 amino acids. The structure of Pgp consists of a long chain which is divided into homologues halves; with each halve containing an ATP-domain and trans membrane domain (TMD) [190]. According to the location of Pgp on the cell membrane, it is believed to help in the absorption, distribution and metabolism of drug in live cells [190]. Many studies conducted in vivo and in vitro demonstrate that Pgp plays a vital role in drug absorption and disposition [191]. Generally Pgp is extensively distributed and expressed by certain organs which have secretory functions including liver, kidneys, intestines and bile ducts [184]. Furthermore, Pgp also plays very important role to preventing toxic substances entering cerebrospinal fluid [192]. In cancer patients, when the chemotherapeutic agents are given for treatment, Pgp functions as a drug pump to extrude drug molecules through the plasma membrane and uncharged drug particle entering via a plasma membrane are removed by passive diffusion. A study conducted by Ambudkar et al (2003) stated that Pgp gene was overexpressed in different kinds of cancer including ovarian, breast, and colon cancer [184].

In OC several proposed mechanisms have explained the issue of drug resistance of OC in earlier literature [193]. For example, members of the ABC family of transporters were recognised earlier as conferring resistance to cytotoxic agents, which has been supported by recent work with advanced technologies [194]. This includes the decrease of expression of Pgp by RNAi which sensitises cells to paclitaxel, and the reduction of expression of MRP2 [91] with ribozymes which confers sensitivity to cisplatin [195]. In addition, overexpression of Pgp in OC cells is strongly related with paclitaxel resistance [196].

1.6 Apoptosis
Apoptosis is defined as programmed cell death (PCD) [196]. A programmed cell is an important process to eliminate aged cells or defunctional normal cells. This activity also normally occurs during development and as a homeostatic mechanism to maintain cell populations in tissues. It also represents a crucial function of regulating the immune system, for example when cells get damaged by disease [197]. Although, apoptosis can be induced by normal factors (physiological) and abnormal factors (pathological) which can trigger apoptosis, not all cells necessarily
die. Apoptosis most often proceeds via a series of distinctive changes (Table 1.6) in morphology and biochemistry of the cells including cleavage and shrinkage of the nucleus, chromatin condensation, outer membrane blebs and expression of apoptotic antibodies collectively termed apoptosis [198].

**Table 1.6 Main differences between apoptosis and necrosis**

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell membrane intact</td>
<td>Damage of cell membrane</td>
</tr>
<tr>
<td>Cell shrinkage</td>
<td>Cell swelling</td>
</tr>
<tr>
<td>Ends with degraded cell</td>
<td>Ends with cell lysis</td>
</tr>
<tr>
<td>Affects single cell</td>
<td>Affects groups of cells</td>
</tr>
<tr>
<td>Phagocytosis by adjacent cells or macrophages</td>
<td>Phagocytosis by macrophages</td>
</tr>
<tr>
<td>No inflammatory signs</td>
<td>Significant inflammatory signs</td>
</tr>
<tr>
<td>Stimulated via physiological process</td>
<td>Induced by non-physiological process</td>
</tr>
<tr>
<td>Lysosomal preservation</td>
<td>Lysosomal release</td>
</tr>
<tr>
<td>Strictly regulated process including activation and enzymatic steps</td>
<td>Uncontrolled ion haemostasis</td>
</tr>
<tr>
<td>ATP-dependent energy requirement</td>
<td>No energy requirement.</td>
</tr>
<tr>
<td>Chromatin aggregation on-random DNA fragmentation</td>
<td>Random digestion of DNA</td>
</tr>
</tbody>
</table>

Table adapted from [199].

### 1.6.1 Caspases and apoptosis

The caspase family represents one of the main executors of programmed cell deaths. These group members are classified as cysteine proteases and remaining intracellular protease is classified as pro-caspase forms or zymogen [200]. Once triggered by stress or damage, these inactive pro-form can be converted to active enzymes following the induction of apoptosis [201]. Caspase enzymes have been classified into three subfamilies as presented in (Table 1.7). Initiation of apoptosis through death receptors occur as a result of the stimulation of initiators like caspase-8 or caspase-10 and both caspases can induce other caspases in a
cascade [202]. Then caspase-effector group such as caspase-3 and 6, will then become activated under a cascade effect. These effector caspases are thought to coordinate the execution phase of apoptosis (executioners) and have a significant influence on the induction of apoptosis [200].

**Table 1.7 Different types of caspase groups according to their function modified from [203].**

<table>
<thead>
<tr>
<th>Initiator caspases</th>
<th>Executioner caspases</th>
<th>Inflammation mediator caspases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-2</td>
<td>Caspase-3</td>
<td>Caspase-1</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Caspase-6</td>
<td>Caspase-5</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>Caspase-7</td>
<td>Caspase-11</td>
</tr>
<tr>
<td>caspase-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>caspase-12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.6.2 Mechanisms of apoptosis

Apoptosis is a complex process. Two different apoptotic pathways (Figure 1.2) have been delineated by many studies: extrinsic and intrinsic pathway.

1.6.2.1 Extrinsic pathway.

The extrinsic outside the cell, when the cell is exposed to such a condition that it decides to die. Death Receptors (cell surface receptors that transmit apoptotic signals initiated by specific ligands) play a central role in this pathway. These death receptors are considered as members of tumour necrosis factor (TNF) family [197]. Studies carried out on extrinsic pathway of apoptosis have well characterized the FasL/FasR and TNF and tumour necrosis factor-receptor (TNF-α/TNFR1) models. Most attention has been paid to the Fas ligand pathway. The Fas ligand and Fas receptor are joined via Fas associated death domain (FADD) adaptor protein, while adaptor protein TNF-receptor-associated death domain protein (TRADD) represents the binding place of the TNF ligand and TNF receptor [204]. The adaptor protein FADD interacts with caspase 8 through the death effector domain, resulting in the death-
inducing signalling complex (DISC). Furthermore, caspase-8 cleaves BH3 interacting-domain death agonist (BID). BID induces the translocation, oligomerization and insertion of Bax and/or BAK into the mitochondrial outer membrane leading to mitochondrial permeabilization [205]. In addition caspase-8 through activation of the DISC activates caspase cascade that eventually leading to activation of effector caspases-3,-6,-7 [206].

1.6.2.2 Intrinsic pathway.

An intrinsic pathway, also known as a mitochondrial pathway can be triggered by various death signals including DNA damage [207]. As a result, pro- apoptotic proteins such as Bax and Bak are stimulated. The pro-apoptotic proteins Bax and Bak are categorized under the Bcl-2 gene family [208]. Both of these create a multiple pore leading to release of several proteins including cytchrome-C, Smac/DIABLO, endonuclease G and Omi/HtrA2 [209]. Cytochrome C triggers the apoptosome complex, Apaf-1 and procaspase-9 in the presence of dATP. While Smac/DIABLO combines with endogenous inhibitor of apoptosis protease family (IAPs), such as survivn, there is also an increase in caspase-3 and caspase-9 activities [210]. The essential cellular proteins such as cytoskeleton proteins and kinases undergo proteolysis leading to caspase-3 activity, thus achieving morphological changes and DNA fragmentation which are the main characteristics of apoptosis [196].
Figure 1.2 Two main intracellular pathways

Figure 1.2 Apoptosis is triggered by two main pathways. The extrinsic pathway involves the induction of death domain receptors whereas the intrinsic pathway involves the mitochondria [196].

1.7 Complementary Alternative Medicine (CAM) and cancer
In recent times, many options for treating cancer there have been available such as radiation, chemotherapy and surgery. As reviewed earlier, all these options have limitation and serious side effects and expose patients to potential risks. Hence, there is a constant search for novel drugs and an urgent need to establish novel ways and methods to discover drugs that help to cure cancer. The fight against cancer involves many aspects including the treatment of side effects due to chemotherapy. Complementary and alternative medicine (CAM) includes a collection of therapies, practices, and products that are not considered part of conventional
medical practice. CAM plays a vital role in the treatment of several diseases and has been used in a parallel fashion with Western medicine [211]. The National Centre for Complementary and Alternative Medicine (NCCAM, 2009) identified complementary and alternative therapies within four domains. Although definitions of CAM vary, generally speaking, complementary therapy is the use of CAM along with conventional therapy; alternative therapies are methods that are used in place of conventional therapy; or CAM, is an integrative therapy, which is a total integrative approach to cancer care, including both conventional and complementary therapies [212].

Due to undesirable side effects and complication conditions like fatigue, nausea and hair loss, conventional chemotherapies are not preferred among cancer affected people [213]. Recently, cancer patients have been using alternative therapies in addition to conventional treatment [189]. Various studies have revealed that cancer patients and survivors are more likely to use CAM than an unaffected individual also in the general population, with up to 83% reporting CAM use [214]. Furthermore, affected people pay more attention to employing CAM in their life style.

A promising field of research is clinical studies that explore cancer preventive proteins that exist in milk. Major milk proteins, especially whey concentrates been reported to act as protective agents against carcinogenesis. One such alternative naturally derived protein is bovine lactoferrin (bLf) which has several biological and therapeutic functions. bLf is non-toxic, economical and its applications in healthcare and therapeutics are expected to be widespread [10]. bLf also has the potential efficacy for anti-carcinogenic applications and has many advantages as a safe and natural compound with no proven side effects [8, 215]. A significant number of studies have shown that orally ingested bLf reduce tumour growth [216, 217].
1.8 Lactoferrin

1.8.1 Structure and function

Historically, Lf was first isolated from bovine milk [217] but this glycoprotein is found in other mammalian milks [218]. Lf (Figure 1.3) is a glycoprotein with a molecular weight of about 80 kDa and contains about 700 amino acids with two symmetrical lobes forming a simple polypeptide chain (N and C lobes). These two lobes are joined together by a hinge region containing parts of an α helix between the amino acids. Each lobe is further subdivided into two domains, N1, N2 and C1, C2 which contains one iron binding site and one glycosylation site [219]. These lobes join and attach to the metal atom with synergy to carbonate ion (CO$_3^{2-}$). The metals elements that can bind are iron (Fe$^{2+}$, Fe$^{3+}$ Mn$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Al$^{3+}$, Ga$^{3+}$ ) [220]. Lf has metal ion binding feature and hence it can be saturated with iron which is referred as holo Lf. According to many biophysical studies, there are three forms of Lf according to its iron saturation state: holo Lf (binds two Fe$^{3+}$ ions), Apo Lf (iron free) and monoferric form (one ferric ion) [221]. Apo Lf is known to have an iron free structure and has an open conformation whilst holo-Lf has an iron binding form and is distinguished by two bound iron molecules located on each of the two lobes forming a closed conformation with greater resistance to proteolysis [222]. Various studies have been conducted justifying that iron saturated lactoferrin (Holo Lf) is more rigid and compact than Apo Lf form. Baker and Baker demonstrated that, metal free Apo-form is more flexible and are prone to thermal denaturation and proteolysis [223]. There are three distinct isoforms forms of Lf. Lactoferrin-α is distinguished by its iron binding nature, but has no ribonuclease activity. The other two forms lactoferrin-β and lactoferrin-γ, do not express any iron binding action but do show ribonuclease activity [224].

Lf is also detected in various body fluids (Table 1.8) such as tears, saliva, vaginal fluid and gastrointestinal juice. Lf has been found in higher concentration in bovine, human colostrum’s and milk [11, 216]. The amount of Lf reported in colostrum is more than what is found in regular milk. In bovine colostrum, the Lf concentration is about 20-fold higher than that in bovine milk [225].
Colostrum is the early milk produced during the first several days of post parturition, containing higher amounts of immunoglobulin’s, and growth factors [226]. The fluid has a distinct composition different from the milk produced during the latter part of lactation. Unique components of colostrum provide immediate immune protection to the newborn. Colostrum is not only a source of nutrients such as proteins, carbohydrates, fat, vitamins and minerals but also contains several biological molecules which are essential for specific function [227, 228]. Various beneficial elements have been found in the bovine colostrum such as immunoglobulin’s (Igs), IgG1, growth factors, insulin-like growth factor-1 (IGF-1), transforming growth factor beta-2 (TGF-β2) and growth hormone (GH) along with Lf, lysozyme and lactoperoxidase. Furthermore, Lf forms the most important elements beside the immunoglobulin’s [229, 230].

Lf possesses various biological functions (Table 1.9) such as antibacterial, antiviral, antiparasatic and anticancer [230]. Several physiological roles have been attributed to Lf, such as regulation of iron homeostasis, host defence against infection and inflammation, regulation of cellular growth, differentiation and protection against cancer. Recent studies and researches have clarified the role of orally taking bLf to the improve intestinal microbial flora, elevated serum ferritin and hematocrit levels; reduce in lower respiratory system diseases and elevated anti-microbial functions [231]. Orally administered bLf has also shown the beneficial effects in other animal infection models including oral candidiasis, influenza virus pneumonia and skin infections due to the herpes virus [232].
Figure 1.3 Three-dimensional structure of biferric lactoferrin

**Figure 3.** The polypeptide chain of Lf. A, Structure of the iron-bound (holo-Lf) is folded into two globular lobes, N and C lobe. Each lobe forms two domains N1 and N2 or C1 and C2. The helix joins the two lobes (H) with the C-terminal. The two iron binding sites are highlighted red. B, structure of the iron free form of Lf (Apo) [223].

**Table 1.8 Distribution of Lf in body fluids**

<table>
<thead>
<tr>
<th>Biological fluid</th>
<th>Amounts reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow’s Milk</td>
<td>&gt; 20-200 µg/ml</td>
</tr>
<tr>
<td>Cows colostrum</td>
<td>&gt; 1500 µg/ml</td>
</tr>
<tr>
<td>Saliva</td>
<td>&gt; 7-10 µg/ml</td>
</tr>
<tr>
<td>Tear</td>
<td>&gt;2200 µg/ml</td>
</tr>
<tr>
<td>Colostral breast Milk</td>
<td>&gt;7000 µg/ml</td>
</tr>
</tbody>
</table>

*Data taken from [233, 234]*
Table 1.9 In vitro and in vivo activities of bLf

<table>
<thead>
<tr>
<th>Functions</th>
<th>Mode of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimicrobial</td>
<td>Growth suppression by sequestration microbes from iron required for metabolism.</td>
<td>[235] [225, 236]</td>
</tr>
<tr>
<td>Antiviral</td>
<td>Prevents first contact between virus and host cell.</td>
<td>[237] [238]</td>
</tr>
<tr>
<td>Immunomodulatory and anti-inflammatory activity</td>
<td>Lipopolysaccharide (LPS) binding, stimulation of (NK), reduction of pro-inflammatory cytokines-cell maturation.</td>
<td>[239] [240]</td>
</tr>
<tr>
<td>Anti-carcinogenic activity</td>
<td>Inhibit the development of metastases <em>in vivo</em>.</td>
<td>[8] [9] [241]</td>
</tr>
<tr>
<td>Iron absorption</td>
<td>Increases solubility and receptor mediated uptake.</td>
<td>[242] [243]</td>
</tr>
</tbody>
</table>

1.8.2 Complexes of lactoferrin

Several research studies have demonstrated that bLf has self-assembled feature and has also been found as dimers, with a molecular weight twice that of the Lf monomer. It also has been found Lf can bind with milk proteins macromolecules such as IgG, albumin, casein, lysozyme and heparin [244, 245]. Moreover, Lf has been found to elute with different molecular weights equivalent to monomers, dimers and trimers, according to chromatographic analysis of bovine milk [246]. Scattering studies have also proven that HLf has revealed a large aggregation, although no molecular level explanation has been presented [247]. Moreover, bLf has been
detected in complex form at large MW, estimated to be more than 80kDa in nonlactating mammary secretion during involution and also bLf has been found attached to other milk proteins [248]. Recently, field experimental study data revealed the majority of bLf produced via mammary glands during 7 and 21 of involution was at high MW of 150kDa which indicates a dimeric form of bLf, while 250kDa might suggest the presence of Lf in trimeric form or union between dimeric Lf with a IgG molecule. In addition, the functions and biochemical features of complexes among Lf and other macromolecules, like Ig and casein are still unclear [249]. A study revealed that fusion of Lf with antimicrobial proteins such as IgA in bovine milk might leads to improved antimicrobial activity [250].

1.8.3 Immunomodulatory functions of lactoferrin

It has been shown that bLf plays a crucial role in immunomodulation that influence both innate and acquired immunity [251]. Furthermore, bLf induces the immune system to produce and express mature lymphocytes. Lf present in different biological fluids such as saliva, tears, nasal and vaginal secretions play a vital function as a first line of defence in boosting the innate immune system in human [252]. An in vivo study conducted by Sfeir et al (2004) demonstrated that an oral intake of bLf increases the response of immune system in mice [253]. Lf is a positively charged protein that can bind with negative charge molecules on the cell surface layer of immune cells [222, 254]. As a result, this association can stimulate the signalling pathways that raise the response state of immune cells such as activation, differentiation and proliferation [255]. Moreover, bLf (Figure 1.4) can bind to host cells and lead to inducing various signalling pathways [256]. bLf has been shown to prevent the expression of inflammatory cytokines that stimulate and encourage immune cells to migrate to inflammation sites [257]. Many studies reported that ingested bLf via an oral route can decrease the expression of pro-inflammatory cytokines and increase anti-inflammatory cytokines [258]. More recent studies have been shown that, oral intake of bLf promotes and encourages the intestine-associated immune functions: increased NK cell, interleukin and interferon production [8]. Furthermore, an oral intake of Lf elevated the level of Th1 cytokine, which can generate CD8*T cells through the stimulation of IL-18 [259]. Orally
administrated bLf also promotes the secretion and production of Th1 and Th2 type cytokines in the digestive system especially in the small intestine of healthy animals [260]. Moreover, Lf receptors have been detected on the surface of lymphocytes cells, platelets and in some bacterial species [260]. Research conducted by Puddu et al in 2009, showed that majority of cells were able to bind to the Lf, including immunogenic cells such as monocyte, macrophage and dendritic cells, which helped in the migration, activation and proliferation of immune cells as well as the expression of cytokines and effector molecules necessary to elicit an immune response in the host [261].

**Figure 1.4 Mechanism of lactoferrin action and the immune system.**

*Figure 4. After oral administration, bLf is partially degraded by the protein digestive enzymes in the stomach and in the small intestine from protease to peptides. The bLf and peptides produced as a result of digestion are absorbed by the mucosa layer present in small intestines and are received by Lf receptors which are found at the surface of the gut cells and immune cells like lymphocyte, macrophage and dendritic cells. This stimulates the immune cells to increase in number and to induce the*
production of the humeral factor such as cytokines, and IL-18. These factors have several biological functions, such as enhancing the immunity, killing infective germs and to removing malignant cells [251, 262].

1.8.4 Lactoferrin as an anticancer: future therapeutic candidate

Many studies have shown that an oral intake of bLf decreases and supresses tumour development [8]. Lf as a natural compound has the capability to enhance the immune system as well as play a role as potential chemo-preventative agent against carcinogenesis. Thereby, bLf can be employed as a non-toxic anti-cancer agent [263]. bLf in native form exerts anticancer function in rodents and humans and has proven to be safe for normal human cells [215]. In vivo studies on oral administration of bLf demonstrate that it activates the immune system and other associated lymphoid tissues in the small intestine [8, 264]. Lf receptors present in the human intestine bind to both bLf and human lactoferrin (hLf) and allow the successful absorption of iron attached to the Lf especially in breast fed infant during their first 6 months [265]. In vivo studies have shown that bLf has supressed colon, oesophagus, and lung and bladder cancer in rats and has induced apoptosis in colon epithelial cancer cells and leukaemia in mice [215]. Research studies have suggested that in vitro purified and saturated bLf has antitumor functions by regulating the function of NK cells functions. Recently, it was reported that the antitumor function of bLf has contributed to the modulation of the NK cell and lymphokine activated killer cell cytotoxicity. In a recent study, bLf was shown to suppress the VEGF which stimulate angiogenesis in an animal model [266]. The pathway through which Lf modulates the suppression of tumour cells is still poorly understood and needs to be investigated. In addition to this 100% iron saturated bovine Lf (Fe-bLf) also showed a decrease in the angiogenesis and an increase in the production of Th1 and Th2 cytokines and hence increased apoptosis [8]. The chemotherapeutic regimen such as paclitaxel, doxorubicin and fluorouracil, when used with Fe-bLf as a combination dosage exhibits significant efficacy against tumour cells In vivo. Importantly, Fe-bLf and its nanoformulation stimulate production of red and white blood cells depleted by chemotherapy, thus serving as a potential cure to the mice [8, 9].
1.8.5 Lactoferrin receptors (LfR)

As mentioned earlier in this literature review, the biological properties of Lf are mediated by specific receptors located on the surface of the target cells. Lf receptors are typical for several types of cells such as mucosal epithelial cells [268], hepatocytes, monocytes, macrophages, polymorphonuclear leukocytes, lymphocytes and some type of bacteria such as *Staphylococcus aureus* [269-270]. LfR can be varied, with sulphate chains of proteoglycans representing the major part of Lf binding sites (80% of total binding), and with the glycan moiety being reported to participate in Lf cell binding, these domains play a vital role in the endocytosis of Lf inside the cells [271]. Some cells have “main receptors”, which enable them to bind not only with lactoferrin, but also transferrin or the Lf of other species. This phenomenon allows Lf to bind to a wide variety of “Lf putative receptors” expressed in various cells and organs and help with the internalisation and absorption of Lf [272].

1.8.6 Lactoferrin and apoptosis

Data from both *in vivo* and *in vitro* has shown that bLf triggers apoptosis in both extrinsic and intrinsic pathways via activation of several of apoptotic genes such as caspase-8, caspase-9, 3 and Fas in colon cancer in rats and leukemia cell [273]. Through the initiation of caspase-8 and 9, effector caspase-3 is upregulated leading to cell apoptosis [274]. However, an earlier study revealed that hLf triggers apoptosis in Jurkat Leukemia cells through JNK associated Bcl-2 pathway via caspase-9 related to the intrinsic pathway [275]. In addition, a study conducted by Mohan *et al* in 2007 stated that, a combination of bLf and tea polyphenol exhibited a strong inhibition of human tongue squamous carcinoma by means of apoptosis via the trigger of caspase-9 and Bcl-2 [276]. Unpublished data from our lab revealed that both and Fe-bLf and Se-bLf have a greater effect in inducing apoptosis in colon cancer cells due to the expression of caspase-9 [277]. Apoptosis was induced via an intrinsic pathway; caspase-3 was upregulated along with Bax, a known pro-apoptotic protein that counters the effect of Bcl-2, an anti-apoptotic protein [277]. Additionally, in breast cancer, bLf is able to reduce the growth of cancer cells, and the addition of exogenous bLf to the culture media of breast cancer cell lines (MDA-MB-231) induced cell cycle arrest at the G1/S transition [278].
1.9 Ion exchange chromatography

Chromatography is a common approach that has been in use for several decades. This method has been widely used for separation and purification of bio-molecules from small peptides to large antibodies from different mixtures. Ion exchange chromatography (IEC) is most popular method for the purification of proteins and other charged molecules is IEC. The separation is due to competition between proteins with different Surface charges for oppositely charged groups on an ion exchanger adsorbent [279]. In cation exchange chromatography positively charged molecules are attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support [280, 281]. Majority of IEC (Figure 5) technique involved five major steps. Equilibration represents initial stage, in terms of pH and ionic strength, allowing the binding of the desired solute molecules. The exchanger groups are associated at this time with exchangeable counter-ions. The second stage is sample application and adsorption, in which solute molecules carrying the appropriate charge displace counter-ion and bind reversibly to the gel. Unbound substances can be washed out from the exchanger bed starting buffer. In the third stage, the substances are removed from the column by changing to elution conditions unfavourable for ionic binding of the solute molecules. This process involves increasing the ionic strength of the eluting buffer. As shown in Figure 4, the desorption is achieved by increasing salt concentration gradient, and solute molecules are released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first. The fourth and fifth stages are the removal from the column of substances not eluted under the previous experimental conditions and equilibration at the starting conditions for the next purification [319]. Furthermore, the separation is completed since different substances have different degrees of interactions with the ion exchanger due to differences in their charges, charge densities and distribution of charges on their surfaces [281].
Figure 1.5. Separation in ion Exchange chromatography

Figure 5 Ion exchange chromatography separates molecules based on differences in their accessible surface charges.
1.10 Hypothesis
It has been shown that bLf is involved in various biological functions and that tends to possess anti-carcinogenic properties. This study hypothesized that bLf could be successfully purified from Australian bovine colostrum. Secondly, this study hypothesizes that bovine lactoferrin could be successfully employed as a therapeutic anti-carcinogenic natural and safe protein to treat different types of cancer, especially ovarian cancer.

1.11 Novelty of the project
The effects of bLf (especially HMW purified in the study) on ovarian cancer and any other cancer cell lines are not yet known. Therefore, this project is going to fill the knowledge gap regarding the action of bLf in a wide range of cancers. However, its effects on targeting the development of ovarian cancer and apoptosis have not been reported to date. The present project discusses the anti-tumourigenesis and apoptosis enhancing effects of the colostrum derived lactoferrin in human ovarian cancer cell based assays. The focus of this project was to elucidate on a biochemical and molecular basis the preclinical anti-cancer efficacy of the HMW-bLf treatment in targeting tumour cell growth and apoptosis.

1.12 Research Question
Can the HMW-bLf target the growth and apoptosis in cancer cells?

1.13 Project aims
1- To purify and physicochemically characterize bovine lactoferrin (bLf) from Australian bovine colostrum.

2- To study in vitro the effects of HMW-bLf on cancer cell growth and cell death by employing OC cell line OVCAR-3.

3- To study the molecular mechanism of HMW-bLf in inducing cancer cell death in OC cell line OVCAR-3.

3- To compare the anti-carcinogenic activity of purified HMW-bLf among ovarian cancer, breast cancer and colon cancer cell models.
Chapter 2: Materials and Methods

2.1 Lactoferrin purification

The bovine lactoferrin was purified by using previously established method [282]. Figure 2.1 shows the flow sheet diagram outlining the steps involved in purification procedures.

2.1.1 Preparation of bovine colostrum whey

The colostrum sample was obtained immediately postpartum from Australian farm cows and frozen at -80 °C. Frozen colostrum was thawed with running water at room temperature every time before use. To collect the whey proteins, the high viscous colostrum sample was diluted with sterile working phosphate buffer prepared by mixing (8g NaCl, 2.2g KCl, 1.44g of Na₂HPO₄, 0.24g KH₂PO₄ in 1 litre and adjusted to pH 7.4). Diluted colostrum sample was skimmed by centrifugation at 3000 x g at 4 °C for 30 mins. The fat (yellow layer) was discarded and the supernatant was collected and kept frozen at -20°C.

2.1.2 Casein precipitation

Caseins contribute to 80% (w/w) of the whole protein mass and can easily be recovered from skim milk via isoelectric precipitation (by addition of or in situ production of acid). The skimmed diluted bovine colostrum whey sample was acidified by 1M HCl until pH 4.6. The precipitated casein was removed by centrifugation (3500 X g for 30 mins at 4°C), the supernatant was collected and the pH was adjusted to 7.4.
Figure 2.1 Flow diagram outlining the details used for purification of lactoferrin from Australian bovine colostrum.
2.1.3 Gel filtration and ion exchange chromatography.

Lactoferrin was purified using a cation exchange chromatography on SP-Sepharose following the procedure of Van Berkel et al [282]. The column was packed with SP Sepharose big beads food grade (Amersham biosciences, 17-0657-03). After packing into the column (15x3 cm) and before being used for the first time, the medium (stationary phase) was washed as follows with 5 column volumes of water followed by 5 column volumes of 1 M NaCl. It was left in 1 M NaCl for 12 h and then in 5 column volumes of water. The diluted skimmed colostrum was diluted again in the ratio of 1:1 with the dilution buffer (0.04 M NaH$_2$PO$_4$, 0.8 M NaCl, 0.04% (v/v) Tween 20, pH 7.4), and then the diluted prepared colostrum sample supernatant was loaded through the column. Following this, the SP-Sepharose was washed with washing buffer (0.02 M NaH$_2$PO$_4$, 0.4 M NaCl, 0.02% (v/v) Tween 20, pH 7.4) to elute the unbound proteins. Lactoferrin was then eluted with the elution buffer (0.02 M NaH$_2$PO$_4$, 1 M NaCl, pH 7.4). The column was run at a flow rate of 3 ml/min. The elution fractions were dialysed against sterile milli-Q water for 24 h. The sample was then freeze-dried to remove water without affecting the structure of the purified protein.
2.2 Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE)

The purity and molecular weight of affinity purified bovine lactoferrin was checked by SDS-PAGE. Protein samples mixed with sample buffer were heated to 94 °C for 5 mins and (10µL at a concentration of 4mg/ml of protein) of each sample was loaded on to each lane of 10% (1.5 mm thick) gel. The electrophoresis was carried out at 200V for 55 mins in a Mini PROTEAN 3© gel tank (Bio-Rad, Gladesville, NSW, Australia) in freshly prepared running buffer. Protein standard markers (Bio Rad, 161-0374) were added to the first lane of the gel. Proteins were stained with Coomassie Brilliant Blue R 250, 0.125% (w/v) in 10% acetic acid and 50% methanol. Destaining was carried out in a solution of 10% acetic acid and 50% methanol in deionised water.

2.3 Western blotting (WB)

Following electrophoresis, the gel was removed and placed in Towbin 0.03% SDS transfer buffer for 15 mins. A Polyvinylidene Fluoride (PVDF) membrane (Amersham Biosciences, Australia) was cut to size (7x8.4 cm) and activated for 30 seconds in 100% Methanol and then immersed into milli-Q water for 10 mins. The membrane was kept in Towbin transfer buffer for 20 min. The membrane sandwich was then assembled as per manufacturer instructions (Bio-Rad). All contents of the sandwich were soaked in cold transfer buffer prior to assembly. Transfers were performed in a Bio-Rad tank in fresh transfer buffer, at 0.35 mA and 100 Volts for 60 mins. Following transfer, the membrane was separated and rinsed three times 1 x Tris Buffer saline (TBS, pH 7.6) for 5 mins. The membrane then was blocked for two hours with porcine gelatine (Sigma- Aldrich) prepared in 1 x TBS and incubated at 37°C with gentle orbital rocking. After blocking, membrane was rinsed three times for about 5 mins each and incubated overnight at 4°C with goat anti-bovine lactoferrin (Bethyl laboratories, USA) primary antibody in a 1:1000 dilution. After overnight incubation, membrane then was then washed three times in 1 x TBS containing 0.1 Tween -20 (TBS-T) for 10 mins each and finally washed one time with 1 x TBS. The membrane was then incubated for 1 hour with anti-goat-HRP conjugate (Sigma-Aldrich) at 1:10,000 dilution at room temperature with gentle rocking. Subsequently, membrane was then washed three times with 1 x TBS-T for 10 mins each followed
by washing with 1 x TBS 5 mins each. After probing, membranes were treated with ECL working solution from developing kit (Amersham Bioscience, Australia) for two mins. Membrane was viewed using the Bio-Rad ChemiDoc XRS Camera. Images were captured and processed using Quantity One® software.

2.4 Differential scanning calorimetry (DSC)

The degree of thermal stability of purified HMW-bLf from colostrum (as identified by SDS-PAGE and WB) was assessed by differential scanning calorimetry (DSC). Absorption or release of thermal energy from samples was monitored using DSC (TA instrument DSC Q200. USA). 5 mg of bLf was measured accurately by sensitive balance and sealed into aluminium pan. DSC scans were programmed in the temperature range of 35–110 °C and at heating rate of 10 °C/mins. Native monomeric bovine lactoferrin (NM-bLf) ~78 kDa was used as a control, along with its iron depleted (Apo-bLf) and iron saturated (Fe-bLf) forms. These samples were prepared in our laboratory.

2.5 Fourier Transformer Infrared Spectroscopy (FTIR) analysis

FTIR was carried out to confirm the chemical constitution of the HMW-bLf purified from colostrum and the native monomeric bovine lactoferrin (NM-bLf), Apo-bLf and Fe-bLf samples were used as controls. FTIR spectroscopy is an excellent classical common technique for structure determination of small molecules and to detect the secondary structure of proteins. Samples were mixed with 200 mg of potassium bromide (KBr) (Sigma- Aldrich) powder. The mixtures were compressed to thin disc by using air hydraulic machine to make the KBr disc containing the sample. FTIR (Bio-Rad, Gladesville, NSW, Australia, Bruker Vertex 70 spectrometer and OPUS 5.5 software) analysis was performed between 4000 and 450cm⁻¹ at a resolution of 4cm⁻¹ averaging 10 scans.
2.6 Iron estimation

The method of measuring iron saturation in lactoferrin was modified from as described by King (1982) and Kanwar et al (2008) [9]. Briefly, to 1 ml of HMW-bLf, iron standard representing the highest iron concentration and blank which was milli-Q water. 50µl of ascorbic acid was added to each sample and allowed to stand for 10 mins. Samples were then centrifuged at 10000 rpm for 20 mins. 500µl supernatants collected from each sample was added into new eppendorf tubes containing 100µl of alkaline acetate solution followed by addition of 75 µl of tripyridyl solution. 200 µl of each solution was then transferred into an optical clear 96 well plate (interpath services, West Heidelberg, Victoria, Australia) and the absorbance was read at 550nm. Percentage of iron was calculated by substituting the resulting absorbance value into the following equation.

\[
\text{Iron (µg/ml) = } \frac{\text{Test} - \text{Blank}}{\text{Standard} - \text{Blank}} \times \text{concentration of standard}
\]

2.7 Gut enzyme intestinal digestion assay

Omnizyme cocktail represents most of the gut enzymes responsible for digestion of proteins, carbohydrates and fats [283]. The Omnizyme, a commercially available proteolytic enzyme tablets were purchased from Rainrock Nutritionals (U.S.A). Omnizyme enzyme was added to purified HMW-bLf to investigate its stability against gut enzymes. The Omnizyme tablet was dissolved in 15 ml of 0.1M NaHCO₃ at pH 7. 2.5µl of Omnizyme solution was added to 50µl of purified HMW-bLf from bovine colostrum at a concentration of 1mg/ml and the mixture was incubated at 37°C. Samples were shaken at 150 rpm. The supernatants were collected at different time intervals of (4 h, 6 h and 8 h) and heated at 42°C for 7 minutes to arrest the enzyme reaction. To analyse the stability of HMW-bLf, NM-bLf against gut digestive enzymes, all samples were analysed by SDS-PAGE.
2.8 *In Vitro* cell bioassays

2.8.1 Cell lines and culture conditions

In this study, three types of cancer cell lines OVCAR-3, MDA-MB-231 and SW480 were used and obtained from American Type Culture Collection (ATCC, supplied by Cryosite, Australia. OVCAR-3 has been derived from *Homo sapiens* (female) ovary adenocarcinoma, MDA-MB-231 has been derived from *Homo sapiens* (female) breast carcinoma while SW480 cell line was derived from *Homo sapiens* (male) colorectal adenocarcinoma. All cell lines were epithelial and had adherent growth properties. ATCC guidelines were followed in maintaining these cell lines and all the glassware and plastic ware used were sterile. The maintenance and subculturing of the cell lines was carried out in S@feMate™ 1.2 class II biosafety cabinet (Euroclone Bioair, Italy) and incubated in Lishen CO₂ incubator at 37°C with 5% CO₂ (Thermoline, Preston south, Victoria).

2.8.2 Cell culture

OVCAR-3 was grown in RPMI-1640 (ATCC) medium with 20% foetal bovine serum (FBS), 0.01mg/ml bovine insulin (Sigma Aldrich) and 1% Penicillin-Streptomycin 100X (Gibco™) and incubated at 37°C in humidified atmosphere of 5% CO₂, MDA-MB-231 grown in L-15 media supplemented with 10% FBS and 1% Penicillin-Streptomycin solution (Gibco™) and cells where incubated at 37°C in a CO₂ free environment. SW480 were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin (GibcoTM) and incubated at 37°C in humidified atmosphere of 5% CO₂.

2.8.3 Cell lines maintenance

Cancer cell lines were cultured in two sizes of culture flasks 25 and 75 cm² (Interpath, Australia) with appropriate amount of growth media. When cells reach confluent levels of 80-90%, they were then subcultured by discarding old media and rinsing with sterile PBS of cell culture grade. Suitable amount of Trypsin-EDTA solution (Sigma-Aldrich, Australia, T3924) was added and flasks were incubated at 37 °C for 5-7 mins, until cells were detached from the flask base. To stop trypsin activity, suitable amount of fresh growth media was then added and cells were aspirated by gentle pipetting to prevent the clumping of cells. Following
centrifugations, single cell suspension was prepared, cell number counted using a haemocytometer and recultured at approximately 1-2 x10^5 cells per ml of culture media.

2.8.4 Lactate dehydrogenase enzyme (LDH assay)

Cytotoxicity caused by treatments of HMW-bLf and other control forms of bLf was measured by release of lactate dehydrogenase (LDH). The cytotoxicity detection kit (Roche Applied Science, Castle Hill, NSW, and Australia) is based on calculating the LDH leakage into the culture medium after 24 h following exposure of cells to different treatments. LDH is present in all cells and is released into supernatant due to cell membrane damage. This can be easily determined by spectrophotometric reader. The assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD. The formation of NADH from the above reaction results in the change in absorbance at 490nm. Two kinds of controls were used in this assay. Negative control used was normal culture media and positive control representing maximum value of LDH release was 1% (v/v) Triton X-100 (Sigma Aldrich, Sydney, Australia) mixed in assay media. The assay was carried out according to the manufacturer’s instructions, where around 2 x 10^4 OVCAR-3, MDA-MB-231 and SW480 cell/ml were seeded in 96 well plates (100 µl/well). Plates were incubated overnight to allow cells to attach to the plate. Entire media was carefully discarded and replaced with the different treatment concentration doses of HMW-bLf 800, 1600, 2400, 3200 µg/ml prepared in assay media. After treatment, cells were incubated in 37°C at 5% CO₂ (MDA-MB-231) was incubated in atmosphere free CO₂ incubator) for 24 h. Each treatment was carried out in triplicate. Plates were centrifuged at 250 x g for 8 mins. 100 µl of supernatant from each well was transferred into another non sterile 96 well plate. 100 µl of LDH reagent mixture was added to all cell supernatant. Plates were then incubated in dark at 25°C for 30 minutes. The absorbance values were measured by using a SH-1000 lab absorbance microplate reader (Corona Electric) at 492 nm with reference wave length at 620 nm. The percentage cytotoxicity calculated by following this equation.
Cytotoxicity (%) = \frac{\text{Absorbance value} - \text{Low control}}{\text{High control} - \text{Low control}} \times 100.

Low control = Untreated cells, High control = Cells treated with 1% Triton X

2.8.5 Cell viability assay

MTT assay was performed to investigate the effect of HMW-bLf and other control forms of bLf on the viability of cancer cells. The assay measures the levels of mitochondrial dehydrogenase activity and directly measure the activity of viable cells. The assay uses a chemical called MTT (3 - [4, 5 - dimethylthiazol -2 -yl] - 2, 5 – diphenyl tetrazolium bromide) provided by (Sigma Aldrich). This chemical, when mixed with mitochondrial dehydrogenase forms crystals converting the solution colour from yellow to purple with insoluble crystals. Cells were seeded in 96-well plates at a density of 2x 10^5 cells per well and allowed to grow for 24 h before treatment. Cells were then treated with different doses of HMW-bLf 800, 1600, 2400, 3200µg/ml and incubated for 24 h. 10 µl of MTT solution was added per well and incubated for 3-5 h. After incubation, cell media was removed and 100µl MTT solvent was added per well to dissolve the purple crystals formed due to the reaction between mitochondria and MTT reagent. The ELISA SH-1000lab absorbance microplate reader (Corona Electric) was used to read the absorbance at 550 nm with a reference absorbance at 690 nm. Absorbance levels in blank and in control wells were used to determine the percentage of proliferation according to the following equation.

\text{Viability} \% = \frac{\text{Treatment value} - \text{cell free sample}}{\text{Mean value of untreated cells}} \times 100

2.8.6 Cells proliferations assay (CyQuant).
To determine the effect of purified HMW-bLf on the levels of proliferation of OVCAR-3, MDA-MB-231 and SW480. Cell proliferation assay was performed using CyQuant assay kit (Invitrogen) as per kit instructions. The CyQuant® assay is based on the measurement of cellular DNA content via fluorescent dye binding. Because cellular DNA content is highly regulated, it is closely proportional to cell number. The extent of proliferation is determined by comparing cell counts for samples treated with bLf samples and untreated controls. Initially viable cells are plated out at a concentration of 2 x 10^5 cells/ml in 96 well microplates and incubated overnight. Treatments with different doses of HMW-bLf were carried out in triplicates. After 24h of incubation, media was removed from the wells. The wells were washed carefully twice with sterile PBS. Cells in the microplate were stored at -80 °C overnight. 200 µl of 1X cell-lysis buffer mixed with CyQuant® GR stock solution (Invitrogen) was added to each well and incubated at room temperature for 5 minutes. Samples were measured using fluorescence microplate reader with filters appropriate for 480 nm excitation and 520 nm emission.

\[
Proliferation \% = \frac{\text{Treatment value}}{\text{Positive control}} \times 100
\]

Positive control = Cells + media 20% FBS

2.8.7 Morphological observations
After cells were treated with purified HMW-bLf and other forms of bLf for each assay, cells were viewed using an inverted microscope (Prism Optical) and images obtained by a digital camera.

2.9 Flow cytometric Analysis
Flow cytometry (FC) was used for the determination of cell death following treatments with bLf samples. In this assay, the cytotoxicity effect of HMW-bLf (different concentrations) and iron saturated bovine lactoferrin (Fe-bLf) as a positive control was determined by propidium iodide (PI staining assay). OVCAR-3, MDA-MB-231 and SW480 were seeded at 2x10^5 in six well plates. Once the cells had
reached appropriate growth levels of ~ 90% confluency, cells were treated with HMW-bLf and incubated for 24h. Cells were detached using trypsin EDTA (Invitrogen) and centrifuged. The supernatant was discarded and cell pellet was washed with sterile PBS and resuspended in 500 µl sterile PBS, then PI solution was added. After 15 mins of incubation at room temperature, the cells were analysed using flow cytometry (BD FACSCantos II).

2.10 Annexin V-FLUOS/PI staining
The detection and quantification of early and late apoptosis in OVCAR-3 treated with HMW-bLf was further analysed using Annexin V-FLUOS staining kit (BD Biosciences, Australia). Annexin V is used to investigate the early apoptosis and the assay was based on the theory of plasma membrane alteration found in early stages of apoptosis, namely Phosphatidylserine (PS) exposed at the external surface cell. Annexin V is a Ca\(^{2+}\) dependent phospholipid binding protein with high affinity for Phosphatidylserine. However, necrotic cells also expose PS due to the loss of membrane integrity therefore apoptotic cells are differentiated from necrotic cells by the use of propidium iodide (PI) that stains DNA of leaky necrotic cells only [325]. Cells were cultured in 6 well plates at density 2×10\(^5\) and incubated at 37°C in 5% CO\(_2\). Cells were then treated with different samples of HMW-bLf for 24h. After incubation, cells were washed with 1× PBS and trypsinised with 0.25% trypsin/ethylenediamine tetra acetic acid (EDTA) (Sigma-Aldrich, Australia) and collected with PBS into centrifuge tubes. After centrifugation, supernatant was then removed and washed with PBS and centrifuged again at 1800 rpm for 5 mins and the pellet then was resuspended in 1x binding buffer. Annexin-V-FLUOS labelling solution and PI solutions were added at 5µl to each test sample and incubated for 15 mins at room temperature in the dark. Samples were analysed using a fluorescence activated cell sorter (BD FACSCantos II). Cell death was analysed using the FACS software output. All the processes for Annexin V-propidium iodide method were followed as per the instructions provided by the manufacturer.

2.11 Cellular internalization of HMW-bLf
Immunofluorescence staining followed by confocal microscopy enabled the visualisation of the internalised HMW-bLf in OVCAR-3, MDA-MB-231 and SW480
cells. For immunofluorescence experiments, cells were cultured on to 8 well slides at a density of 2 x 10^5 cells/ml and incubated overnight. Once cells reached 80% of confluence, then cells were treated with HMW-bLf 1600 µg/ml. Following incubations after 2 and 4 h, media was removed and cells were washed 2 times with 1X PBS and fixed with 4% paraformaldehyde prepared in 1 X PBS pH 7.4 for 20 minutes. Cells were then permeabilized with 0.2% (v/v) TritonX-100 for 10 mins. 2% (v/v) normal rabbit serum (Vector Labs Burlingame, CA, USA) was used to block for nonspecific binding of secondary antibody for 20 mins at 25°C. After washing three times with PBS, cells were incubated at 37°C with 1:200 goat antibovine lactoferrin antibody (Bethyl Laboratories USA) for 1 h. After washing, cells were then incubated with 1:160 secondary antibody, anti-goat IgG-FITC for 1 h (Sigma-Aldrich, Australia). Mounting medium with propidium iodide (PI) (Vector Labs, Burlingame, CA, USA) was added per well in case of MDA-MB-231 and SW480, and in case of OVCAR-3 mounting medium with DAPI (Vectashield®) was added to the well followed by application of a 22 x 66 mm cover slip (Proscitech, Thuringowa, Qld, Australia) on the slides. Slides were sealed with nail polish to prevent over drying and viewed under Leica SP5 confocal microscope (Leica Microsystems, Melbourne, Australia), with images captured using (LAS version-software) and then processed in Adobe Photoshop CS3.

2.12 Caspase-3 activation assay
Caspase-3 activation assay was carried out to evaluate the caspase-3 activation levels in cells following treatments. Cells were treated with different bLf samples after completion of incubation period, culture media were removed and centrifugation was carried out to discard any debris. In 96 well plates, 100 µg/ml proteins (in triplicate) were added from each sample supernatant. Then 50 µl of mixture reagent (Dithiothreitol (DTT) in Radio-immunoprecipitation assay RIPA buffer solution) was added. Finally, 6 µl of substrate acetyl-Asp-Glu-Val- Asp p-nitroanilide (Sigma Aldrich, Australia-A2559) dissolved in Dimethyl Sulfoxide (DMSO) 1mg /100 µl was added to all samples. The plate was then incubated for 180 mins at room temperature in dark. Level of caspase-3 expression was quantified by using a SH-1000lab absorbance microplate reader (Corona Electric) at 405 nm [284].
Caspase-3 activity was calculated in millimolar/ml by using the equation:
\[
\text{Caspase-3 activity (mM/ml) = O.D. at 405nm x dilution factor (100) x E (millimolar constant) x time of incubation in mins x volume of sample}
\]
- Value of millimolar constant is 10.5
- Time was 3 hours so 180 mins
- Volume of the sample is the amount of sample added for loading 100µg of the protein.

2.13 Gene expression studies
Total RNA from OVCAR-3 cells was obtained after 24 hours treatments with HMW-bLf at 1600 and 3200µg/ml concentration using Apo-bLf, Fe-bLf and NM-bLf at highest concentration 3200µg/ml as control in 6 well plate using TRIZOL reagent (Invitrogen, Mulgrave, VIC, Australia). Briefly, 1 ml of TRIZOL reagent was added per well. The solution in each well was mixed properly and the plate was incubated for 5 min. The samples were collected in an eppendorf tubes and again incubated for 5 min at 15-30ºC for complete dissociation of nucleoprotein complexes. 200µl of chloroform per 1 ml of TRIZOL was added and tubes were then shaken vigorously for 20 sec. Samples were then centrifuged in Heraus Fresco 17 microcentrifuge (Thermo Scientific Pty Ltd, Australia) at 12000xg for 15 mins at 4ºC. Following centrifugation, the mixture separated into an aqueous phase and organic phase. The RNA remained in the aqueous phase therefore; the aqueous phase was collected into a separate eppendorf tube after adding 500µl of Isopropyl alcohol per 1 ml of TRIZOL. Again the samples were centrifuged at 12000xg for 10 min at 4ºC. RNA formed a gel like pellet and hence the supernatant was removed carefully following the addition of 1 ml 75% ethanol per 1 ml TRIZOL, samples were mixed using a vortex. After mixing, samples were again centrifuged at 7500xg for 5 min at 4ºC. The isolated RNA pellet was dried after removing the supernatant and was dissolved in 30µl of RNase free water. RNA concentration was determined by taking the absorbance at 260 nm using a Corona SH-1000Lab absorbance microplate reader.
RNA concentration was calculated multiplying the absorbance value by 40μg/ml. The isolated RNA was used immediately for cDNA synthesis.

2.13.1 cDNA Synthesis
The SuperScript III First Strand Synthesis system from (Invitrogen, Mulgrave, Vic, Australia) was used to synthesize first strand cDNA from isolated RNA. The procedure was followed as per the instructions in the kit. In brief, 2μg of isolated RNA from each treatment was mixed with 1μl of 50ng/μl random hexamers; 1μl of 10nM dNTP mixture and diethylpyrocarbonate (DEPC) treated nuclease free water to make the final volume to 10μl. RNase free tubes were used for the experiment. The mixture was then incubated at 65°C for 5 minutes. After the incubation, 10μl of cDNA synthesis mixture was added to the above tube. The cDNA synthesis contains 2μl of 10X RT buffer, 25mM MgCl2, 2μl of 0.1M DTT, 1μl of RNase OUT and 1μl of SuperScript III RT. The final mixture is then heated at 25°C for 10 minutes. cDNA synthesis was performed in the first step by incubating the tubes at 50°C for 50minutes using PCR Thermal Cycler Dice™ (TaKaRa). The reaction was then terminated by heating the samples at 85°C for 5 minutes followed by incubation on ice for 2-3minutes. 1μl of RNase H was added to each tube and incubated at 37°C for 20 minutes to remove the residual RNA. The synthesised cDNA was then stored at -80°C and used as a template for further real time PCR reactions.

2.13.2 Reverse Transcription PCR
Reverse transcription PCR (RT-PCR) was carried out to amplify the cDNA for the gene of interest. The PCR master mix was kept at room temperature and vortexed well before use. All reaction mixtures were kept on ice since they are temperature sensitive. The following components were used to prepare RT-PCR mixure. 1μl of 10X Taq buffer, 0.2μl of 10mM dNTP, 0.4μl of 10μM reverse primer, 1.6μl of 25mM MgCl2, 0.25μl of 0.25mM DNA Taq polymerase, 1μl cDNA template and 5.15μl RNase free water. The mixture was then mixed well and centrifuged for 10 seconds. TaKaRa PCR Thermal Cycler Dice™ was used to run RT-PCR. An initial incubation at 95°C for 5 minutes denatured the cDNA template followed by 40 repeats of second cycle at 95°C for 30 seconds. Binding of primer sequences to the 5’ end of
the template takes place in the annealing temperature (50°C for 30 seconds) followed by extension of the amplification which takes place at 72°C for 1 minute. After 40 cycles, the reaction undergoes final step of extension during the incubation at 72°C for 10 minutes. Amplified cDNA was then run on an agarose gel electrophoresis for further analysis.

2.13.3 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Quantitative Real Time PCR is used to amplify and simultaneously quantify the targeted DNA template at the same time. qRT-PCR iQ5 (Bio-Rad, Gladesville, NSW, Australia) was used to run qRT-PCR products. Depending on the annealing temperatures for various primers an appropriate cycle was created using the software. Annealing temperatures of various primers used are mentioned in the (Table 2.1). The qRT-PCR mixture contains 7.5μl of SYBR® green pre-mix from (Bio-Rad, Gladesville, NSW, Australia), 0.4μl forward and reverse primer, 1μl of cDNA template and RNase free water in PCR plates (Bio-Rad, Gladesville, NSW, Australia). The plate was then briefly centrifuged to and incubated in an iQ5 instrument. The first cycle was set at 95°C for 5 minutes followed by 60 repeats of cycle 2 with first step at 95°C for 30 seconds. Second cycle varies with primers used in the reaction for 45 seconds and third step at 72°C for 45 seconds. The quantification process takes place in the third step of cycle 2. Therefore, third cycle of final extension is set for 10 minutes at 72°C. Graph is then plotted using the values obtained from the software. 2^{-ΔΔCt} was calculated by getting the difference between treatments and untreated and comparing with that of a house keeping gene of respective treatments. Amplified gene is then run on an agarose gel electrophoresis. Experiments were carried out thrice to obtain the reproducibility of the results.
Table 2.1 Annealing temperatures of genes with forward and reverse primer sequence.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence (5’-3’)</th>
<th>Reverse Sequence (5’-3’)</th>
<th>Annealing Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivin</td>
<td>CCA CCG CAT CTC TAC ATT CA</td>
<td>TAT GTT CCT CTA TGG GGT CG</td>
<td>50</td>
</tr>
<tr>
<td>Actin</td>
<td>CTC ACC GAG CGC GGC TAC A</td>
<td>CTC CTG CTT GCT GAT CCACAT</td>
<td>50</td>
</tr>
<tr>
<td>Bcl2</td>
<td>TTC TTT GAG TTC GGT GGG GTC</td>
<td>TGC ATA TTT GTT TGG GGC AGG</td>
<td>75</td>
</tr>
<tr>
<td>Bax</td>
<td>GGC CCA CCA GTC CTG AGC AGA</td>
<td>GCC ACG GCG TCC CAA AGT</td>
<td>55</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>CTC GGT CTG GTA CAG ATG TCG</td>
<td>GGT TAA CCC GGG TAA GAA TGT GCA</td>
<td>55</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>GGG ACA GGA ATG GAA CAC AC</td>
<td>CAG CAA GGG AAG GGC ACT TC</td>
<td>52</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>CTG CGA ACT AAC AGG CAA GC</td>
<td>CTA GAT ATG GCG TCC AGC TG</td>
<td>52</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>AGTGACAGGATGTTGCCC GTTGC</td>
<td>GCATCTATCCCCCCTAAAG TGG</td>
<td>59</td>
</tr>
<tr>
<td>Cytochrome-c</td>
<td>TGGGTGATGAA AAGG</td>
<td>CGGCTGTGTAAGAGTATG C</td>
<td>55</td>
</tr>
<tr>
<td>Fas</td>
<td>GAA GGA CAT GGT TTA GAA GTG</td>
<td>ACT TAG TGT CAT GAC TCC AGC</td>
<td>55</td>
</tr>
</tbody>
</table>
The primer sequences used in above table provided by GeneWorks, Australia and Sigma Aldrich Pty Ltd, Australia (Hindmarsh, SA Australia). Sequences were obtained from [285].

2.14 Detection of apoptosis related proteins expression in OVCAR-3 using FACS analysis.

Detection of apoptosis-related proteins such as survivin, Bcl-2, Bcl-xL, Bad, caspases-7, 8 and 9 were carried out. Additionally, low density lipoprotein (LRP) and transferrin (TFR) receptors were also analysed post treatments. OVCAR-3 cells were cultured on to 6 well plates at a density of 2 x 10^5 cells/ml and incubated overnight. Once cells reached 80% of confluency, cells were treated with HMW-bLf, Apo-bLf, Fe-bLf and NM-bLf at 3200 µg/ml. Following incubation for 24 h, both adherent and floating cells were harvested and washed twice with PBS. Cells were then fixed with 4% paraformaldehyde prepared in 1 X PBS pH (7.4) for 20 mins. Cells were then permeabilized with 0.2% (v/v) TritonX-100 for 10 mins (Tfr and LRP-1 receptors did not undergo to this step). 2% (v/v) Bovine serum albumin (BSA) (Sigma Aldrich, Australia) was used to block for nonspecific binding of antibodies for 30 mins at 37°C. After washing three times with PBS, cells were incubated at 37°C with 1:100 primary antibody for 1 hour (Table 2.2). Cells were then washed three times with sterile PBS, then incubated with secondary antibody 1:100 anti-goat IgG-FITC for 1 hour (Sigma-
Aldrich, Australia) followed by washing two times with PBS. The concentrations of primary and secondary antibodies were pre-standardized in the lab. Data acquisition was carried out using FACSCantos II flow cytometer and results were analysed.

**Table 2.2 Primary and secondary antibodies used for flow cytometry analysis of various apoptosis related proteins and lactoferrin receptors.**

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivin</td>
<td>Mouse anti-Survivin (Monoclonal)</td>
<td>Anti-mouse FITC</td>
</tr>
<tr>
<td></td>
<td>Santa-Cruz (1:100)</td>
<td>Sigma, Aldrich 1:100</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Anti-Bcl2 mouse (Monoclonal)</td>
<td>Anti-mouse FITC</td>
</tr>
<tr>
<td></td>
<td>Santa-Cruz (1:100)</td>
<td>Sigma, Aldrich 1:100</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Anti-Bcl-xL mouse (Monoclonal)</td>
<td>Anti-mouse FITC</td>
</tr>
<tr>
<td></td>
<td>Santa-Cruz (1:100)</td>
<td>Sigma, Aldrich 1:100</td>
</tr>
<tr>
<td>BAD</td>
<td>Anti-BAD mouse (Monoclonal)</td>
<td>Anti-mouse FITC</td>
</tr>
<tr>
<td></td>
<td>Santa-Cruz (1:100)</td>
<td>Sigma, Aldrich 1:100</td>
</tr>
<tr>
<td>Bax</td>
<td>Anti-Bax mouse (Monoclonal)</td>
<td>Anti-mouse FITC</td>
</tr>
<tr>
<td></td>
<td>Santa-Cruz (1:100)</td>
<td>Sigma, Aldrich 1:100</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>Anti-caspase-7 mouse (Monoclonal)</td>
<td>Anti-mouse FITC</td>
</tr>
<tr>
<td></td>
<td>Santa-Cruz1:100</td>
<td>Sigma, Aldrich 1:100</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Anti-caspase-8 mouse (Monoclonal)</td>
<td>Anti-mouse FITC</td>
</tr>
<tr>
<td></td>
<td>Santa-Cruz1:100</td>
<td>Sigma, Aldrich 1:100</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>Anti-caspase-9 mouse (Monoclonal)</td>
<td>Anti-mouse FITC</td>
</tr>
<tr>
<td></td>
<td>1:100 Santa-Cruz</td>
<td>Sigma, Aldrich 1:100</td>
</tr>
<tr>
<td>LRP-1</td>
<td>Anti-LRP-1 mouse (Monoclonal) Santa-Cruz (1:100)</td>
<td>Anti-mouse FITC Sigma, Aldrich 1:100</td>
</tr>
</tbody>
</table>

2.15 Treatment of drug resistance OVCAR-3 with HMW-bLf

OVCAR-3 represents an appropriate model system to study drug resistance in ovarian carcinoma. To reproduce levels of resistance in OVCAR-3 as per published finding [245] and ATCC guide lines, OVCAR-3 was treated with different concentration of Melphalan (Sigma –Aldrich- Australia, M2011). Cells were adjusted to 2×10⁵/ml and then plated in 6 well flat bottom plates, and incubated for 24-48h at 37°C in a humidified atmosphere of 5% CO2. After incubation, media were removed and cells were then washed in 1x phosphate buffered saline (PBS). PBS was removed and then cells were treated with different concentration of Melphalan (0.3 μM, 0.6 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM, 15 μM and 20 μM). Culture media plus Melphalan concentration doses were changed regularly every 48 h for 10 days. Cells were then treated with Melphalan at lower concentration which did not cause anticancer effects plus HMW-bLf (0.3 μM, 0.6 μM, 1.25 μM/ml, 2.5 μM/ml, 5 μM/ml +HMW-bLf1600µg/ml) for 24 h. To study the morphological changes and cell growth, cells were viewed using an inverted microscope (Prism Optical) and images were obtained by using digital camera.

2.16 Investigations of Pgp expression in OVCAR-3.

Pgp is also known as multidrug resistance protein 1 (MDR1). A MDR1 phenotype associated with the overexpression of Pgp was seen in many drug resistant cancer cell lines [328]. Cells were cultured in 6 well plates as mentioned in previous experiments and then cells were treated with HMW-bLf at 1600µg/ml, 2400µg/ml, 3200 μg/ml. Afters 24 h, cells were collected and washed twice with PBS. Cells were then fixed with 4% paraformaldehyde prepared in 1 X PBS pH (7.4) for 20 mins. 2% (v/v) Bovine serum albumin (BSA) (Sigma Aldrich, Australia) was used to block for nonspecific binding of antibodies for 30 mins at 37°C. After washing three times with PBS, cells were incubated at 37°C with mouse anti-Pgp primary antibody for 1 h 1:100 (Santa Cruz biotechnology, Australia). Cells were then washed three times
with sterile PBS, then incubated with secondary antibody 1:100 anti-mouse IgG-FITC for 1 h (Sigma-Aldrich, Australia) followed by washing two times with PBS. Data acquisition was carried out using BD FACS Canto II flow cytometer and results were analysed.
Chapter 3: Results

3.1 Molecular and immunological characterization of purified protein

The purity and molecular weight of purified lactoferrin sample was verified and confirmed as shown in Figure 3.1 (A) by SDS-PAGE and (B) by Western blot. The molecular weight of the bLf was compared using prestained protein standards Bio-Rad (Catalogue no.161-0374). The purified fractions from bovine colostrum sample show a high molecular weight bands at 250 kDa in SDS-PAGE. Interestingly, no band was observed at ~ 78-80 kDa which is known molecular weight of monomeric bLf. The HMW protein bands on SDS-PAGE were later identified as bLf by Western blot.

Figure3.1 (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of main fractions obtained by ion exchange chromatography. 10µl from each samples fractions were loaded into a10% SDS gel. M, marker. Lane 1; Fourth elution fraction. Lane 2; Fifth elution fraction. Lane 3; Sixth elution fraction. Lane 4; Seventh elution fraction. Figure 3.1(B). Western blot of bLf fractions was carried out to identify the purified protein using goat anti-bLf antibody (Bethyl Labs). M- Marker. lane1, 2, 3; lyophilized elution fractions.
3.2 Internal chemical constitution of HMW-bLf by using (FTIR)

FTIR represents excellent tool to investigate any changes that occur in secondary structure of protein. Figure 3.2 shows a comparison between the FTIR spectra of HMW-bLf, NM-bLf, Apo-bLf and Fe-bLf, FTIR spectroscopy demonstrated a number of bands representing different amide groups. The characteristic stretching and bending vibrations arise due to amide I are addressed at \((1600–1700 \text{ cm}^{-1})\), amide II \((1480–1575 \text{ cm}^{-1})\), amide III \((1229–1301 \text{ cm}^{-1})\) and signal band obtained at 700 cm\(^{-1}\) can be assigned to \(\text{–NH}_2\) bond. As bLf is classified as a glycoprotein, the broad structure from 900–1200 cm\(^{-1}\) is due to C–O,C–C stretches and C–O–H, C–O–C deformation of carbohydrates [286]. The obtained results demonstrated that purified HMW-bLf in the study, has the same identical chemical structure as a glycoprotein as compared to the ~78-80kDa NM-bLf and Apo-bLf.

![FTIR Spectra Comparison](image)

**Figure 3.2.** Comparison of the FTIR spectra was carried out to investigate the chemical constitution of the HMW-bLf, Apo-bLf and NM-bLf. Presence of amide I, II, III peaks indicated the similar structure of HMW-bLf and Apo-bLf.
3.3 Stability of HMW-bLf against digestion enzymes

Stability of HMW-bLf was analysed using a digestive enzyme cocktail (Omnizyme). Digestion of bLf protein was confirmed using commercially available Omnizyme tablets as described earlier in chapter (2). Omnizyme digestion assay (Figure 3.3) revealed that HMW-bLf in lanes 2, 3 and 4 showed excellent stability, with digested forms appeared at bands 150 and 100kDa at various intervals of time (4, 6 and 8 hours) as compared to NM-bLf ~78-80kDa. In case of NM-bLf in lanes 5, 6 and 7 the samples have completely disappeared at ~78kDa and digested to its peptides appearing at 50, 37 and 25kDa. Lane 8 represents untreated HMW-bLf used as a control.

Figure 3.3 Omnizyme digestion of HMW-bLf purified from bovine colostrum and NM-bLf at different time intervals. Commercially available Omnizyme tablet was dissolved in milli-Q water and was added to bLf samples. All the samples were incubated at 37°C at 150 rotations per minute (rpm) for different time intervals. The samples were collected at regular interval of 4 h, 6 h and 8 h and heated at 42°C for
7 minutes to arrest the enzyme reaction. To analyse the stability of HMW-bLf and NM-bLf, all samples were analysed by SDS-PAGE. The red arrows indicated different degraded peptides after molecules digestion with Omnizyme enzyme. M, Marker. Lane 1, HMW-bLf + Omnizyme 4 h. Lane 2, HMW-bLf + Omnizyme 6 h. Lane 3, HMW-bLf + Omnizyme 8 h. Lane 4, NM-bLf + Omnizyme 4 h. Lane 5, NM-bLf + Omnizyme 6 h. Lane 6, NM-bLf + Omnizyme 8 h. Lane 7, HMW-bLf alone.

3.4 Iron content in HMW-bLf
Iron percentage estimation assay (Figure 3.4) showed negligible amount of iron element in HMW-bLf. The value was found to be estimated around 0.47% as compared with the standard value that represents 100% of iron concentration. Also, very little amount to moderate of iron values 1.1 % and 22 % was detected in Apo-bLf (iron free form of bLf) and NM-bLf respectively. Furthermore, this assay demonstrated significant high level of iron in iron saturated bLf (Fe-bLf) which was 94 %.
Figure 3.4.A Percentage of iron element in HMW-bLf purified from bovine colostrum, Apo-bLf, Fe-bLf and NM-bLf derived from ~78-80 kDa bLf standard represents 100% of iron. Assay was carried out twice. Amount of the NM-bLf was tested in the lab and the value is presented here. Figure 3.4, B Image of lyophilized bLf forms showed different colour according to the iron amount.
3.5 Level of thermal stability of HMW-bLf

Thermal stability of lyophilized powder forms of HMW-bLf, Fe-bLf, Apo-bLf and NM-bLf Figure 3.5.A-D was studied by using differential scanning calorimetry (DSC). The thermograms obtained show one denaturation peak for different bLf forms at different temperatures. HMW-bLf shows only one denaturation transition peak at 89°C which indicated highest temperature when compared with Fe-bLf, Apo-bLf and NM-bLf with denaturation peaks at 82°C, 77°C and 75°C, respectively.

![Differential scanning calorimetry (DSC) thermograms obtained showed HMW-bLf expressed highest temperature resistance when compared with other bLf forms as seen in the above graphs. The DSC was programmed in the temperature range of 35 –110 °C and at heating rate of 10 °C/min. (A) HMW-bLf isolated from bovine colostrum, (B) Fe-bLf, (C) Apo-bLf, (D) NM-bLf.](image)

Figure 3.5. Differential scanning calorimetry (DSC) thermograms obtained showed HMW-bLf expressed highest temperature resistance when compared with other bLf forms as seen in the above graphs. The DSC was programmed in the temperature range of 35 –110 °C and at heating rate of 10 °C/min. (A) HMW-bLf isolated from bovine colostrum, (B) Fe-bLf, (C) Apo-bLf, (D) NM-bLf.
3.6 Effects of HMW-bLf on cell cytotoxicity of OVCAR-3 cells

LDH assay was carried out to investigate the cytotoxicity effect of HMW-bLf and compared with other bLf forms, used as controls at different concentrations after 24 hours incubation on OVCAR-3. Cells were treated with HMW-bLf (800, 1600, 2400, 3200 μg/ml), Apo-bLf and Fe-bLf (3200 μg/ml) and NM-bLf (1600, 2400, 3200 μg/ml) with inclusion of negative control (untreated cells) and positive control (Triton-X treated cells).

As shown in Figure 3.6, HMW-bLf even showed a significant (p <0.05) cell cytotoxicity of 20% at the lowest concentration tested (800 μg/ml). The level of cytotoxicity increased further with increasing concentrations of HMW-bLf which induced highly significant (p ≤ 0.01) cytotoxicity 33.5%, 36% and 40% at 1600, 2400, 3200 μg/ml, respectively. Fe-bLf at highest concentration of 3200μg/ml induced 35% cytotoxicity which again was found to be statistically highly significant (p ≤ 0.01). Less cytotoxicity effect was appeared with Apo-bLf (3200 μg/ml) at 11% as compared with untreated cells. For NM-bLf, 25% of cell cytotoxicity was observed with 3200μg/ml.
Figure 3.6  Cell cytotoxicity in OVCAR-3 cells after 24 h bLf treatments. Cells were treated with various concentrations of HMW-bLf, with concentrations of 800, 1600, 2400 and 3200 μg/ml NM-bLf 1600, 2400 and 3200 μg/ml, Apo-Lf, and Fe-bLf with highest concentration 3200 μg/ml for 24 h. Both positive control absorbance value (Triton-X 100 treated cells) and absorbance value of untreated cells termed as low control was used to calculate the percentage of cytotoxicity for the treatments. The cytotoxicity percentage of low control (negative control) was 0. Please refer to the formula on page 55. All treatments were tested in triplicate and assay was repeated 2 times. Results are expressed as mean values (±SD) and a Student’s t-test was performed for evaluating statistical significance. A value of (p<0.05) denotes statistical significance, whereas (p ≤0.01) denotes results that are highly significant. A value of (p≥0.05) denotes statistically non-significance.

* = P < 0.05 compared with no treatment group.

** = P ≤ 0.01 compared with no treatment group.

P ≥ 0.05 statistically non-significant.

3.7 Cell viability studies on OVCAR-3

In order to determine the effect of various concentrations of HMW-bLf and other bLf forms on the cell viability of OVCAR-3, MTT assay was employed. Figure 3.7 shows the percentage of cell viability after 24h treatment with HMW-bLf (800, 1600, 2400, 3200 μg/ml), Apo-bLf and Fe-bLf (3200 μg/ml), and NM-bLf (1600, 2400, 3200 μg/ml). Data obtained revealed that at moderate concentrations of HMW-bLf (1600 μg/ml and 2400 μg/ml) cell viability was statistically highly significantly (p < 0.01) reduced to 67 and 59%, respectively. With highest concentration of HMW-bLf used at 3200 μg/ml, viability reduced further severely (29% viability; p ≤0.01). Additionally, strong effects with Apo-bLf and Fe-bLf at 3200 μg/ml were observed as well and cell viability decreased to 53% and 45% (p ≤0.01), respectively. In the case of NM-bLf treatments with 1600 μg/ml 2400 μg/ml, and 3200 μg/ml dose dependent effect on OVCAR-3 cell viability was observed with 53%, 36%, and 33%, viable cells respectively.
Figure 3.7 Cell growth/viability (MTT) assay of OVCAR-3 cells after treatments for 24 h with HMW-bLf at 800, 1600, 2400, 3200 μg/ml, with Apo-bLf and Fe-bLf at highest concentration 3200μg/ml, and with NM-bLf at 1600, 2400, 3200μg/ml. Negative control was blank media and positive control included untreated OVCAR-3 cells with media containing 20% FBS. All treatments were tested in triplicate and assay was repeated 2 times. Data expressed as mean values (±SD) and a Student’s t-test was performed for evaluating statistical significance. A value of (p < 0.05) denotes statistical significance, whereas (p ≤0.01) denotes results that are highly significant. A value of (p≥0.05) denotes statistically non-significance.

* = P < 0.05 compared with no treatment group.

** = P ≤ 0.01 compared with no treatment group.

P ≥ 0.05 statistically non-significant.
3.8 Effect of HMW-bLf on cell proliferation of OVCAR-3

The cell proliferation level was further assessed in OVCAR-3 post treatments for 24 h with HMW-bLf (800, 1600, 2400, 3200 μg/ml), NM-bLf (1600, 2400, 3200 μg/ml), Apo-bLf and Fe-bLf at highest doses (3200μg/ml) using the CyQuant assay kit (Invitrogen, Australia). Untreated cells were used as negative control and positive control included 20% FBS treated cells. Figure 3.8.1 shows the statistically significant ($p \leq 0.01$) effect of HMW-bLf at doses 800, 1600, 2400 μg/ml with 70%, 80% and 84% reduction in cell proliferation, respectively. At highest concentration used, both HMW-bLf and Fe-bLf (3200μg/mL) reduced cell proliferation again at statistically highly significant ($p \leq 0.01$) levels showing only 5.8% and 18.5% cell proliferation, respectively. Also, NM-bLf 3200μg/ml causing near similar reduction as that of Fe-bLf and 17.3% cell proliferation was observed.

![CyQuant assay in OVCAR-3](image)

**Figure 3.8.** CyQuant proliferation assay was performed on OVCAR-3 cells after treatments for 24 h with HMW-bLf at 800, 1600, 2400, 3200 μg/ml, with Apo-bLf and Fe-bLf at highest concentration 3200μg/ml and with NM-bLf at 1600, 2400, 3200μg/ml. Negative control (Untreated cells with media containing 10% FBS) and positive control (cells with media contain 20% FBS) were included in the assay. All treatments were tested in triplicate and assay was repeated 3 times. Data
expressed as mean values (±SD) and a Student’s t-test was performed for evaluating statistical significance. A value of (p<0.05) denotes statistical significance, whereas (p ≤0.01) denotes results that are highly significant and the value of (p≥0.05) denotes statistically non-significance.

** = (p ≤ 0.01) compared with positive control group.

3.9 Cellular internalization of HMW-bLf in OVCAR-3 cells
To investigate the internalization of HMW-bLf in OVCAR-3, confocal microscopy was carried out to determine the level and localization of HMW-bLf in cancer cells. The results obtained indicated that there was a rapid uptake of HMW-bLf by OVCAR-3. HMW-bLf was localised on to cell membrane, internalized to nuclei of the tumour cells. Figure 3.9 shows confocal microscopy images of OVCAR-3 after 2 and 4 h of treatment. Specific binding of HMW-bLf with cell membrane, cytoplasm and nuclei was observed in all cells at different time intervals compared to untreated cells which showed no immunofluorescence detection of bLf specific antibody.
Figure 3.9. Cellular internalization of HMW-bLf in OVCAR-3. OVCAR-3 cells were seeded in 8 chamber slides and treated when 80% confluent. Cells were treated with HMW-bLf (1600µg/ml) for different time intervals (2 and 4 hours). Cells were then probed with bLf specific primary goat anti-bovine lactoferrin antibody (Bethyl Laboratories) in 1:200 dilution. Secondary antibody, anti-goat IgG-FITC produced in rabbit (Sigma-Aldrich) was used at 1:160 dilution. Cells were then washed and Vector mounting medium with DAPI was added to all wells. Slides were visualized by using Leica SP5 confocal microscope (Leica Microsystems, Melbourne, Australia) and images were processed in Adobe Photoshop CS3. (A-D) Untreated cells for comparison of fluorescence are showing only nuclei staining by DAPI (blue) and no
immune reactivity for bLf. (E-H) Internalisation of HMW-bLf in OVCAR-3 cells after 2 hours (FITC staining-green) with nuclei staining (DAPI-blue) (I-L) Internalization of HMW-bLf in OVCAR-3 after 4 h cells (FITC staining-green) with nuclei staining (DAPI-blue).

3.10 Effect of HMW-bLf on OVCAR-3 cell death
The ability of HMW-bLf to induce cell death of OVCAR-3 was further confirmed with propidium iodide (PI) staining, Fe-bLf at 3200µg/ml was used as a positive control. The dead cells after staining with PI were subsequently quantified using the flow cytometry. As observed from the (Figure 3.10.A-C) the HMW-bLf was effective in a concentration dependant manner (1600, 2400 & 3200 µg/ml) in inducing the cell death in OVCAR-3. While the highest dose of Fe-bLf (3200µg/ml) was also found to induce significant cell death. Treatment of cells with HMW-bLf at 1600, 2400 & 3200 µg/ml concentrations induced statistically highly significant (p ≤ 0.01) 37%, 57% and 75% cell death in OVCAR-3 cells, respectively. Similar effect was seen with Fe-bLf at 3200 µg/ml as well, and 66% cell death was observed (p ≤ 0.01). The morphological observations of cells post treatment also confirmed the dose dependent cytotoxicity effect of HMW-bLf. High numbers of floating and dead cells were observed under microscope (40X) in the well with highest concentration treatment.
Figure 3.10.A

- Untreated OVCAR-3
- OVCAR-3+HMW-bLf 1600μg/ml
- OVCAR-3+HMW-bLf 2400μg/ml
- OVCAR-3+HMW-bLf 3200μg/ml
- OVCAR-3+Fe-bLf 3200μg/ml
Figure 3.10.B

% Cell death in OVCAR-3 24h

Treatments

Figure 3.10.C

Untreated OVCAR-3
HMW-bLf 1600μg/ml
HMW-bLf 2400μg/ml
HMW-bLf 3200μg/ml
Fe-bLf 3200μg/ml
Figure 3.10. A, B, C  Fluorescence-activated cell sorting (FACS) of OVCAR-3 cells used in this study to investigate the cell death percentage by using propidium iodide (PI) staining after 24h treatments with HMW-bLf at (1600, 2400, 3200 µg/ml). Fe-bLf at highest concentration 3200µg/ml was used as a positive control and compared with untreated cells. Image (A.) Dots blot and histogram from FACS of OVCAR-3, (B) Graphical representation shows the percentage of fluorescence detected in OVCAR-3, (C) Morphological observations of cells after 24h of treatments in OVCAR-3 at 40X magnification.

3.11. HMW-bLf induces significant apoptosis in OVCAR-3

The annexin V-binding assay is one of the most sensitive and widely used techniques to detect and distinguish between early apoptosis and late apoptosis, as well as between apoptosis and necrosis. The assay indicated that treatments on OVCAR-3 induced apoptosis highly significantly (p ≤ 0.01) than necrosis (Figure 3.11.A,B) where cells were treated with HMW-bLf at 1600, 2400, 3200 µg/ml, and with highest concentration of Fe-bLf at 3200 µg/ml. Untreated cells were used as control. The result obtained after 24 h incubation period showed early apoptotic cells’ percentage increased with increasing HMW-bLf concentrations, while percentage of live cells decreased in a dose dependent manner of HMW-bLf at 1600, 2400, 3200 µg/ml with 63.5%, 54% and 27%, respectively. HMW-bLf at 2400, 3200 µg/ml and Fe-bLf at 3200 µg/ml had mostly highly significant effect (p ≤ 0.01) to induce early apoptosis with 38 %, 56% and 43 %, respectively. In HMW-bLf at 1600 µg/ml which represents the minimum concentration dose used in this experiment, 21 % apoptotic cells were at a significant level of (p < 0.05) when compared with untreated OVCAR-3 cells. The necrotic levels were observed as 8 % with 2400µg/ml HMW-bLf, similar necrotic cell levels were determined, 11% with highest concentration groups of HMW-bLf and Fe-bLf as well.
Figure 3.11.A
Figure 3.11.A, Dot plots of annexin V staining based flow cytometric analysis of OVCAR-3 cells in the early stages of apoptosis, cells were located on the top right quadrant of the dot-plot as single positive annexin V-binding cells.

Figure 3.11.B, Histogram from FACS analysis shows the percentage of early apoptotic, late apoptotic and necrotic OVCAR-3 cells when treated with HMW-bLf and Fe-bLf for 24 h. Graphed data was presented as ± SEM; of two independent experiments.

* P < 0.05, ** P ≤ 0.01, compared with untreated cells and P ≥ 0.05 statistically non-significance.
3.12 RNA extraction and purity

Total RNA from OVCAR-3 cells was isolated after 24 h treatments with HMW-bLf at 1600, 3200µg/ml and with Apo-bLf, Fe-bLf and NM-bLf at highest concentration of 3200µg/ml, Untreated cells were used as control. The isolated pure RNA was used to make cDNA for gene expression studies.

3.12.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Actin is a globular multi-functional protein found in all eukaryotic cells. Actin plays crucial roles in such vital cellular mechanism such as cell signalling, maintenance of cell junctions cell division, cytokinesis, cell shape, cell motility and muscle contraction [287]. It is also one of the most important housekeeping gene which is commonly used to compare the variation in the expression of other genes. Figure 3.12.1 shows almost nearly similar amount of actin gene expression in each sample from OVCAR-3. Obtained bands from 1% agarose gel revealed that equal amount of base pairs and hence equal amount of cDNA among the various treatments.

![Figure 3.12.1 Gel electrophoresis of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) products, showing the amounts of actin present in each RNA sample from OVCAR-3 cells. The reaction mixture including 10X Taq buffer, 10mM dNTP, 10µM forward and reverse primers, 25mM Mgcl₂, 0.25 mM DNA Taq Polymerase and cDNA was prepared for each samples. Denaturing step at 95°C, annealing step at 50°C and extension step at 72°C was carried out in a TaKaRa PCR Thermal Cycler Dice. The PCR product was run on agarose gel for quantitative analyses. The images were captured and processed using BioRad ChemiDoc XRS camera and Quantity One software under UV illumination. Lane 1 (Marker), lane 2 (Untreated OVCAR-3), lane 3 (HMW-bLf 1600 µg/ml), lane 4 (HMW-bLf 3200 µg/ml), lane 5 (Apo-bLf 3200 µg/ml), lane 6 (Fe-bLf µg/ml), Lane 7 (NM-bLf 3200 µg/ml).]
3.12.2 Study of post treatment changes in different genes’ expression levels in OVCAR-3 cells

qRT-PCR was used to quantify the mRNA expression using cDNA synthesized from the total RNA extract from OVCAR-3 cells after treatment for 24 hours with HMW-bLf 1600 μg/ml and 3200 μg/ml, Apo-bLf, Fe-bLf and NM-bLf at 3200 μg/ml. Untreated cells were used as a control to investigate the changes in gene expression.

Recently, survivin was shown to inhibit apoptosis and accelerate cancer cell proliferation as well [288]. Data obtained from qRT-PCR as seen in Figure 3.12.2.A revealed that survivin gene expression was down regulated by 0.65 and 0.62 folds in HMW-bLf1600 and NM-bLf 3200μg/ml treatments respectively. Furthermore, survivin gene was reduced by 0.9 folds, 0.86 folds and 0.8 folds with Fe-bLf, HMW-bLf and Apo-bLf at 3200 μg/ml treatments, respectively when compared with untreated OVCAR-3 cells. Bcl-2 represents another anti-apoptotic protein with a crucial function in cell death, also inhibits all DNA repair system [289]. Figure 3.12.2.A showed that expression of anti-apoptotic gene Bcl-2 was reduced by 0.3 fold and 0.7 folds with HMW-bLf at 1600 μg/ml and NM-bLf at 3200 μg/ml, respectively. While the expression levels of Bcl-2 gene showed highly significant (p < 0.001) down regulation with the other of bLf forms and highest effect seen with HMW-bLf.

Bax is announced as a pro-apoptotic effectors protein member from Bcl-2 family that can trigger apoptosis when overexpressed. It was observed Figure 3.12.2.A that treatments with Fe-bLf and NM-bLf led to 1.5 and 1.2 fold up regulation, respectively. The highly significant (p < 0.001) up regulation of Bax gene was observed with HMW-bLf (1600 μg/ml) and Apo-bLf (3200 μg/ml) with up to 22 and 13 folds, respectively. Cytochrome-C is known to induce or potentiate apoptotic processes predominantly by triggering or enhancing the activity of caspases. Figure 3.12.2.A showed that the cytochrome-C gene level was up regulated in all treatment used, and highest expression was reported with HMW-bLf (3200μg/ml) when compared with untreated OVCAR-3 and other treatments. Caspase-7 (Figure 3.12.2.B) classified as an effectors caspase was up regulated in all treatments group at gene expression levels, while the expression was down-regulated with Apo-bLf. Caspase-8, an apoptotic gene of the caspase cascade, regulated via extrinsic pathway, and
absence of caspase-8 expression leads to tumour growth [290]. As shown in Figure 3.12.2.B, caspase-8 expression was up regulated (p < 0.001) in all treatments; 3 folds, 2.5 folds, 1.8 folds and 1.3 folds with NM-bLf, Apo-bLf, HMW-bLf and Fe-bLf at highest concentration 3200µg/ml, respectively. Caspase-9 is an initiator apoptotic gene of the caspase cascade, regulated by the intrinsic pathway, Figure 3.12.2.B shows that its gene was up regulated to 1.7 folds with Apo-bLf and 0.9 folds, 0.6 folds with HMW-bLf and Fe-bLf at 3200µg/ml, respectively compared to (2^ΔΔCt) value of untreated cells, Caspase-3 is considered as a key mediator in the proteolytic cleavage cascade activated during apoptosis. Figure 3.12.2.B shows the expression of caspase-3 at highest level with HMW-bLf 3200µg/ml to 4.6 folds, where the expression was increased to 2.2 folds with Fe-bLf 3200µg/ml and 1 and 0.7 folds in case of Apo-bLf and NM-bLf at 3200µg/ml, respectively.

As seen in Figure3.12.C, the gene expression levels of death receptors factor, Fas and Fas-ligand (Fas L) were up regulated significantly (p < 0.005) in all treatments groups, especially with HMW-bLf and Fe-bLf at 3200µg/ml. Epidermal growth factor receptor (EGFR) gene levels in OVCAR-3 cells after 24 treatments as shown in Figure 3.12.2.C were down regulated with all treatments, except with HMW-bLf 1600 µg/ml where expression was seem to be up regulated but it was not statistically significant. mRNA expression of TNF-related apoptosis-inducing ligand (TRAIL) was also shown to be up regulated in OVCAR-3 post treated with bLf. Strongest up regulation was seen in Apo-bLf, HMW-bLf and Fe-bLf at 3200 µg/ml with 14 folds, 11 folds and 9 folds, respectively as compared with control cells with (2^ΔΔCt).

Low density lipoprotein-1 (LRP-1) gene expression (Figure 3.12.2.D) was 1.1 fold increased with HMW-bLf and NM-bLf, where is a slightly elevated expression was seen with other treatment groups as compared to control group. Transferrin receptor (TfR) in OVCAR-3 Figure 3.12.2.D was seen up regulated significantly (p < 0.005) with only Fe-bLf to 1.2 folds. The expression was negligibly up regulated with HMW-bLf and Apo-bLf treatments. On the other hand, the lactoferrin receptor (LfR) gene expression was found to be up regulated highly significantly (p ≤ 0.001) with HMW-bLf and Apo-bLf and very slight increases of LfR gene expression were seen with Fe-bLf and NM-bLf Figure 3.12.2.D.
Figure 3.12.2.A

**Survivin**

- Untreated cells
- HMW-bl 16000 μg/ml
- HMW-bl 20000 μg/ml
- Apolbl 20000 μg/ml
- Fe-bl 20000 μg/ml
- NMA-bl 20000 μg/ml

**Bcl-2**

- Untreated cells
- HMW-bl 16000 μg/ml
- HMW-bl 20000 μg/ml
- Apolbl 20000 μg/ml
- Fe-bl 20000 μg/ml
- NMA-bl 20000 μg/ml

**Bax**

- Untreated cells
- HMW-bl 16000 μg/ml
- HMW-bl 20000 μg/ml
- Apolbl 20000 μg/ml
- Fe-bl 20000 μg/ml
- NMA-bl 20000 μg/ml

**Cytochrome C**

- Untreated cells
- HMW-bl 16000 μg/ml
- HMW-bl 20000 μg/ml
- Apolbl 20000 μg/ml
- Fe-bl 20000 μg/ml
- NMA-bl 20000 μg/ml
Figure 3.12.2.B
Figure 3.12.2.C

![Graphs showing the expression levels of Fas, Fas-L, Trail, and EGFR](image)
Figure 3.12.2 Gene expression analyses using quantitative Real Time Polymerase Chain Reaction (qRT-PCR) in OVCAR-3 cells. Cells were grown in 6 well plates at a density of $2 \times 10^4$ cells/ml and left for 48 h in complete growth medium. Cells were then treated with HMW-bLf at 1600 and 3200µg/ml, Apo-bLf, Fe-bLf and NM-bLf at 3200 µg/ml. The total RNA was isolated using TRIzol reagent (Invitrogen). The reaction mixture using SYBR green premix (Bio Rad), forward and reverse primers and cDNA was prepared and all the genes were amplified using qRT-PCR iQ5 (Bio Rad) depending upon individual annealing temperatures of respective primers. The graphs were plotted by calculating $2^{\Delta\Delta C_t}$. The quantitative data by graphs in image A to D shows the variation in expression levels of various genes. The graph is plotted by comparing each individual values with its actin values. The experiments are repeated two times in duplicate and representative data is shown here.
* P < 0.05, ** P ≤ 0.01, compared with untreated cells and P ≥ 0.05 statistically non-significant.

3.13 Flow cytometry analysis to determine the changes in apoptosis related biomarkers expression

Detection of apoptosis-related proteins’ expression such as survivin, Bcl-2, Bcl-xL, BAD and Bax caspase-7, 8 and 9; cell receptors such as LRP1, transferrin receptor, was further investigated in OVCAR-3 after treatment with HMW-bLf, Apo-bLf, Fe-bLf and NM-bLf at highest concentration of 3200µg/ml for 24h using flowcytometry. This assay was carried out to confirm the finding of qRT-PCR analysis (section 3.12). As shown in Figure 3.16.A-J from obtained flow cytometer data, bLf forms including HMW-bLf, Apo-bLf, Fe-bLf and NM-bLf showed significant down-regulation of expression of anti-apoptotic proteins such as survivin, Bcl-xL and Bcl-2 the treatments further led to significant up-regulation of Caspases,-7, -8, -9 and BAD levels, and with LRP1 and transferrin receptors.
Figure 3.13.A

Survivin expression in OVCAR-3

Treatments

% survivin +ve expression OVCAR-3, 24h

% survivin +ve cells

- Untreated OVCAR-3
- HMW-blf 3200µg/ml
- Apo-blf 3200µg/ml
- Fe-blf 3200µg/ml
- Cblf 3200µg/ml
Figure 3.13.B

**Bcl-xL expression in OVCAR-3**

- **Untreated OVCAR-3**
- **OVCAR-3 + HMW-blf 3200μg/mL**
- **OVCAR-3 + Apo-blf 3200μg/mL**
- **OVCAR-3 + Fe-blf 3200μg/mL**
- **OVCAR-3 + NM-blf 3200μg/mL**

**% Bcl-xL -ve expression in OVCAR-3, 24h**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>% Bcl-xL -ve cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated OVCAR-3</td>
<td></td>
</tr>
<tr>
<td>HMW-blf 5000μg/mL</td>
<td></td>
</tr>
<tr>
<td>Apo-blf 3200μg/mL</td>
<td><strong>25</strong> (p&lt;0.01)</td>
</tr>
<tr>
<td>Fe-blf 3200μg/mL</td>
<td><strong>25</strong> (p&lt;0.01)</td>
</tr>
<tr>
<td>NM-blf 3200μg/mL</td>
<td><strong>15</strong> (p&lt;0.05)</td>
</tr>
</tbody>
</table>
Figure 3.13.C

Bcl-2 expression in OVCAR-3

Unpublished data

% Bcl-2 -ve expression in OVCAR-3, 24h

Treatments
Figure 3.13.D

BAD expression in OVCAR-3, 24h

% BAD +ve expression in OVCAR-3, 24h

Treatments

- Untreated OVCAR-3
- HMW-bLf 3200μg/ml
- Apo-bLf 3200μg/ml
- Fe-bLf 3200μg/ml
- NM-bLf 3200μg/ml
Figure 3.13.E

**Bax expression in OVCAR-3, 24h**

- Untreated OVCAR-3
- OVCAR-3 + HMW-bLf 3200μg/ml
- OVCAR-3 + Apo-bLf 3200μg/ml
- OVCAR-3 + Fe-bLf 3200μg/ml
- OVCAR-3 + NM-bLf 3200μg/ml

**% Bax +ve expression in OVCAR-3, 24h**

- Untreated OVCAR-3
- HMW-bLf 3200μg/ml
- Apo-bLf 3200μg/ml
- Fe-bLf 3200μg/ml
- NM-bLf 3200μg/ml

Treatments
Figure 3.13.F

Caspase-7 expression in OVCAR-3, 24h

Untreated OVCAR-3

OVCAR-3 + HMW-bLf 3200μg/ml

OVCAR-3 + Apo-bLf 3200μg/ml

OVCAR-3 + Fe-bLf 3200μg/ml

OVCAR-3 + NM-bLf 3200μg/ml

% Caspase-7 +ve expression in OVCAR-3, 24h

Treatments

0 10 20 30 40 50 60
% Casp-7 +ve cells

Untreated OVCAR-3  HMW-bLf 3200μg/ml  Apo-bLf 3200μg/ml  Fe-bLf 3200μg/ml  NM-bLf 3200μg/ml
Figure 3.13.H

Caspase-9 expression in OVCAR-3, 24h

Caspase-9 +ve expression in OVCAR-3, 24h

% Casp-9 +ve cells

Treatments

Untreated OVCAR-3
HMW-blf 3200µg/ml
Apo-blf2400µg/ml
Fe-blf3200µg/ml
NM-blf3200µg/ml
Figure 3.13.1

**LRP-1 expression in OVCAR-3, 24h**

- Untreated OVCAR-3
- OVCAR-3 + HMW-blf 3200μg/ml
- OVCAR-3 + Apo-blf 3200μg/ml
- OVCAR-3 + Fe-blf 3200μg/ml
- OVCAR-3 + NM-blf 3200μg/ml

**% LRP-1 +ve expression in OVCAR-3, 24h**

- Untreated OVCAR-3
- HMW-blf 3200μg/ml
- Apo-blf 3200μg/ml
- Fe-blf 3200μg/ml
- NM-blf 3200μg/ml

Treatments
Figure 3.13.J

**TfR expression in OVCAR-3, 24h**

- Untreated OVCAR-3
- OVCAR-3 + HMW-bLf 3200μg/mL
- OVCAR-3 + Apo-bLf 3200μg/mL
- OVCAR-3 + Fe-bLf 3200μg/mL
- OVCAR-3 + NM-bLf 3200μg/mL

**% TfR +ve expression in OVCAR-3, 24h**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>% Transferrin+ve cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated OVCAR-3</td>
<td>5</td>
</tr>
<tr>
<td>HMW-bLf 3200μg/ml</td>
<td>20 **</td>
</tr>
<tr>
<td>Apo-bLf 3200μg/ml</td>
<td>25 **</td>
</tr>
<tr>
<td>Fe-bLf 3200μg/ml</td>
<td>35 **</td>
</tr>
<tr>
<td>NM-bLf 3200μg/ml</td>
<td>15 **</td>
</tr>
</tbody>
</table>
**Figure 3.13.A-J** Detection of apoptosis-related proteins such as survivin, Bcl-2, Bcl-xL, Bad, caspases-7, 8, 9; cell receptors LRP1, transferrin receptor, investigated in OVCAR-3 post treatment with HMW-bLf, Apo-bLf, Fe-bLf and NM-bLf at higher concentration of 3200µg/ml for 24 using flow cytometry. Each experiment result presented with histogram of various treatments group and graph represents percentage of expression.

* P < 0.05, ** P ≤ 0.01, compared with untreated cells.

3.14 Caspase -3 activity in OVCAR-3 cells post treatment with HMW-bLf

N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide, a caspase-3 substrate was used to measure the caspase-3 activity in the OVCAR-3, by measuring the absorbance at 405nm. The cells were treated with HMW-bLf (1600, 2400 and 3200 µg/ml) and with highest dose of Apo-bLf and Fe-bLf (3200 µg/ml) for 24 h. Caspase-3 has substrate selectivity for Asp-GFlu-Val-Asp peptide sequence, the released caspase-3 in the supernatant was measured and graph was plotted as shown in Figure 3.14 below. In OVCAR-3 all treatments were observed to induce nearly same levels of caspase-3 activity expression about 0.15 millimolar/ml (p < 0.05) after 24 h as compared with untreated cells (0.122 millimolar/ml).
Figure 3.14 Caspase-3 activity was detected using N-Acetyl-Asp-Glu-Val-Asp pnitroanilide, a caspase-3 substrate obtained from Sigma Aldrich. OVCAR-3 cells were treated with different forms of bLf and the supernatant was collected after 24 hours. Expression of caspase-3 was observed in all treated cells. The minimum caspase-3 activity was observed with untreated cells. Data expressed as mean percentages (±SE) obtained from 3 repeat wells in each group. Experiments was carried out twice

* = P < 0.05 compared with no treatment group.

3.15 Drug resistance study of OVCAR-3 with Melphalan and HMW-bLf
To investigate the concentration levels of Melphalan drug at which OVCAR-3 cells can grow and show drug resistance, cells were treated for 10 days with Melphalan at various concentrations and untreated OVCAR-3 was used as control. Morphological changes in the cells were viewed qualitatively using an inverted microscope (Prism Optical) and images were obtained using digital camera Figure 3.15.A. With concentration of 5 µM/ml cells started to float and detached. At lower concentrations of Melphalan 0.3 µM, 0.6 µM/ml, 1.25 µM/ml, 2.5 µM/ml, OVCAR-3 cells were still
attached with normal morphological features. In other treatment group, at 10 µM/ml, 15 µM/ml majority of the cells were dead and found with decreasing viable cell numbers. Strongest effect was noticed with 20 µM/ml cells where is more than 95% stated to lose their characteristic morphology by rounding up and getting detached and dead as compared with untreated OVCAR-3.

Following previous experiment, to investigate any enhancement of anticancer efficacy of drug in combination with HMW-bLf, HMW-bLf at moderate concentration 1600 µg/ml was used with low concentration of Melphalan. Cells were treated with combination of HMW-bLf at 1600 µg/ml plus Melphalan at different concentration 0.3 µM, 0.6 µM/ml, 1.25 µM/ml, 2.5 µM/ml in 6 well plate for 24 h. The morphology was observed and cytotoxic effect was indicated as round shaped dead detached floating cells. Figure 3.15.B and C, OVCAR-3 cells got detached and distinguished by rounding of cells in treatment group of 0.6 and 1.25 µM. Higher effect was noticed in 2.5 µM and 5µM treatments with maximum cells detached as compared with untreated cells. Most of the cells showed the presence of apoptotic bodies on the cell surface which was observed more in the treatment with Melphalan plus HMW-bLf.
Figure 3.15.A Morphological changes: OVCAR-3 cells treated with Melphalan at concentrations of 0.3 µM, 0.6 µM, 1.25 µM/ml, 2.5 µM/ml, 5 µM/ml, 10 µM/ml, 15 µM/ml and 20 µM/ml for 10 days. Images were taken of cells in culture wells with an inverted microscope with a mounted digital camera. All images were at 40X magnification.
Figure 3.15.B Morphological changes: OVCAR-3 cells treated with Melphalan 0.3µM, 0.6 µM, 1.25 µM/ml, 2.5 µM/ml, 5 µM/ml + HMW-bLf1600µg/ml. Images were taken of cells in culture wells with an inverted microscope with a mounted digital camera. All images were taken at 40X magnification.

3.16 Investigation of Pgp expression of OVCAR-3 after treated with HMW-bLf
OVCAR-3 cells were treated in 6 well plates with HMW-bLf 1600, 2400, 3200 µg/ml and Apo-bLf at 3200 µg/ml for 24 hours. Pgp expression was studied by using flow cytometry. HMW-bLf induced increase in Pgp expression. As seen in Figure 3.1.6.A-B, cells expressed Pgp without treatment and it was increased slightly in 11.5% cells with HMW-bLf 1600 µg/ml and to 14% Pgp +ve cells with HMW-bL 2400 µg/ml treatments. Treatment with highest concentration of HMW-bLf induced significant expression levels of Pgp with 34.5% of Pgp +ve cells (p ≤ 0.01).
Figure 3.16.A

Figure 3.16.B
Figure 3.16. A, B Flow cytometric analysis of Pgp expression from OVCAR-3 after treated with HMW-bLf 1600, 2400, 3200 µg/ml for 24 hours by using (FACS). A, Dot blot and histogram of various treatments group. B, graph represents percentage of Pgp expression. * P < 0.05, ** P ≤ 0.01, compared with untreated cells and P ≥ 0.05 statistically non-significance

Results Section B:

3.17 Effects of HMW-bLf and other bLf forms on cell cytotoxicity of MDA-MB-231 cells and SW480 cells

Cells were treated with HMW-bLf (800, 1600, 2400, 3200 µg/ml) and Apo-bLf and Fe-bLf (3200 µg/ml) and different concentrations of NM- bLf (1600, 2400, 3200 µg/ml) with inclusion of negative control (untreated cells) and positive control (Triton-X). The HMW-bLf was effective in a concentration dependant manner (800, 1600, 2400 & 3200 µg/ml) in inducing the cell cytotoxicity in MDA- MB- 231 and SW480. In MDA- MB- 231 cytotoxicity values of HMW-bLf and Fe-bLf at higher concentration 3200ug/ml were statistically highly significant (p ≤0.01) with 90% and 76%, cytotoxicity respectively. Moreover significant (p <0.05) effect of Apo-bLf with 66%, HMW-bLf at 2400ug/ml with 50% and NM-bLf at 2400 & 3200 µg /ml with 51and 60%, respectively as compared with untreated group was observed. The cytotoxicity of HMW-bLf and NM-bLf on SW480 and induced LDH release at higher concentration (3200 µg/mL) Figure 3.17.2 was observed most statistically highly significant (p ≤0.01) with 83% and 58% respectively. On the other hand, Apo-bLf and Fe-bLf at the same concentration (3200 µg/ml) induced cytotoxicity values of 30% (p <0.05) and 45% (p <0.01), respectively.
Figure 3.17.1 Cell cytotoxicity assay in MDA-MB-231 cells after 24 h bLf treatments. Cells were treated with various doses of HMW-bLf, (800, 1600, 2400 and 3200 μg/ml), NM-bLf (1600, 2400 and 3200 μg/ml), and Apo-bLf, Fe-bLf with highest concentration of 3200μg/ml for 24 h. Both positive control absorbance value (Triton-X 100 treated cells) and and absorbance value of untreated cells termed as low control was used to calculate the percentage of cytotoxicity for the treatments. The cytotoxicity percentage of low control (negative control) was 0. Please refer to the formula on page 56. All treatments were in triplicates and assay was repeated three times. Results are expressed as mean values (±SD) and a Student’s t-test was performed for evaluating statistical significance. A value of (p < 0.05) denotes statistical significance, whereas (p ≤ 0.01) denotes results that are highly significant

* = P < 0.05 compared with no treatment group.

** = P ≤ 0.01 compared with no treatment group
Figure 3.17.2 Cell cytotoxicity in SW480 cells after 24 h bLf treatments. Cell cytotoxicity assay was performed on SW480. Cells were treated with various concentrations of HMW-bLf (800, 1600, 2400 and 3200 μg/ml), NM-bLf (1600, 2400 and 3200 μg/ml), and Apo-Lf, Fe-bLf with higher concentration 3200μg/ml for 24 h, Both positive control absorbance value (Triton-X 100 treated cells) and and absorbance value of untreated cells termed as low control was used to calculate the percentage of cytotoxicity for the treatments. The cytotoxicity percentage of low control (negative control) was 0. Please refer to the formula on page 55. All treatments were in triplicates and assay was repeated three times. Results are expressed as mean values (±SD) and a Student’s t-test was performed for evaluating statistical significance. A value of (p < 0.05) denotes statistical significance, whereas (p ≤0.01) denotes results that are highly significant. A value of (p≥0.05) denotes statistically non-significance.

* = P < 0.05 compared with no treatment group.

** = P ≤ 0.01 compared with no treatment group.
3.18 HMW-bLf decreases cell viability/growth of MDA-MB-231 and SW480 cells

As shown in Figure 3.18.1 HMW-bLf showed highly significant effect and \( (P \leq 0.01) \) decreased cell viability in MDA-MB-231 at all concentrations used (800, 1600, 2400, 3200 µg/ml) to 77%, 61%, 53%, 30%, respectively. It was apparent therefore that HMW-bLf induced decreased cell viability in MDA-MB-231 cells after 24 h in a dose-dependent manner. Furthermore, highly significant results \( (P \leq 0.01) \) were obtained with Apo-bLf and Fe-bLf at (3200 µg/ml) with cell viability reduced to 37% and 22% respectively. Similar effects on cell proliferation were seen with NM-bLf as well in a dose dependent manner with 59%, 48% and 42% at 1600, 2400, 3200 µg/ml \( (P \leq 0.01) \). (Figure 3.18.2) showed decrease in cell viability of SW480 after 24h treatments by HMW-bLf (800, 1600, 2400, 3200 µg/ml) in a dose dependent manner. Highly significant results \( (P \leq 0.01) \) were obtained at 2400 and 3200 µg/ml of HMW-bLf with 43% and 31% respectively. Moreover Fe-bLf at highest concentration showed strong effect \( (P \leq 0.01) \) on reducing cell viability to 33% as compared to 100% viability with positive control.
Figure 3.18.1

![24h MTT assay, MDA-MB-231](image)

Figure 3.18.2

![24h MTT assay, SW480](image)
Figure 3.8.1-2  Cell viability (MTT) assay of MDA-MB-231 and SW480 cells after treatments for 24 h with HMW-bLf at (800, 1600, 2400, 3200 μg/ml), Apo-bLf and Fe-bLf at highest concentration 3200μg/ml and with NM-bLf at (1600,2400,3200μg/ml). Negative control was blank media and positive control included untreated cells with media containing 20% FBS. Data expressed as mean values (±SD) and a Student’s t-test was performed for evaluating statistical significance.

A value of (p ≤ 0.01) denotes results that are highly significant.

** * = P ≤ 0.01 when compared with positive control value.

3.19 HMW-bLf targets cell proliferation in MDA-MB-231 and SW480 cells

The cell proliferation levels were assessed in MDA-MB-231 and SW480 after treatments for 24 with HMW-bLf (800, 1600, 2400, 3200 μg/ml), NM-bLf (1600, 2400,3200 μg/ml), Apo-bLf and Fe-bLf at highest doses (3200μg/ml) using the CyQuant assay kit (Invitrogen, Australia). As shown in Figure 3.19.1 and 3.19.2 HMW-bLf at a highest dose 3200 μg/ml (p ≤0.01) decreased cell proliferation by 90% and 85% (p ≤0.01) in MDA- MB- 231 and SW480 cells, respectively as compared with other doses and positive control. When observed for the highest dose of Fe-bLf, the proliferation was reduced to 60% in both the MDA- MB- 231 and SW480 cell lines (p <0.05). Also, it was observed from the data that NM-bLf was less effective compared to the HMW-bLf where its potency was reported to affect the proliferation to near 55% and 55%, respectively in MDA-MB-231 and SW480 cells at the highest concentration used.
Figure 3.19.1-2  
CyQuant cell proliferation assay was performed on MDA-MB-231 and SW480 cells after treatments for 24 h with HMW-bLf at 800, 1600, 2400, 3200 μg/ml, Apo-bLf and Fe-bLf at highest concentrations of 3200μg/ml and with NM-bLf at 1600, 2400, 3200μg/ml. Negative control (untreated cells with 10% FBS) and high control (positive control) was cells with 20% FBS. All treatments were tested in triplicate and assay was repeated 3 times.

Data expressed as mean values (±SD) and a Student’s t-test was performed for evaluating statistical significance. A value of (p <0.05) denotes statistical significance, whereas (p ≤0.01) denotes results that are highly significant.

* = P < 0.05 when compared with positive control values.* * = P ≤ 0.01 when compared with positive control values. P ≥ 0.05 statistically non-significant.

3.20 HMW-bLf was internalized to cell cytoplasm and nuclei of MDA-MB-231 and SW480 cells

The cellular internalization of HMW-bLf in MDA-MB-231 and SW480 was studied by confocal microscopy. The images obtained indicated that there was a rapid uptake of HMW-bLf by cancer cell lines used in this experiment. HMW-bLf localised on to cell membrane, internalized to nuclei of cancer cells. Figures 3.20.1 and 3.20.2 show representative images of MDA-MB-231 and SW480 (stained green by FITC) and cell nuclei (stained red with PI). Specific binding of HMW-bLf with cell membrane, cytoplasm and nuclei was observed in all cells after 2 and 4 h compared to untreated cells that showed no immunofluorescence detection of bLf specific antibody.
Figure 3.20.1 Cellular internalization of HMW-bLf in MDA-MB-231 cells. Breast cancer cells MDA-MB-231 were seeded in 8 chamber slides and treated when 80 % confluent, cells were treated with HMW-bLf (1600µg/ml) for different time intervals (2 and 4 h). Then the cells were probed with bLf specific goat-anti-bovine lactoferrin primary antibody (Bethyl Laboratories) in 1:200 dilution. Secondary antibody anti-goat IgG-FITC produced in rabbit (Sigma-Aldrich) was used at 1:160 dilution. Cells were then washed and Vector mounting medium with PI was added to all wells. Slides were visualized by using Leica SP5 confocal microscope (Leica Microsystems, Melbourne, Australia) and images were processed in Adobe Photoshop CS3 (A-C) Untreated cells for comparison of fluorescence, showing only nuclei staining by PI (red) and no immune reactivity for bLf.
Figure 3.20.2 Cellular internalization of HMW-bLf in SW480 cells. Colon cancer cells SW 480 were seeded in 8 chamber slides and treated when 80 % confluent, cells were treated with HMW-bLf (1600µg/ml) for different time intervals (2 and 4 h). Then the cells were probed with bLf specific goat-anti-bovine lactoferrin primary antibody (Bethyl Laboratories) in 1:200 dilution. Secondary antibody anti-goat IgG-FITC produced in rabbit (Sigma-Aldrich) was used 1:160 dilution. Cells were then washed and Vector mounting medium with PI was added to all wells. Slides were visualized by using Leica SP5 confocal microscope (Leica Microsystems, Melbourne, Australia) and images were processed in Adobe Photoshop CS3 A-C) Untreated cells for comparison of fluorescence, showing only nuclei staining by PI (red) and no immune reactivity for bLf.
3.21 HMW-bLf induces cell death in MDA-MB-231 and SW480 cells

The cytotoxicity effects of HMW-bLf to induce cell death in MDA-MB-231 and SW480 cells were further confirmed with (PI) staining. Fe-bLf was used as a positive control at 3200 µg/ml. The dead cells after PI staining were subsequently quantified using the flow cytometry. Similar to its effects on OVCAR-3 cells, the HMW-bLf was effective in a concentration dependant manner (1600, 2400 & 3200 µg/ml) in inducing the cell death in these two cell lines tested. The highest dose of Fe-bLf (3200µg/ml) was found to be nearly effective as the high dose HMW-bLf employed. As observed from the (Figure 3.21.A-B) the cytotoxic effect of high dose of HMW-bLf was statistically highly significant (p ≤ 0.01) in achieving cell death percentages of 82% and 76.5% in MDA-MB-231 and SW480 cells, respectively. The cell death rates with Fe-bLf treatments were 79% and 70% in MDA-MB-231 and SW480 cells respectively. The morphological observation of cells post treatment also confirmed the dose dependent cytotoxicity effect of HMW-bLf. High numbers of floating and dead cells were observed under microscope (40X) in the well with highest concentration treatment.
% Cell death in MDA-MB-231, 24h

Treatments

Untreated cells
HMW-bLf 1600µg/ml
HMW-bLf 2400µg/ml
HMW-bLf 3200µg/ml
Fe-bLf 3200µg/ml
Figure 3.21.A (1-3), B (1-3), Fluorescence-activated cell sorting (FACS) of MDA-MB-231 and SW480 cells to investigate the cell death percentage by using PI after 24h.
treatments with HMW-bLf at (1600, 2400, 3200 µg/ml). Fe-bLf at highest concentration 3200µg/ml was used as positive control, untreated cell represents negative control. Image (A.1) Dot blot and Histogram from FACS of MDA- MB- 231, (A.2) graphical representation shows the percentage of fluorescence detected in MDA- MB- 231, (A.3) Image shows morphological changes of cells after 24h of treatments in MDA- MB- 231. Image (B.1) Dots blot and histogram from FACS of SW480, (B.2) Graphical representation shows the percentage of fluorescence detected in SW480, (B.3) Microscope images shows morphological observation of cells after 24h of treatments in SW480. All images were at 40X magnification.

3.22 HMW-bLf induces caspase-3 activity in MDA-MB-231 and SW480
The tumour cells MDA-MB-231 and SW480 were treated with HMW-bLf (1600, 2400 and 3200 µg/ml) and with highest dose of Fe-bLf (3200 µg/ml) for 24 h. Caspase-3 released in the supernatant was measured and graph was plotted. In MDA-MB-231 Figure 3.22.1 Fe-bLf at 3200 µg/ml shows maximum caspase-3 activity with 0.623 millimolar/ml (p ≤ 0.01). Caspase-3 activity in HMW-bLf treatments at 2400, 3200 µg/ml was statistically highly significant (p ≤ 0.01) with 0.598 millimolar/ml. In case of SW480 Figure 3.22.2 HMW-bLf at 2400, 3200 µg/ml showed maximum activity of caspase-3 activity (p < 0.05) with 0.21, 0.23 millimolar/ml, respectively. Also significant caspase-3 expression was observed in Fe-bLf with 0.17 millimolar/ml activity.
Figure 3.22. (1.2) Caspase-3 activity was determined spectrophotometrically using N-Acetyl-Asp-Glu-Val-Asp pnitroanilide, a caspase-3 substrate (Sigma Aldrich). MDA-MB-231 and SW480 cells were treated with different forms of bLf and the supernatants were collected after 24 hours. The minimum caspase-3 activity was observed with untreated cells. Experiments were carried twice.

Data represents ± SEM; * P < 0.05 and ** P ≤ 0.01, compared with untreated cells.
Chapter 4: Discussion

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008. The World Health Organization (WHO) also estimated that by the year 2030, cancer will kill 12 million people per year [3]. Numerous strategies have been established to reduce the toxic side effects caused by chemotherapeutic drugs. One such hopeful approach is the use of naturally sourced and derived non-toxic anti-cancer proteins. To develop an effective anti-cancer treatment, a number of anti-cancer proteins have been identified, studied and tested successfully [291]. Bovine lactoferrin (bLf) is a multifunctional iron-binding glycoprotein found at high concentrations in bovine and human colostrum and milk. It has been identified as a key element in the host defence system. Lf is an important component of the innate immune system, and is also secreted as a natural defence protein in most exocrine fluids and found in different biological fluids of mammals [292]. Lf plays an important role in the regulation of iron homeostasis, host defence against infection, cancer and inflammation [8, 9]. Lf also regulates the cellular growth, differentiation and protection against cancer development and metastasis. A significant number of studies have already proven that orally delivered bLf reduces tumoure growth [8, 262].

Recently, a novel approach for industrial production and application of bovine Lf in milk products has been developed [293]. Lf can be purified from bovine milk by many purification methods. In general, Lf chromatography isolation methods can be classified into four categories: the ion-exchange chromatography method, the affinity chromatography method, the size exclusion chromatography method and the batch chromatography method. However, no well-documented industrial scale of Lf purification exists for any Lf purification procedure is considered proprietary by the company that developed it. Most of these industrial scale methods are carried out by ion-exchange chromatography or by affinity chromatography [294]. Because Lf has a cationic nature according to its amino acid composition, it can be purified by cation-exchange chromatography. A cation-exchange chromatography was the selected method for the production of bLf by manufacturing companies [295]. In this project,
bLf has been purified with a molecular weight of 250kDa. Nevertheless, few scattering studies proved that Lf aggregates with different molecular weights equivalent to monomers, dimers and trimers, according to chromatographic analysis of bovine milk [245]. Recently, data from a field experimental study revealed that the majority of bLf produced via mammary glands during 7 and 21 of involution was at a high MW of 150kDa which indicates a dimer form of Lf, and 250kDa might suggest the presence of Lf in trimeric form [296].

Epithelial Ovarian Cancer (EOC) is an extremely fatal disease, mainly due to ineffective early detection methods and the clearance of late stage disseminated disease [297]. Most women present with advanced disease and, despite excellent responses to initial surgery and chemotherapy, 5-year survival statistics remain poor [298]. Similarly, the high rate of chemo-resistance revealed high rates of recurrence and mortality [299]. Despite various treatment options such as surgery and chemotherapy drugs, OC still remains a fatal disease among females worldwide. A successful and safe treatment for OC is still being explored. There are some methods of treatment, but none of these options have been successful in eliminating the cancer or preventing the recurrence of the cancer. To eradicate OC, non-toxic, safe and highly effective treatment is required. Several studies conducted in our laboratory proved that bLf possesses an anticancer function without any toxic side effect on normal cells [8, 9, 300, 277, 311]. According to the percentage of iron content, Lf exists in two forms: holo Lf (binds two Fe^{3+} ions), and Apo Lf (iron free) [222]. Furthermore, the amount of iron elements was measured in HMW-bLf purified from Australian bovine colostrum was much depleted and more like Apo-bLf, when compared with the iron standard (100%) and other forms of bLf including monomeric NM-bLf (22%) and Fe-bLf 94%, provided from our lab.

Further, the data obtained from the FTIR experiment to check the internal chemical structure revealed that the purified HMW-bLf was almost comparable with Apo-bLf with minor difference. Exploring heat stability of bLf is very important because this protein has been used as a bioactive component. In order to develop a practical method for pasteurization of bLf, the heat stability has been studied previously [301]. These findings indicated that NM-bLf is significantly more resistant to thermal
denaturation at pH 4 relative to higher pH, concurring with a previous study of Apo-Lf using immunoelectrophoretic and HPLC techniques, but in contrast to a study utilising differential scanning calorimetry, where native Lf was reportedly more thermo stable at basic pH [301, 302]. The heat-sensitivity of Apo-bLf and Fe-bLf was higher in milk than in a phosphate buffer. In addition, several factors can affect heat the stability of Lf such as pH, salts, and whey proteins [303]. In this current study the thermal behaviour of HMW-bLf, and other bLf forms such as Apo-bLf, Fe-bLf and NM-bLf was investigated using DSC. The thermograms obtained showed that value of maximum heat absorption temperature of denaturation was highest (89°C) with HMW-bLf. This result suggested that HMW-bLf may be due to its structural integrity as a trimer at high temperature. In other bLf forms the iron saturated form (Fe-bLf) was more resistant to heat compared with Apo-bLf and NM-bLf. Similar findings have also been reported suggesting that an increase in the protein stability depends upon the degree of iron saturation [304, 305]. Earlier studies on the relative effect of proteases, on the iron-saturated and Apo-forms have shown that Apo-Lf is much more susceptible to gut digestion than the corresponding iron-saturated form [306, 307]. On the other hand, a study noticed that Apo-bLf was largely unaffected by trypsin [307]. In the current study HMW-bLf appeared highly resistant against a digestive enzyme cocktail in vitro, even after 4, 6, and 8 h. We found HMW-bLf existed majorly as trimers with the original band location at 250kDa with degraded forms to dimers at 150kDa, 100kDa and even fewer as digested 50kDa peptide fragments. The resistance to gut enzymes is beneficial in that it can lead to an increase in the bioavailability of HMW-bLf to the tumour site when given orally as compared to NM-bLf. NM-bLf demonstrated rapid hydrolysis with peptide fragments indicated at 50kDa, 37kDa and 25kDa. In an earlier study conducted in vitro, it was found that incubation of Lf with trypsin causes the Lf molecule to break down to fragments with molecular sizes of 20, 30, 40 and 50 kDa [308].

Bovine Lf has been shown to decrease the cell viability of breast cancer cell lines HS578T and T47D with increased apoptosis about 2-fold for both cell lines, with proliferation rates decreasing as well [309]. A similar effect of bLf was seen in vivo with colon, lung and squamous cancer cells [260, 310]. The unpublished data from
our laboratory showed maximum anticancer effect of Apo-bLf and Fe-bLf at the concentration of 3200 μg/ml in the breast cancer cell lines MDA-MB-231, MCF-7, Caco-2 and SW480 cells [277, 311].

The current study showed for the first time the anticancer efficacy of HMW-bLf purified from Australian bovine colostrum using ovarian cancer, breast and colon cancer cell lines model in vitro. Cytotoxicity (LDH release) assay and PI staining showed highest cytotoxicity of OVCAR-3, MDA- MB- 231 and SW480 in vitro at HMW-bLf concentration of 3200μl/ml after 24 hours. Furthermore, earlier findings from several animal studies have suggested that bLf can inhibit the development of azoxymethane (AOM)-initiated colon tumours in the rat when given as a dietary supplement [312]. It has also been shown to reduce growth and metastasis of solid tumours [8, 9, 313]. Data obtained in the current study from cell proliferation and viability assays using cyQuant and MTT, respectively have confirmed the LDH results, and demonstrated the opposite effects. HMW-bLf at higher concentration showed the highly significant decrease in cell viability with OVCAR-3. Furthermore, same efficacy was observed with other bLf forms, especially in case of Fe-bLf at 3200μg/ml. On the other hand, MDA-MB-231 viability was decreased significantly with all bLf concentrations. Higher effect on cell viability was seen with HMW-bLf at highest concentration at 3200μg/ml. Similar observations were observed with SW480 cells, whereas the HMW-bLf at 2400, 3200μg/ml shows highly significant decrease in cell viability and proliferation. Further observation into the effects of HMW-bLf on cell morphology revealed that cells, after 24 h incubations exhibited poor growth with altered morphology. Three cell lines used in this study showed detachment and decreased number of cells at highest concentration of HMW-bLf. High levels of cellular fragmentation apoptotic bodies were also present in groups treated with 2400 and 3200 μg/ml, thus indicating a dose dependant cytotoxic effect.

An interesting property of Lf is its ability to target the nucleus, as first described in neutrophilic leukocyte cells [314]. The cellular internalisation of hLf has proven to be assisted by the presence of lactoferrin receptores identified in the brush border membranes of intestinals cells [315]. It was demonstrated that recombinant hLf
internalized into differentiated Caco-2 via receptors and localized to the nuclei of the cells.

Previous data proposed that transferrin receptor (TfR) and low density lipoprotein receptor-related protein receptors (LRPs) play a crucial role in facilitating the internalization of bLf inside cells [316]. Studies conducted in our laboratory involved both colon and breast cancer cells which demonstrated that bLf had internalised into the membrane and cytoplasm [263]. In the current project study, the internalisation of HMW-bLf after incubation of 2 and 4 h with cancer cells, was observed on the surface of cell membrane, cytoplasm and nuclei in all the three types of cells at different time intervals compared to untreated cells which showed no immunofluorescence detection of bLf specific antibody. The rapid uptake of HMW-bLf 1600 µg/ml into the cytoplasm and nuclei of cancer cells may explain the strong cytotoxicity effect of HMW-bLf at this highest concentration which leads to initiate gene transcription and transcription factors within the cell to increase cell cytotoxicity and apoptotic signals. Other studies have shown that internalization of bLf to the cell and nucleus can regulate gene transcription [317]. However, further studies would be necessary to investigate the exact level of binding and endocytosis exhibited by HMW-bLf.

The main limitation to the effective treatment of OC is the development of resistance to the currently preferred chemotherapeutic regimen of carboplatin plus paclitaxel [318]. Since OVCAR-3 was established from patient refractory to cytotoxicity chemotherapy, it represents an appropriate model to investigate drug resistant and cross resistance in OC. OVCAR-3 is resistant to several chemotherapeutic agents, including Melphalan until the toxic dose (5x10^{-6} µM) [319]. Our findings proved that OVCAR-3 as seen in a drug resistance assay, demonstrates the resistance of tumour cells when treated with different concentrations of Melphalan less than (5x10^{-6} µM). In OC research it was reported that overexpression of Pgp correlated with tumour development [320]. Furthermore, OC tumour cells presented resistance against paclitaxel due to overexpression of Pgp [185, 321]. In this study, an experiment was carried out to assess the expression of Pgp due to treatment of OVCAR-3 by HMW-bLf using flow cytometry. We noticed an increase in the
expression of Pgp at 1600 µg/ml and 2400 µg/ml, where extreme overexpression of Pgp at 3200 µg/ml was seen. The overexpression of these transmembrane receptors is caused by the entry of a foreign drug into the cell system. If the drug molecule is a substrate for P-gp, it causes structural alterations in the membrane transporter with the utilisation of ATP molecules [322]. This structural alteration helps the transporter pumps to efflux any drug that enters the cells. In case of cancer cells and drug resistant tumours where this transporter is highly overexpressed, the entry of HMW-bLf could cause a further increase in the expression of these proteins in order to cause efflux of the anticancer molecule. This holds true in OVCAR-3 as it is a drug resistant cell line [322]. Further investigations are required to approve the real scenario.

Apoptosis is a mechanism that plays a crucial role for cell suicide and is regulated by programmed cellular signalling pathways. Apoptosis can happen by one of the two representative pathways: the death receptor-mediated (extrinsic) pathway and the mitochondrial (intrinsic) pathway. Moreover, induction of caspases plays a significant factor in either pathway including initiator caspase like caspase-8 and capase-9, and the executioner of apoptosis, caspase-3 [323]. The efficacy of any medicine used for cancer treatment is based on its capacity to induce apoptosis. As mentioned earlier, bLf is known to induce the intrinsic pathway of apoptosis in oral carcinoma [275]. An in vivo study conducted by Fujita et al in 2001 investigated the mechanism pathway of bLf on colon carcinoma. They concluded that bLf supressed the growth of the tumour through caspase-8 signalling pathways. Additionally, Mohan et al (2007) reported that bLf is known to activate the intrinsic pathway of apoptosis in oral carcinomas [276]. Therefore, the main highlight of this project study was to clarify the mechanism of HMW-bLf induced apoptosis in OC.

Our data obtained from Annexin-V FLOUS/PI staining by using flow cytometry suggest that HMW-bLf has a potential effect of inducing apoptosis in OVCAR-3 even more than necrosis after 24 h treatments at the concentration used in this study, especially at the highest concentration of HMW-bLf (3200 µg/ml). Caspase-3 is a key element in apoptosis execution and it can be activated by several other active pathways in the formation of pro-caspase-3 and also because it has catalytic
specificity for a various cellular substrates [324]. One reliable indication of the induction of apoptosis is detection of cells that express the active form of caspase-3 [325, 326]. In fact, the presence of cells that are positive for active caspase-3 is considered a sign of apoptosis stimulation.

Caspase-3 activity was evaluated in three cell lines, after treatment with HMW-bLl. This observation proved that HMW-bLl induces significant expression caspase-3 in all three cell lines used in the current study. bLl has been shown to up-regulate the sensitivity of death receptor Fas in colon mucosa [327]. Fas was announced to trigger the extrinsic pathway of apoptosis, which also involves caspases-8 and 3. Fujita et al in 2004 proved that bLl induced Fas expression in rats inoculated with colon tumour cells also had an up-regulated effect of active forms of caspase-3 and caspase-8 as well [273]. Fas and Fas ligand gene expression of OVCAR-3 treated with HMW-bLl for 24 hours was investigated. Both Fas and Fas-L showed strong up-regulation with HMW-bLl and Fe-bLl at 3200μg/ml treatment, as compared to untreated cells.

Caspases-3,-7, -8 and -9 showed their highly significant (p< 0.001) up-regulated gene expressions after treatments with HMW-bLl when compared with untreated OVCAR-3. Similar results were confirmed from protein expression studies carried out by flow cytometric analysis. These results specify that HMW-bLl was able to stimulate a mechanism, an intrinsic pathway via activating caspase-9 and extrinsic pathway via up-regulating of caspase-8. Data from the current study suggested that HMW-bLl induces apoptosis.

Tumour necrosis (TNF)-related apoptosis-inducing ligand (TRAIL) represents a member of the TNF family of cytokines that promotes apoptosis [328]. TRAIL induces apoptosis through interaction with its receptors leading to apoptosis when overexpressed [329, 330]. Our finding from gene expression experiments revealed that TRAIL expression from OVCAR-3 was a strongly up-regulated expression with HMW-bLl treatment. Along with TRAIL receptors in OVCAR-3 cells, qRT-PCR and flow cytometry data obtained from protein expression studies showed HMW-bLl at its highest concentration showed highly significant (p< 0.001) with up-regulation of LRP
in OVCAR-3. The data obtained from qRT-PCR showed down-regulation of gene expression of Bcl-2, whereas the gene expression was up-regulated with pro-apoptotic Bax. Gene expression was also seen up-regulated in cytochrome-C (inner mitochondrial protein) and showed the activation of an intrinsic pathway of apoptosis in OVCAR-3 cells when treated with HMW-bLf and other bLf forms.

Survivin represents the most significant member from an inhibitor of apoptosis protein (IAP) family [331]. The main role of survivin is the inhibition of caspase activation resulting in the blocking of apoptosis pathway, also interaction with the microtubules of the mitotic spindle during the G2/M phase of cell cycle [332]. Survivin is highly expressed in most of cancer types but its expression is totally absent in differentiated normal tissue [333], thus the proposal about the contribution of survivin gene reactivation in carcinogenesis [331, 334]. There is large evidence that over-expression of survivin is related with poor response to chemotherapy and decrease overall survival in leukemic patients [335]. Because, survivin is very important in cancer cell survival and hence blocking of survivin expression are significant goals for any novel anti-tumour therapies [333, 336]. Unpublished studies from our laboratory have shown that bLf is able to down regulate the survivin expression in colon cancer cells. This may be the reason for induction of apoptosis in cancer cells by Lf.

Gene expression study presented successful down-regulation in survivin expression from OVCAR-3 after treated with HMW-bLf and other bLf forms as compared with untreated cells. The significant (p< 0.001) effect on the gene expression of survivin was shown by Fe-bLf, HMW-bLf and Apo-bLf at 3200µg/ml.

There are numerous reports on EGFR as an adverse prognostic indicator in different sorts of tumour cells. The EGFR family of receptor tyrosine kinases remains of significant interest regarding ovarian cancers [337]. In this present study, our finding from qRT-PCR data shows significant down-regulation of EGFR gene expression due to treatment of OVCAR-3 with HMW-bLf and other bLf forms at highest concentrations (3200µg/ml). Flow cytometry was used to study the expression of pro-apoptotic and anti-apoptotic proteins by employing antigen antibody interactions
and measurement of signal using fluorescently labelled secondary antibody. As shown in Figure 3.16 the data from A-J from flow cytometric analysis, HMW-bLf showed down-regulation of expression of anti-apoptotic proteins such as survivin, Bcl-xL and Bcl-2 while further treatment led to up-regulation of caspases-7,-8,and -9, and Bad levels, along with LRP1 and transferrin receptors. These results confirmed q-RT-PCR gene expression data.

4.1 Proposed cellular mechanism involved in apoptosis of OVCAR-3 after treatment by HMW-bLf

Data from the current project proposed that HMW-bLf seems to induce both extrinsic and intrinsic pathways in cell death signalling pathways mechanisms. In the extrinsic pathway HMW-bLf up-regulates Fas which binds to Fas ligand (Fas-L) death receptor protein (present in cytotoxic T-cells or free in circulation), which later induces the intracellular death signalling pathway proteins. Fas-L triggers the activation of caspase-8 from procaspase-8. The caspase-8 protein activates Bid to t-Bid which leads to the down regulation of IAP proteins such as Bcl-2 and Bcl-xL. The t-Bid then induces release of cytochrome-C from mitochondria [203]. Activation of caspase-8 also triggers the effector caspase-3 via possible formation of the death inducing signalling complex (DISC) which finally initiates the apoptosis process.

In the intrinsic pathway, HMW-bLf is proposed to internalize in OVCAR-3 via up-regulation of LfR, TfR and LRP-1 receptors and initiates apoptosis by the down-regulation of anti-apoptotic proteins such as survivin, Bcl-2 and Bcl-xL and the the up-regulation of pro-apoptotic proteins like Bax and Bad. These pro-apoptotic proteins further leads to loss of mitochondrial membrane potential and release of the cytochrome-C and Smac/Dialbo from the mitochondria. Cytochrome-C further leads to activation of Apaf-1 and procaspase-9 to form a complex that activates caspase-9. The caspase-9 further activates both caspase-7 and caspase-3 which are the effector caspases that finally triggers the apoptosis.
Figure 4.1 Schematic diagram showing the proposed molecular mechanism of HMW-bLf action in inducing OVCAR-3 cell death. Cell death mechanism involved in the activation of extrinsic pathway for cellular apoptosis, is mediated via up regulation of death receptors (Fas and TRAIL) followed by activation of (caspase-8, caspase-7 and caspase-3). On the other hand, internalization of HMW-bLf facilitated through the up-regulated LfR, LRP-1 and TIR receptors leads to down regulation of anti-apoptotic genes Bcl-2, Bcl-xL and survivin while leads further up-regulation of apoptotic genes (BAX and BAD) activates the intrinsic/mitochondrial pathway of apoptosis that triggers the executioner caspases (caspase-3 and-7). The red arrows represented in the Figure 4.1 are experimental findings in this project, whereas black arrows represent the work of other researchers [196].
Conclusion

In the current study, bLf has been successfully purified from Australian bovine colostrum by ion exchange chromatography (IEC). Interestingly, the molecular weight of purified bLf was at 250 kDa and named here high molecular weight-bLf (HMW-bLf). This HMW-bLf possesses unique features such as high thermal stability and resistance against a gut digestion enzyme cocktail better than the native monomeric form of bLf used in this study. Furthermore HMW-bLf exhibited significant cytotoxic effects and also reduced cell proliferation significantly (p≤ 0.001 ) in OVCAR-3 and other cell lines used in this study. These were (MDA-MB-231 and SW480) especially at highest concentration (3200µg/ml). These three cell lines showed up regulation of caspase-3 expression when treated by HMW-bLf for 24h. Furthermore, immunofluorescence assay using confocal microscopy showed high binding and internalization activity of HMW-bLf at 2 and 4 h in all cell line involved in this study. For the first time, HMW-bLf indicated inducement through both apoptosis pathways (extrinsic and intrinsic) in OVCAR-3. Flow cytometry and qRT-PCR data revealed that HMW-bLf triggers up-regulation of expression levels of pro-apoptotic proteins such as Bax, Bad, FAS, FAS-L, TRAIL, Cytochrome-C, caspase-7, caspase-8, caspase-9 and caspase-3, and down regulation of anti-apoptotic proteins such as Bcl-2, Bcl-xL and survivin, and also EGFR. Up-regulation of gene expression was also observed in LRP1, TIR, and LRP-1 receptors.

The novel bioassay based findings from this study clearly proved the potential of HMW-bLf as a strong and promising therapeutic agent for targeting different kinds of cancers such as ovarian, breast and colon cancers. This study also indicates the future potential of the natural therapeutic HMW-bLf molecule for replacing the painful and toxic chemotherapeutic options.
The interesting and novel findings of the present study open the door for further strategies to investigate this natural multifunctional bio macromolecule employed as an anti-cancer therapeutic agent. These promising results should be validated in other ovarian cancer cell lines such as SKOV-3, SKOV-21 and SW626. Western blotting to confirm further the expression of receptors (LRP1, TfR, and LRP-1) and of the pro-apoptotic and anti-apoptotic genes found activated during HMW-bLf inducing apoptosis observed in this study, should also be carried out. The molecular mechanism of apoptosis should be further studied by analysis of gene and protein expression of other molecules such as (Smac/Diablo and Disc). Further study needs to be carried out to prove the safety of HMW-bLf when used orally, which can be achieved by treating normal ovarian cells with HMW-bLf in vitro to investigate the cytotoxic effects. To further confirm these results obtained from the present study and to achieve further understanding in this area, additional in vivo allograft and xenograft animal cancer model studies would be necessary.
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Appendix

Assay Medium (OVCAR-3)
RPMI 1460 medium, 0.01mg/ml bovine insulin, 1 X Penicillin Streptomycin solution.

Assay Medium (MDA MB 231)
Leibovitz’s L-15 growth medium, 1 X Penicillin Streptomycin solution.

Assay Medium (SW480)
DMEM, 1 X Penicillin Streptomycin solution.

PBS (Phosphate Buffered Saline)
1.1 g Na₂HPO₄, 0.32 g NaHPO₄, 9g NaCl, Up to 1L with autoclaved Milli-Q water. Saline was then autoclaved for sterility.

RIPA Buffer
0.6057 g Tris base, 0.877 g NaCl, 10 ml Nonident P-40, 5 ml 10% Na-deoxycholate, 1 ml 10%. SDS, pH to 7.5, adjusted to 100 mL with H₂O.

Running Buffer
3.03 g Tris Base, 14.4 g Glycine, 1.0 g SDS, autoclaved Milli-Q water up to 1 L.

SDS Sample Buffer
1.25 ml 0.5M Tris base, 2.5 ml glycerol, 2 ml 10% (w/v) bromophenol blue and 3.55 Milli-Q water. 100 μl of β-mercaptoethanol added to 900 μl of sample buffer prior to use.

TBS
2.42 g Tris base, 8 g NaCl, make up to 1L with autoclaved Milli-Q water, pH 7.6.

TBS-T

161
2.42 g Tris base, 8 g NaCl, 1ml Tween-20, make up to 1L with autoclaved Milli-Q water, pH 7.6.

**Towbin Transfer Buffer**

3.03 g Tris Base, 14.4 g Glycine, 1.0 g SDS, 200 ml methanol, autoclaved Milli-Q water up to 1 L.

**TBE**

108 g Tris Base, 56 g Boric acid, 20ml 0.5M EDTA, up to 1L with autoclaved Milli-Q water.