EVALUATIONS ON EFFICACY AND IMMUNOSTIMULATORY PROPERTIES OF TROPOMYOSIN RECEPTOR KINASE C TARGETED PEPTIDOMIMETIC LIGAND-DIOIDO-BORON DIPYRRROMETHENE HYBRIDS IN PHOTODYNAMIC ANTICANCER THERAPY

KUE CHIN SIANG

FACULTY OF MEDICINE UNIVERSITY OF MALAYA KUALA LUMPUR

2016
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ABSTRACT

Photodynamic therapy (PDT) utilises the administration of photosensitiser (PS) along with focal light activation at specific wavelengths to generate singlet oxygen species to kill tumour cells. Currently, most of the available photosensitisers have poor tumour selectivity. This has led to poor therapeutic outcome. In order to improve the tumour selectivity of the PS, a Tropomyosin receptor kinase C (TrkC) receptor based active targeting ligand-PS complex was synthesised. TrkC is being targeted due to its overexpression in cancer including breast, melanoma, pancreatic, neuroblastoma etc. In this study, a synthetic isoleucine-tyrosine-isoleucine-tyrosine based TrkC receptor ligand (IY-IY) was linked to a model photosensitiser diiodo-boron dipyrromethene I$_2$-BODIPY to form a TrkC targeting PS derivative IYIY-I$_2$-BODIPY. Thereafter, tumour targeting properties, in vivo antitumour efficacy and immune stimulatory properties of conjugates in TrkC positive (4T1) and TrkC negative (67NR) breast cancer models in pre and post-PDT scenarios were evaluated. Data showed that IYIY-I$_2$-BODIPY, but not a scrambled control (YIYI-I$_2$-BODIPY) and free drug (I$_2$-BODIPY) selectively induced photocytotoxicity in a dose-dependent manner in 4T1 cells upon irradiation. Bio-distribution studies in 4T1 mouse model showed that IYIY-I$_2$-BODIPY accumulated high in tumours at 1 h post intravenous administration and maintained high up to 6 h, at a level which was approximately 2-fold higher compared to YIYI-I$_2$-BODIPY. Antitumour activity of IYIY-I$_2$-BODIPY in 4T1 showed 96% reduction in tumour volume at day-6 post PDT at 10 mg/kg. Moreover, 71% of IYIY-I$_2$-BODIPY treated mice were “healed” from aggressive breast cancer for up to 90 days post-PDT with no evidence of metastasis, indicating complete remission. This observation was neither found in YIYI-I$_2$-BODIPY nor I$_2$-BODIPY treated mice. The selectivity of IYIY-I$_2$-BODIPY was further confirmed when 67NR breast tumours in mice showed only slight tumour reduction followed by tumour re-growth upon PDT using all three
compounds. At a similar therapeutic dosage, IYIY-I2-BODIPY (strong response) and YIYI-I2-BODIPY (weak response), but not I2-BODIPY, in non-irradiation condition selectively increases the pro-inflammatory cytokines IL-2, IL-6, IL-17 and suppresses immunosuppressive cytokines TGF-β at 2 h post administration. Moreover, the conjugates increase both CD4 and CD8 T-lymphocyte populations with phenotype of IFN-γ (Th1, CTL) and IL-17 (Th17, Tc17), and decreases immunosuppressive granulocytic myeloid-derived suppressor cells (G-MDSC) and regulatory T cells, which were known to be highly increased in cancer. Only IYIY-I2-BODIPY induced tumor growth delay (~20% smaller size) in mice when administrated daily for 5 days. When illuminated with light to produce effects associated with PDT, IYIY-I2-BODIPY induced even stronger immune responses. In addition, IYIY-I2-BODIPY and light treated mice had higher levels of immune effector T-cells compared to photoirradiated YIYI-I2-BODIPY and I2-BODIPY controls. Adoptive transfer of splenocytes and lymphocytes from IYIY-I2-BODIPY treated survivor mice that were photoirradiated gave significantly delayed tumour growth in recipient mice. Our data provide evidences that TrkC ligand conjugate, alone and in combination with PDT modulates immune responses that are conducive to suppressing tumour growth. Thus this conjugate can act as an immune-stimulatory PDT agent with potential applications in cancer treatment.
ABSTRAK
Terapi fotodinamik (photodynamic therapy, PDT) menggunakan molekul fotosensitif dengan sinaran cahaya yang mempunyai gelombang spesifik untuk menjana oksigen molekul singlet reaktif yang berupaya memusnahkan sel-sel tumor dan meningkatkan sistem imunisasi pada masa yang sama. Kebanyakan molekul fotosensitif yang digunakan di tahap klinikal mempunyai kelemahan dalam selektiviti tumor, mengakibatkan hasil terapi yang tidak memuaskan. Oleh yang demikian, penasaran reseptor TrkC dengan menggunakan ligan bermolekular kecil (IYIY) yang berkonjugasi dengan diiodo-boron dipyrromethene (IYIY-I2-BODIPY) telah dihasilkan. Kelampauan expresi TrkC dalam kebanyakan kanser, termasuk payudara, melanoma, pankreas, neuroblastoma dan lain-lain jenis kanser menjadikannya sebagai sasaran dalam pengajian ini. Konjugasi IYIY-I2-BODIPY dalam pemilihan tambatan dan fotositotoksisiti terhadap TrkC in vitro telah pun dijalankan. Dalam pengajian ini, penilaian IYIY-I2-BODIPY dalam keberkesan antitumor dan kebolehannya sebagai pendorong imunisasi di kanser payudara tikus TrkC positif (4T1) dan TrkC negatif (67NR) sebelum dan selepas PDT. IYIY-I2-BODIPY secara terpilih telah meningkatkan fotositotoksisiti di sel 4T1 semasa proses penyinaran. Pengajian bio-distribusi pada tikus membuktikan IYIY-I2-BODIPY banyak terkumpul di 4T1-tumor secepat 1 jam dan kuantitinya tetap sehingga 6 jam selepas suntikan intravena, lebih kurang 2 kali ganda dibandingkan dengan YIYI-I2-BODIPY. Aktiviti antitumor IYIY-I2-BODIPY dalam 4T1 menunjukkan pengurangan saiz tumor sebanyak 96% pada hari ke-6 selepas PDT (10 mg/kg). Tambahlan lagi, 71% daripada tikus yang dirawati dengan IYIY-I2-BODIPY “sembuh” daripada kanser selepas 90 hari proses PDT tanpa sebarang tanda-tanda metastasis, menunjukkan penyembuhan yang sempurna. Pemantauan ini tidak didapati samada di tikus yang dirawati dengan YIYI-I2-BODIPY atau I2-BODIPY. Kemampuan pemilihan IYIY-I2-BODIPY terhadap TrkC dibuktikan dengan selanjutnya apabila pengurangan saiz yang sikit didapati di TrkC negatif tumor payudara 67NR
selepas PDT, diikuti dengan penumbuhan semula tumor, bersamaan dengan YIYI-I₂-BODIPY. Pada dos terapi yang sama, IYIY-I₂-BODIPY (kuat) dan YIYI-I₂-BODIPY (lemah) tapi bukan I₂-BODIPY, meningkatkan sitokin pro-inflamatori IL-2, IL-6, IL-17 dan mengehadkan sitokin penekan-imunisasi TGF-β selepas 2 jam kompaun inokulasi dalam keadaan tanpa penyinaran. Kedua-dua konjugasi ini juga meningkatkan kumpulan CD4+ dan CD8+ T-sel dengan fenotip IFN-γ (Th1, CTL) and IL-17 (Th17, Tc17), dan mengurangkan populasi *immunosuppressive granulocytic myeloid-derived suppressor cells (G-MDSC)* dan *regulatory T-cells* yang biasanya akan meningkat dalam kanser. Tambahan, tikus yang dirawat dengan IYIY-I₂-BODIPY menyumbang kepada penglewatan penumbuhan tumor apabila inokulasi setiap hari sebanyak 5 hari.

Rawatan IYIY-I₂-BODIPY bersama dengan PDT meningkatkan reaksi imunisasi yang lebih kuat berbanding dengan rawatan konjugasi bersendirian. Tambahan lagi, tikus yang dirawat dengan IYIY-I₂-BODIPY dan PDT mempunyai memori T-sel yang tinggi berbanding dengan kontrol yang difotoradiasi. Pemindahan adoptif sel imunisasi dari tikus yang sembuh daripada rawatan photonradiasi IYIY-I₂-BODIPY kepada tikus penerima menunjukkan kesan yang ketara dalam pengurangan saiz tumor. Data penyelidikan kami membuktikan bahawa IYIY-I₂-BODIPY bersendiriannya atau bersamaan dengan PDT memyumbangkan kepada peningkatan imunisasi yang dapat mengehadkan penumbuhan tumor. Oleh yang demikian, konjugasi ini dapat bertindak sebagai agen PDT perangsang-imunisasi yang berpotensi dalam aplikasi rawatan kanser.
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<td>photodynamic therapy</td>
</tr>
<tr>
<td>PgR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-Myristate 13-Acetate</td>
</tr>
<tr>
<td>PS</td>
<td>photosensitizer</td>
</tr>
<tr>
<td>PSMA</td>
<td>prostate-specific membrane antigen</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SOG</td>
<td>singlet oxygen generation</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TM</td>
<td>tumour microenvironment</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T</td>
</tr>
<tr>
<td>Trk</td>
<td>tropomyosin receptor kinase</td>
</tr>
<tr>
<td>YIYI</td>
<td>tYrosine-Isoleucin-tYrosine-Isoleucin</td>
</tr>
</tbody>
</table>
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CHAPTER 1: INTRODUCTION

1.1 Overview

Conventional cancer therapies such as radiation, chemotherapy and most of the clinical approved anticancer drugs have shown promise in treating certain cancers. However, these anticancer therapies often lack killing selectivity on tumour cells, and have long been associated with immune response silencing in cancer patients (Zitvogel et al., 2008). An ideal goal of anticancer therapy is to selectively destroy the tumour cells while allowing normal cells to remain unharmed, and at the same time to stimulate anti-tumour immune response in the host immune.

Photodynamic therapy (PDT) is considered as an effective alternative treatment option for superficial cancer. The drug used in PDT is called photosensitiser (PS), which is non-toxic to cells unless activated by the light at specific wavelengths. Upon activation, PS will generate the cytotoxic singlet oxygen species in presence of oxygen molecules, and the generated singlet oxygen will have direct killing activity on tumour cells, disrupt tumour vasculature and elevates systemic immune response. It is less toxic to normal tissues upon activation because only the local tumour lesion is irradiated to activate the administered photosensitiser, and stimulates tumour antigen specific immune responses (Brackett & Gollnick, 2011; Castano et al., 2006). PDT has become one of the clinical treatment options for early stages of certain cancer types such as nasopharyngeal, gastroenterological, brain and gynecological cancer (Dolmans et al., 2003; Huang, 2005). Other than PDT, targeted delivery of therapeutic agent (active or passive targeted cancer therapy) is another anticancer therapy approach to increase drug accumulation in tumour sites (Srinivasarao et al., 2015; Torchilin, 2010). In active
targeted therapy, the designed conjugate (composed of targeting ligand linked to cytotoxic agent) targets the cell surface molecules that are overexpressed in cancers (generally survival or metastasis biomarkers including folate receptor, estrogen receptor, prostate specific membrane antigen, sigma-2 receptor, tropomyosin receptor kinase, etc.), with minimum binding to normal cells. The conjugate will then be internalised by receptor mediated internalisation, which in turn causes the drug to be released intracellularly for its cytotoxic action once it is degraded by lysosomes.

Among the cell surface molecules overexpressed in cancer cells, Tropomyosin receptor kinase (Trk) is selected in this study due to its limited treatment option (chemotherapy using Trk inhibitors). Trk consists of three common receptor tyrosine kinases, TrkA-C (Segal, 2003). Trk receptor and their natural ligands (neurotrophins) interaction has also been reported not only to be associated with cancer progression (Nakagawara, 2001), but also can modulate immune responses. Among the Trk receptors, TrkC is highly correlated with cancer growth of different types. Immunohistochemistry studies showed that TrkC is a useful biomarkers for prognosis of tumour progression and invasion (Vaishnavi et al., 2015) in neuroblastoma (Brodeur et al., 1997; Yamashiro et al., 1997), glioblastoma (Kumar & de Vellis, 1996; Wang et al., 1998), thyroid cancer (McGregor et al., 1999), melanoma (Xu et al., 2003) and breast cancer (Blasco-Gutierrez et al., 2007; Jin et al., 2010). In immunology, neurotrophins were reported to modulate cytokines secretion such as increasing interleukin (IL)-6 and IL-4, and impairing transforming growth factor-β signaling (Jin et al., 2007). In immune cells, neurotrophins and Trk were known to regulate the balancing of CD4+ T-lymphocytes subtypes by promoting growth of Th2 but not Th1, due to the relatively high TrkC expression in Th2 compared to Th1 (Rezaee et al., 2010; Sekimoto et al., 2003; Vega et al., 2003).
A synthetic peptidomimetic ligand which is selective to TrkC receptor has been designed (Kamkaew & Burgess, 2013). The preliminary study has shown that the synthetic TrkC receptor targeted peptidomimetic ligand (Isoleucine - tYrosine - Isoleucine - tYrosine, IYIY) selectively binds to TrkC receptor in TrkC transfected NIH-3T3 cells. Moreover, when comparing the effect of IYIY and neurotrophin-3 (TrkC natural ligand), both were able to induce signaling cascades to promote neuronal cell growth and differentiation (Brahimi et al., 2014; Chen et al., 2009). Based on the interesting preliminary findings, a TrkC targeted peptidomimetic ligand-photosensitiser conjugate, which is IYIY-diiodo-boron dipyrromethene (IYIY-I\(_2\)-BODIPY) has been constructed (Kamkaew & Burgess, 2013). The ligated conjugate was hypothesised to have a better targeting in TrkC+ cancer than the unconjugated free photosensitiser diiodo-boron dipyrromethene (I\(_2\)-BODIPY). On top of that, systemic immune responses might also be affected upon the administration of the IYIY-I\(_2\)-BODIPY conjugate. In this study, the efficacies of IYIY-I\(_2\)-BODIPY conjugate including \textit{in vitro} binding selectivity, biodistribution, antitumour activity as well as immunological impacts in TrkC expressing (4T1) and non-TrkC expressing (67NR) murine breast cancer models, under non-irradiated (without PDT) and irradiated (PDT) conditions will be carried out. The breast cancer model is used in this study due to its distinct TrkC expression among metastatic and non-metastatic cell lines. Moreover, tumour can be induced orthotopically at the mammary gland, and hence it can be monitored easily, especially during photodynamic therapy.

The above study is important because this is the first compound designed to target TrkC tumour in active targeting, and may expand the understanding on the systemic impact of active targeted ligand-drug conjugate in high TrkC expressing cancer.
1.2 Aim and Objectives

The aim of this study is to investigate the binding selectivity, biodistribution, antitumour efficacy and systemic immune responses of TrkC receptor targeted conjugate (Isoleucine - tYrosine - Isoleucine - tYrosine, IYIY-I2-BODIPY) in comparison to the non-TrkC receptor targeted scrambled control tYrosine – Isoleucine – tYrosine - Isoleucine, YIYI-I2-BODIPY (reversion of amino acid arrangement) and the free drug I2-BODIPY in TrkC expressing (4T1) and non-TrkC expressing (67NR) murine breast tumour model under non-irradiated and irradiated condition.

The specific objectives of this study are as follows:

i. To compare the in vitro photocytotoxicity and targeting selectivity of IYIY-I2-BODIPY, YIYI-I2-BODIPY and I2-BODIPY in 4T1 and 67NR cell lines at different treatment time points.

ii. To determine the in vivo toxicity profile (maximal tolerated dose, MTD) of IYIY-I2-BODIPY, YIYI-I2-BODIPY and I2-BODIPY in healthy BALB/c mice.

iii. To compare the biodistribution pattern and antitumour efficacy of IYIY-I2-BODIPY and YIYI-I2-BODIPY in 4T1 tumour bearing BALB/c mice.

iv. To quantify the cytokines secretion from T helper cells in IYIY-I2-BODIPY, YIYI-I2-BODIPY and I2-BODIPY in 4T1 tumour bearing BALB/c mice under non-irradiated and irradiated condition.

v. To characterise the immune cell populations, including myeloid cells (innate immune responses) and T-lymphocytes (adaptive immune responses) in 4T1 tumour bearing BALB/c mice under non-irradiated and irradiated condition.

vi. To study the populations of effector T-cells and long-term immunity in IYIY-I2-BODIPY, YIYI-I2-BODIPY and I2-BODIPY treated 4T1 tumour bearing BALB/c mice post photodynamic therapy.
2.1 Active Targeting in Cancer

Chemotherapy of cancer destroys tumour tissues, or at least restricts its growth and metastatic spread. In general, chemotherapy drugs are either small molecule inhibitors with low molecular weight that interfere the up-regulated biochemical pathway in cancer cells or monoclonal antibodies that bind to cellular surface proteins (mechanistic or direct targeted therapy). These therapeutic agents tend to diffuse into all tumour and healthy tissues with low selectivity. In fact in drug discovery, many potent cytotoxic compounds fail as medicines due to their poor tissues selectivity and induced intolerable side-effects. Targeted delivery of therapeutic agent can overcomes the limitations of conventional cancer chemotherapy due to their (i) ability to identify the location of the primary and metastasis tumour, and (ii) selectively accumulate and kill the cancer cells than normal cells.

There are generally two types of drug targeting to deliver therapeutic cargoes to tumour sites (Torchilin, 2010). Passive targeting uses nano-carriers to ferry the drugs to tumour tissues via selective extravasation from the leaky tumour vasculature, an effect called enhanced permeability and retention. Active targeting links targeted moieties such as ligands and monoclonal antibodies (targeting agent) with cargoes (cytotoxic agent or imaging probes) to bind molecules selectively overexpressed on tumour cell-surfaces. The cargoes will then be internalised into tumour cells. Figure 2.1 outlines the passive targeting drug delivery and active targeting ligand-drug conjugate.
Figure 2.1: Passive and active targeting for cancer therapeutic.

Passive targeting relies on enhanced permeability and retention effect to deliver the drugs-encapsulated nano-structures or vesicles to tumour tissues via leaky tumour vasculatures. The acidic tumour microenvironment causes the drugs to be released in tumour cells. Active targeting generally using monoclonal antibody or receptor ligand as the targeting agent, conjugated with cargoes (e.g. cytotoxic agent, imaging agent, drug-encapsulated nanocarriers) to bind receptors that are overexpressed on tumour cells. Diagram modified from Salim et al., 2014.

Conjugation of receptor ligands either to the anticancer therapeutic agents for therapeutic purpose, or radiolabeled imaging probes for diagnostic purpose may increase the binding affinity of conjugates to the cancer cells’ surface receptor. Binding of the ligand to respective receptor enhances the endocytic internalisation by the cancer cells (Alexis et al., 2008; Byrne et al., 2008) and increases the tumour accumulation and residence time of the drug (Cheng et al., 2012; des Rieux et al., 2013; Koshkaryev et al., 2013).
2.1.1 Rationale of Active Targeting

There are two important aspects to consider when designing an active targeting ligand-drug conjugate, which are binding selectivity and sizes. Both aspects are reflected in the biodistribution of ligated conjugate (Bertrand et al., 2014). In order to have a high binding selectivity towards the targeted lesion, recognition of ligands by the targeted cellular biomolecules is important. Targeting ligands employed so far in the development of targetable ligand-drug conjugates include natural ligands such as hormones, vitamins, sugar derivatives, peptides, monoclonal antibodies as well as synthetic small molecules that possess high binding affinity to the targeted cellular substrate (Figure 2.2). The targeted substrates are proteins, sugars or lipids on overexpressed on cancer cells, which can reduce the binding on non-targeted tissues.
Figure 2.2: Common classes of ligands and cargoes used for active targeting in cancer.

The common targeting agents (red) used are vitamins (biotin, folate), hormones (estradiol, DHT, progestin), peptide ligands, glucose derivatives, and synthetic small molecule ligands. These targeting agents are known to have high binding affinity to their respective receptors. The common cargoes (purple) used for anti-cancer therapy are alkylating agents (chlorambucil, isosfamide), antibiotic (mitomycin-C, geldanamycin), antimetabolites (5-fluorouracil), apoptotic agents (Bim, Smac), DNA intercalating/damaging agents (histone deacetylase, cisplatin), mitotic inhibitors (paclitaxel, maytansinoids, desacetylvinblastine monohydrazone, tubulysin b hydrazide), photosensitisers (Boron-dipyrromethene, pheophorbide-a), protein kinase inhibitor (mitogen activated protein kinase kinase inhibitor) and topoisomerase inhibitor (indenoisoquinolines, camptothecin). For imaging of cancer using PET/SPECT/CT/Optical or fluorescence imaging system, the common agents (yellow) used are bromine-77, carbon-11, copper-64, fluorine-18, gallium-68, indium-111, iodine-123, technetium 99m, and near Infrared dye. Diagram obtained from Kue et al. 2016.

Another aspect to consider is the size of the ligand-drug conjugate. The pivotal issue is that the construct should preferentially accumulate in the tumour tissue. Size matters for the ease of penetration into tumours, and the ideal size is usually small (molecular weight < 5000) (Srinivasarao et al., 2015; Vlashi et al., 2013). For these reasons, small molecules can have significant advantages over antibodies due to the reason of pharmacokinetics (Vlashi et al., 2013). In general, high molecular weight active targeting agent has low tumour penetration, tumour retention and slow clearance compared to smaller targeting agent. The details comparison between high molecular weight protein (antibodies) and small peptides used in active targeting as delivering agent will be discussed in following section 2.1.2.
2.1.2 Advantages of Small Molecule in Active Targeting

Active targeting in cancer has been increasingly recognised as an effective strategy to elevate the therapeutic efficacy of anticancer drugs. Monoclonal antibodies (mAb) have been extensively studied as a potential targeting agent due to their specificity in antigen binding. To date, many mAbs have been used clinically to target therapeutic agents to tumour tissue (Scott et al., 2012) for treatment purposes, but they have serious limitations. Paramount amongst these is that mAb has low penetration into tumours (Cabral et al., 2011; Dreher et al., 2006; Jain & Stylianopoulos, 2010) due to its relative big size (> 4 nm) to leave the blood vessels and efficiently diffuse into tumour tissue. Furthermore, mAbs that diffuse into tumour tissue tend to interact with the antigens that are located on the surface of the perivascular tumour cells. This prevents their permeation into the deeper tumour mass (Dennis et al., 2007) - a phenomenon known as “antigen barrier” (Adams et al., 2001; Rudnick et al., 2011; Saga et al., 1995).

In addition, the clearance of mAbs from the body is relatively slow, resulting in undesired exposure to normal tissues such as excretory organs (Baluk et al., 2003; di Tomaso et al., 2005; O'Connor, 2007). Moreover, the antibody tends to be taken up by macrophages via surface Fc receptor (Kamps & Scherphof, 1998), which leads to the low deposition of mAbs in the target lesion. mAbs can also trigger antibody-dependent complement-mediated cytotoxicity (Sapra & Allen, 2003), as well as immunogenicity (van Schouwenburg et al., 2010) to cause hypersensitivity (Carrasco-Triguero et al., 2013).

In comparison, small molecule targeting entities are not or less constrained by the factors outlined above for mAbs. For instance, fluorescence studies have shown that small molecules folate-rhodamine conjugate can saturate folate receptors on tumours
within five minutes of intravenous injection (Vlashi et al., 2009). The antigen-barrier can still perceptibly impact folate-small molecule conjugates, but it has a negligible effect at saturating doses (Vlashi et al., 2009). This implies that small molecule conjugates can be ideal for rapid accumulation in solid tumours, and for brisk clearance afterwards. They can be synthesised in large scale and tend to be cheaper than mAbs.

2.1.3 Parameters Determining the Efficacy of Active Targeting Ligand-Drug Conjugate

Practically, after ligand binds to its target receptor to form a ligand-receptor complex, the complex will be internalised via receptor mediated endocytosis, then passes through an acidified endosome for sorting. The receptor in endosome is either recycled back to surface or underwent enzymatically degradation upon lysosome fusion, depending on the cellular requirement. In fact, this ligand-targeted internalisation happens more rapidly compared to untargeted complexes (Bareford & Swaan, 2007) (Figure 2.3).
Figure 2.3: Conjugate uptake and receptor trafficking pathway.

Once the conjugate binds to receptor, the receptor–ligand complexes are internalised and fused with early endosome. An early endosome is formed, followed by budding off separation to form late endosomes with receptors and targeted ligands in separate units. The vesicle with receptors will return to the plasma membrane (recycling). The late endosome with targeting ligands will fuse with lysosome for subsequent degradation. Diagram obtained from Kue et al., 2016.

There are four factors known to regulate receptor internalisation and trafficking, which then determine the efficacy of active targeting agents for cancer therapy: (i) type and binding rate of ligand-drug conjugate to receptor, (ii) rate of receptor recycling, (iii) receptor occupancy and saturation, and (iv) rate of drug release from ligand-drug conjugate upon internalisation (Bandara et al., 2014; Paulos et al., 2004a).

The rate of receptor internalisation, trafficking and recycling to cell surface is important for the selection of optimal durations of ligand-drug conjugate infusion. The correlation between the rate of receptor internalisation and the receptor occupancy was investigated by Roettger and colleagues (Roettger et al., 1995). They found that high level of receptor occupancy may induce receptor internalisation and ended in lysosome for degradation. In contrast, low level of receptor occupancy may stimulate the trafficking of the internalised receptor to recycling endosomes and subsequently back to cell surface.

The type of linker used in ligand-drug conjugates for successful release of cargo in endosomes is another key factor determining the efficacy of ligand-drug conjugate. Studies have revealed that linkers with shorter cleavage half-lives or pH sensitive N-ethoxybenzylimidazole (NEBI) linker can influence the rate and efficiency of cargo
release in acidic endosomes (Cao & Yang, 2014; Yang et al., 2006). For instance, endosome cleavable pH sensitive acyl hydrazone bond (Leamon et al., 2006) and reducible disulfide bond (Vlahov et al., 2006; Yang et al., 2006) were used to design different folate-drug conjugates for comparison. Results revealed that reduction-mediated release of the cargo from the disulfide bond was highly efficient within endosomes of cancer cells, with cleavage half-life of 1 hour and complete cleavage within 6 hours (Vlahov et al., 2006; Yang et al., 2006). Conversely, pH sensitive acylhydrazone bond has a cleavage half-life of 5.5 hours under acidic endosome environment (pH 5.5) (Leamon et al., 2006).

2.1.4 Small Molecule Conjugates for Active Targeting in Cancer

Research on small molecules (synthetic or natural ligands) as active-drug targeting in cancer was started as early as 1990. These ligands have been designed to target either cytotoxic or fluorescence agents to up-regulated molecules on plasma membrane of cancer cells for therapeutic and diagnostic (imaging) purposes, respectively. The receptors reported so far include folate, biotin, estrogen, progesterone, glucose transport system, androgen and integrin receptors. Recently, number of new synthetic ligands has been developed to target drugs to new receptors, such as sigma-2, prostate-specific membrane antigen (PSMA), carbonic anhydrase IX, cholecystokinin and TrkC receptor. Table-2.1 summarises all the small molecule ligand-drug conjugates for cancer therapeutic that target different surface receptors arranged according to cancer type.

Table 2.2 summarises all the small molecule ligand-imaging probe conjugates for diagnostic imaging based on cancer type.
Table 2.1 Clinical and Preclinical Status of Active Targeting Agents in Cancer Therapeutics

<table>
<thead>
<tr>
<th>cancer type</th>
<th>small molecules conjugates</th>
<th>target</th>
<th>study stages</th>
<th>status</th>
<th>graft / tumour</th>
<th>Remarks</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>breast cancer</td>
<td>folate-tubulysin B hydrazide (EC1456)</td>
<td>FR</td>
<td>in vivo</td>
<td>preclinical</td>
<td>xenograft</td>
<td>100% cure in large size tumour (750 mm³) and syngeneic M109 murine lung carcinoma</td>
<td>(Reddy et al., 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>clinical</td>
<td>ongoing</td>
<td>advanced solid tumour</td>
<td>active, recruiting participants</td>
<td>NCT01999738</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>phase I/II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNGRC-doxorubicin</td>
<td>CD13</td>
<td>in vivo</td>
<td>preclinical</td>
<td>xenograft</td>
<td>MDA-MD-435</td>
<td>tumours were 0.2-0.25 of the size of control tumours, reduced metastasis, prolonged survival, less toxic</td>
<td>(Arap et al., 1998)</td>
</tr>
<tr>
<td>RDG-4C-doxorubicin</td>
<td>Integrin</td>
<td>in vivo</td>
<td>preclinical</td>
<td>xenograft</td>
<td>MDA-MD-435</td>
<td>compared to Dox-treated control, all mice outlived by &gt; 6 months, had smaller tumours, less lymph spreading and lung metastasis, had widespread tumour cell death, less toxic to liver and heart</td>
<td>(Arap et al., 1998)</td>
</tr>
<tr>
<td>RGD-paclitaxel</td>
<td>Integrin</td>
<td>in vivo</td>
<td>preclinical</td>
<td>xenograft</td>
<td>MDA-MD-435</td>
<td>showed integrin specific accumulation, highest tumour uptake at 2 hours post injection, best tumour/background ratio after 4 hours post injection, no efficacy data</td>
<td>(Chen et al., 2005)</td>
</tr>
<tr>
<td>Kidney Cancer</td>
<td>Monovalent AAZ-DM1</td>
<td>Calix</td>
<td>In Vivo</td>
<td>Preclinical</td>
<td>Xenograft</td>
<td>promote tumour shrinkage and delay tumour growth, 22X higher accumulation in tumour vs scrambled control</td>
<td>(Krall, Pretto, Decurtins, et al., 2014)</td>
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<tr>
<td>Divalent AAZ-DM1</td>
<td>Calix</td>
<td>In Vivo</td>
<td>Preclinical</td>
<td>Xenograft</td>
<td>SKRC52</td>
<td>75% tumour reduction vs initial size, 33% full remission up to 90 days</td>
<td>(Krall, Pretto, &amp; Neri, 2014)</td>
</tr>
<tr>
<td>EC17 + EC90 Vaccine + GPI0100 Adjuvant</td>
<td>FR</td>
<td>Clinical</td>
<td>Completed</td>
<td>-</td>
<td>Moderate antitumour activity, 4% PR, 54% SD, 43% PD after first cycle. 53.6% SD after three cycles.</td>
<td>(Amato et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>EC17 + EC90 + GP10100 + IL-2 + IFNα</td>
<td>FR</td>
<td>Clinical</td>
<td>Terminated</td>
<td>-</td>
<td>Moderate antitumour activity, 29% SD (123-340 days), 4% PR (71 days)</td>
<td>(Amato et al., 2014) NCT00485563</td>
<td></td>
</tr>
<tr>
<td>ST7456CL1</td>
<td>Integrin</td>
<td>In Vivo</td>
<td>Preclinical</td>
<td>Xenograft</td>
<td>A498 carcinoma</td>
<td>Inhibit tumour growth by 39%</td>
<td>(Alloatti et al., 2012)</td>
</tr>
<tr>
<td>CRL-L1-Tubulysin B Hydrazide</td>
<td>CCK2R</td>
<td>In Vivo</td>
<td>Preclinical</td>
<td>Xenograft</td>
<td>HEK293</td>
<td>Eliminate all tumour lesions and prolong survival in CCK2R positive tumour</td>
<td>(Wayua et al., 2015)</td>
</tr>
<tr>
<td>Substrate</td>
<td>Inhibitor</td>
<td>Inhibitor type</td>
<td>Treatment</td>
<td>Results</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SV119-Bim</td>
<td>Sigma-2</td>
<td>SV119</td>
<td>in vivo</td>
<td>preclinical syngeneic Panc02 significantly reduced tumour growth treated for 12 days (two days once) and approximately 50% of mice survive without clinical toxicities (Spitzer et al., 2011)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWIV-134</td>
<td>Sigma 2</td>
<td>SV119</td>
<td>in vivo</td>
<td>preclinical syngeneic KCM, xenograft CEPAC tumour regression within 7 days continuous treatment and regrowth when treatment discontinued (Spitzer et al., 2011)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glufosfamide</td>
<td>GLUT</td>
<td>Clinical</td>
<td>phase I/II</td>
<td>completed</td>
<td>5.8% PR, 32.4% SD (median OS 5.3 months, PFS 1.6 months) (Briasoulis et al., 2000; Briasoulis et al., 2003; Hashim et al., 2014) NCT0005053</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glufosfamide + gemcitabine as first line therapy</td>
<td>GLUT</td>
<td>Clinical</td>
<td>phase III</td>
<td>completed</td>
<td>31% SD in glufosfamide+gemcitabine, 19% SD in gemcitabine alone (Ciuleanu et al., 2009) NCT00099294</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glufosfamide + gemcitabine</td>
<td>GLUT</td>
<td>Clinical</td>
<td>phase I/II</td>
<td>completed</td>
<td>52.6% SD (70% SD for 4 months, 30% SD for 6 months) (Chiorean et al., 2008) NCT00102752</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1, continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Compound</th>
<th>GLUT</th>
<th>Phase</th>
<th>Status</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-small cell lung cancer</strong></td>
<td>Glufosfamide</td>
<td>GLUT</td>
<td>clinical phase II</td>
<td>completed</td>
<td>2.7% PR, 49% SD (median OS 5.8 months)</td>
</tr>
<tr>
<td></td>
<td>EC145 ± docetaxel</td>
<td>FR</td>
<td>clinical phase II</td>
<td>ongoing</td>
<td>active but yet recruiting participants</td>
</tr>
<tr>
<td><strong>Ovarian cancer</strong></td>
<td>SW-III-123</td>
<td>Sigma 2</td>
<td>in vivo</td>
<td>preclinical xenograft SKOV3</td>
<td>median OS 86.5 days, 10 days higher than control and ligand alone</td>
</tr>
<tr>
<td></td>
<td>Glufosfamide + platinum based chemotherapy</td>
<td>GLUT</td>
<td>clinical phase II</td>
<td>terminated</td>
<td>renal function limits the enrolment. No confirmed tumour responses reported</td>
</tr>
<tr>
<td></td>
<td>D-glucose- succinic acid-adriamycin (2DG-SUC-ADM)</td>
<td>GLUT-1</td>
<td>in vivo</td>
<td>preclinical xenograft SKOV3</td>
<td>high accumulation in tumour post 2 hours administration, detectable up to 48 hours in tumour, 68.8% tumour growth inhibited vs free drug 47.1%</td>
</tr>
<tr>
<td></td>
<td>EC145</td>
<td>FR</td>
<td>clinical phase II</td>
<td>completed</td>
<td>42% DCR (Median OS: 14.6 months for 100%FR, 9.6 months for 10-90%FR, 3 months for 0%FR expression).</td>
</tr>
<tr>
<td></td>
<td>Epofoleate BMS-753493</td>
<td>FR</td>
<td>clinical phase I/IIa</td>
<td>discontinued</td>
<td>solid tumours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>majority was ovarian cancer (n=16) studied with other solid tumours, best overall response is 19-23% SD, 50% PD</td>
</tr>
</tbody>
</table>

(Giaccone et al., 2004) NCT00005055
(Garg et al., 2014) NCT01577654
(Threshold Pharmaceuticals NCT00442598
(Cao et al., 2013) NCT00507741
(Morris et al., 2014) NCT00507741
(Peethambaram et al., 2015) NCT00550017
<table>
<thead>
<tr>
<th>Compound</th>
<th>Target(s)</th>
<th>Study Type</th>
<th>Study Details</th>
<th>Outcome</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-[c(RGDfK)₂]-DOXO-1, E-[c(RGDfK)₂]-DOXO-2</td>
<td>Integrin</td>
<td>in vivo</td>
<td>preclinical, NIH:OVCA R-3 within xenograft model</td>
<td>no or only moderate antitumour efficacy compared to doxorubicin</td>
<td>(Ryppa et al., 2008)</td>
</tr>
<tr>
<td>cyclo[DKP-t3-RGD]-paclitaxel</td>
<td>Integrin</td>
<td>in vivo</td>
<td>preclinical, xenograft IGROV-1/Pt1 within in vivo model</td>
<td>dose-related antitumour effect observed, better tumour volume inhibition than paclitaxel, no deaths or significant weight losses</td>
<td>(Colombo et al., 2012)</td>
</tr>
<tr>
<td>melanoma</td>
<td>GLUT</td>
<td>in vivo</td>
<td>preclinical, syngeneic B16F0 within xenograft model</td>
<td>80-90% inhibition in tumour sizes at 20 days post tumour inoculation, 57% inhibition in free drug</td>
<td>(Miot-Noirault et al., 2011)</td>
</tr>
<tr>
<td>glioblastoma</td>
<td>glufosfamide</td>
<td>GLUT</td>
<td>clinical phase II, discontinued, phase II, completed</td>
<td>no significant antitumour activity</td>
<td>(van den Bent et al., 2003)</td>
</tr>
<tr>
<td>head and neck cancer</td>
<td>glufosfamide</td>
<td>GLUT</td>
<td>clinical phase I, ex vivo biopsy sample for colony formation assay</td>
<td>31% of primary tumour specimen that resist to cisplatin was sensitive to glufosfamide.</td>
<td>(Dollner et al., 2004)</td>
</tr>
<tr>
<td>EC145</td>
<td>FR</td>
<td>clinical</td>
<td>phase I, completed</td>
<td>100% SD (95-211 days)</td>
<td>(Lorusso et al., 2012)</td>
</tr>
<tr>
<td>nasopharyngeal cancer</td>
<td>EC0489</td>
<td>FR</td>
<td>in vivo, preclinical, xenograft KB cell</td>
<td>100% cure at dosage of more than 2 µmol/kg, higher tolerability and clearance via urine</td>
<td>(Leamon et al., 2011)</td>
</tr>
</tbody>
</table>

**Table 2.1, continued**
<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Drug Description</th>
<th>GLUT Type</th>
<th>Clinical Phase</th>
<th>Preclinical Status</th>
<th>In Vivo Activity</th>
<th>Authors (Year)</th>
</tr>
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<tbody>
<tr>
<td>Colorectal</td>
<td>Fuorodeoxyglucose-chlorambucil (FDG-chlorambucil)</td>
<td>GLUT</td>
<td>Preclinical</td>
<td>Syngeneic</td>
<td>75-90% inhibition at 26 days post tumour inoculation, 66% inhibition in free drug</td>
<td>Miot-Noirault et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CT-26</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glufosfamide</td>
<td>GLUT</td>
<td>Clinical</td>
<td>Completed</td>
<td>Minor tumour shrinkage, 57% SD</td>
<td>Shimizu et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phase I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Folate-methyl-β-cyclodextrin (FA-M-β-CyD)</td>
<td>FR</td>
<td>Preclinical</td>
<td>Syngeneic</td>
<td>100% regression in tumour and survive up to 140 days at 5 mg/kg.</td>
<td>Onodera et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CT-26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>EC 1169 (PSMA inhibitor-tubulysin B hydrazide)</td>
<td>PSMA</td>
<td>Preclinical</td>
<td>Xenograft</td>
<td>71% tumour regression, 29% cure with disease free more than 90 days</td>
<td>Reddy et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LNCaP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC0225</td>
<td>FR</td>
<td>Clinical</td>
<td>Ongoing</td>
<td>Recruiting participants</td>
<td>NCT02202447</td>
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<td></td>
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<td>Phase I</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>11β-dichloro</td>
<td>AR</td>
<td>Preclinical</td>
<td>Xenograft</td>
<td>90% inhibition of tumour volume at 30 mg/kg (5 days daily for 7 consecutive weeks)</td>
<td>Marquis et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LNCaP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ST7456CL1</td>
<td>Integrin</td>
<td>Preclinical</td>
<td>Xenograft</td>
<td>Increased the life span by 34% and reduced metastasis by 64% compared with vehicle control</td>
<td>Alloatti et al., 2012</td>
</tr>
<tr>
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<td></td>
<td>PC3</td>
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Table 2.1, continued

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>Target</th>
<th>Mode</th>
<th>Phase</th>
<th>Preclinical Data</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>urinary bladder</td>
<td>EC0905</td>
<td>FR</td>
<td><em>in vivo</em></td>
<td>preclinical</td>
<td>canine invasive urothelial carcinoma</td>
<td>56% PR, 44% SD. Median OS is 115 days.</td>
</tr>
<tr>
<td>liver</td>
<td>RGD-4C-doxorubicin</td>
<td>Integrin</td>
<td><em>in vivo</em></td>
<td>preclinical</td>
<td>allograft MH134</td>
<td>suppressed tumour growth more than free dox, prominent tumour cell death, complete tumour cell necrosis in 40% of cases</td>
</tr>
</tbody>
</table>

TrkC, Tropomyosin receptor kinase-C; ER, Estrogen receptor; GLUT, Glucose transport system; FR, folate receptor; AR, androgen receptor; CaIX, carbonic anhydrase-9; PSMA, prostate specific membrane antigen

PR, partial remission; SD, stable disease; OS, overall survival; PFS, progression free survival; PD, progressive disease; DCR, complete remission + partial remission + stable disease

a conducted syngeneic S180 sarcoma model with 64% inhibition in tumour sizes compared to Adriamycin with 50% inhibition.
b other solid tumours are colorectal (n=12), lung (n=7), breast (n=6), endometrium (n=2), prostate (n=3), pancreas (n=2), kidney (n=2), one each for the uterine, anal, urothelial, head and neck, hepatocellular, melanoma, uterine leiomyosarcoma, gastric cancer, gastrointestinal stromal tumour, chondrosarcoma, testicular cancer, mesothelioma, thymoma, sinus cancer, and small bowel cancer.
c conducted for one patient for NSCLC (SD), thymic cancer (SD), gallbladder cancer (PR>5 months with fluorouracil, cisplatin, gemcitabine), gastric cancer (SD) and uterine corpus-endometrial cancer.
d conducted on colorectal (SD, 4. 6 months), breast (SD, 4 months), leiomyosarcoma (SD, 4 months) and mesothelioma (SD, 4 months).
Table 2.2 Small Molecule Conjugates for Cancer Imaging in the Clinic and in Preclinical Animal Models

<table>
<thead>
<tr>
<th>cancer type</th>
<th>small molecules conjugates</th>
<th>target</th>
<th>study stages</th>
<th>status</th>
<th>graft / tumours</th>
<th>Remarks</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>prostate cancer</td>
<td>$^{123}$I-MIP-1072 and $^{125}$I-MIP-1095</td>
<td>PSMA</td>
<td>clinical</td>
<td>completed</td>
<td>recurrent metastatic</td>
<td>localised in tumour lesion post 1-4 hours administration. $^{123}$I-MIP-1072 has 5-fold rapid clearance compared to $^{125}$I-MIP-1095</td>
<td>(Barrett et al., 2013) NCT00712829</td>
</tr>
<tr>
<td></td>
<td>$^{99}$mTc-MIP-1404 and $^{90}$mTc-MIP-1405</td>
<td>PSMA</td>
<td>clinical</td>
<td>phase I/II</td>
<td>metastatic</td>
<td>20% increase in accuracy and sensitivity compared to $^{123}$I-MIP-1072 and $^{125}$I-MIP-1095</td>
<td>(Babich et al., 2012) NCT01261754</td>
</tr>
<tr>
<td></td>
<td>EC0652 ($^{99}$mTc-DUPA)</td>
<td>PSMA</td>
<td>clinical</td>
<td>ongoing</td>
<td>advanced, metastatic</td>
<td>no reported toxicity and 7/9 showed high affinity localisation in cancer lesion</td>
<td>(Gardner et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>$^{18}$F-DCFBC</td>
<td>PSMA</td>
<td>clinical</td>
<td>ongoing</td>
<td>primary and metastatic</td>
<td>66% $^{18}$F-DCFBC PET patient was concordant with conventional imaging, and able to detect early bone metastasis.</td>
<td>(Cho et al., 2012) NCT01815515 NCT01417182 NCT01496157</td>
</tr>
<tr>
<td></td>
<td>$^{125}$I-RISAD-P</td>
<td>AR</td>
<td>in vivo</td>
<td>preclinical</td>
<td>TRAMP for prostate cancer</td>
<td>high uptake in tumour and proportional to time and sizes. Studied for radiotherapy and showed delayed tumour growth by sizes.</td>
<td>(Kortylewicz et al., 2015)</td>
</tr>
<tr>
<td>Breast</td>
<td><strong>18F-ISO1</strong></td>
<td>Sigma-2</td>
<td>clinical phase I</td>
<td>ongoing</td>
<td>primary cancer</td>
<td>tumour cell proliferation status significant correlated with control proliferation marker (Ki-67). MTD of 550 MBq.</td>
<td>NCT02284919</td>
</tr>
<tr>
<td>--------</td>
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<td>--------------------------------------------------------------------------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td><strong>18F-3f and 123I-3f</strong></td>
<td>Sigma-2</td>
<td><em>in vivo</em></td>
<td>preclinical</td>
<td>syngeneic clone 66 breast tumour</td>
<td>high tumour to normal tissue ratios, rapid clearance from non-targeted organs (5-120 minutes)</td>
<td>(Tu et al., 2010)</td>
</tr>
<tr>
<td></td>
<td><strong>18F-fluoroestradiol</strong></td>
<td>ER</td>
<td>clinical phase I/II</td>
<td>ongoing</td>
<td>metastatic breast and desmoid tumour</td>
<td>recruiting patients</td>
<td>NCT02374931 NCT01957332</td>
</tr>
<tr>
<td></td>
<td><strong>(18F-FES)</strong></td>
<td>ER</td>
<td>clinical phase II</td>
<td>ongoing</td>
<td>primary and metastatic</td>
<td>3-fold higher tumour to background ratio compared to 18F-FES</td>
<td>(Turcotte et al., 2012)</td>
</tr>
<tr>
<td></td>
<td><strong>4FMFES</strong></td>
<td>ER</td>
<td>clinical phase I</td>
<td>discontinued</td>
<td>primary</td>
<td>low affinity and poor localisation.</td>
<td>(Jonson et al., 1999)</td>
</tr>
<tr>
<td></td>
<td><strong>β-18F-FMOX</strong></td>
<td>ER</td>
<td>clinical phase I</td>
<td>discontinued</td>
<td>primary</td>
<td>50% uptake in breast and was not receptor-mediated.</td>
<td>(Dehdashti et al., 1991)</td>
</tr>
<tr>
<td></td>
<td><strong>18F-FENP</strong></td>
<td>PgR</td>
<td>clinical phase I</td>
<td>discontinued</td>
<td>primary</td>
<td>significant tumour to normal breast, muscle and blood ratio, insignificant in PgR+ and PgR- uptake.</td>
<td>NCT00968409 (Dehdashti et al., 2012)</td>
</tr>
<tr>
<td></td>
<td><strong>18F-FFNP</strong></td>
<td>PgR</td>
<td>clinical phase I</td>
<td>completed</td>
<td>carcinoma</td>
<td>all 36 malignant breast tissue uptake, but not in normal breast tissue.</td>
<td>(Kue et al., 2015)</td>
</tr>
<tr>
<td></td>
<td><strong>IYIY-BODIPY</strong></td>
<td>TrkC</td>
<td><em>in vitro</em> staining</td>
<td>preclinical</td>
<td>human tissue microarray BRC962</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian</td>
<td>Isotope</td>
<td>Ligand/Target</td>
<td>Clinical Phase</td>
<td>Status</td>
<td>Notes</td>
<td></td>
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<td>------------------</td>
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<td>----------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>111\textsuperscript{In}-DTPA-folate</td>
<td>FR</td>
<td>Integrin</td>
<td>clinical phase I/II</td>
<td>completed</td>
<td>primary, recurrent, 100% sensitivity and concordant with conventional imaging for primary ovarian cancer; less effective in recurrent endometrium cancer.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99m\textsuperscript{Tc}-EC20</td>
<td>FR</td>
<td>Integrin</td>
<td>clinical phase II</td>
<td>completed</td>
<td>recurrent, 87% sensitive in FR (10-90%) expression.</td>
<td></td>
<td></td>
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<tr>
<td>18\textsuperscript{F}-FPTP</td>
<td>PgR</td>
<td>Integrin</td>
<td>in vivo</td>
<td>preclinical</td>
<td>uterus and ovary imaging, high uterus and ovarian uptake at 1 hour and 3 hours post injection.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>111\textsuperscript{In}-DOTA-E-[c(RDGfK)]\textsubscript{2}</td>
<td>FR</td>
<td>Integrin</td>
<td>in vivo</td>
<td>preclinical xenograft NIH:OVCAR-3</td>
<td>tumour uptake peaked at 6-7.5% dose/g at 1-2 hours post injection, rapid renal excretion, considerable uptake in liver and spleen, receptor binding demonstrated, tumour growth delay observed</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Kidney**

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Ligand/Target</th>
<th>Clinical Phase</th>
<th>Status</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>99m\textsuperscript{Tc}-EC20</td>
<td>FR</td>
<td>CCK2R</td>
<td>clinical phase I</td>
<td>completed</td>
</tr>
<tr>
<td>CRL-LS288</td>
<td>CCK2R</td>
<td>Integrin</td>
<td>in vivo</td>
<td>preclinical xenograft CCR2R transfected HEK293 cells</td>
</tr>
<tr>
<td>Tumour Type</td>
<td>Tracer</td>
<td>CD13</td>
<td>Status</td>
<td>Tumour Model</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------</td>
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<td>----------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Non-small cell lung cancer</td>
<td>$^{68}$Ga-NOTA-NGR</td>
<td>CD13</td>
<td>in vivo</td>
<td>preclinical</td>
</tr>
<tr>
<td>Liver</td>
<td>$^{99m}$Tc-EC20</td>
<td>FR</td>
<td>clinical</td>
<td>ongoing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>phase II</td>
<td>unknown</td>
</tr>
<tr>
<td>Liver</td>
<td>$^{68}$Ga-DOTA-NGR</td>
<td>CD13</td>
<td>in vivo</td>
<td>preclinical</td>
</tr>
<tr>
<td>Liver fibrosarcoma</td>
<td>$^{99m}$Tc-NGR</td>
<td>CD13</td>
<td>in vivo</td>
<td>preclinical</td>
</tr>
<tr>
<td>Liver fibrosarcoma</td>
<td>$^{64}$Cu-DOTA-NGR1, $^{64}$Cu-DOTA-NGR2</td>
<td>CD13</td>
<td>in vivo</td>
<td>preclinical</td>
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Table 2.2, continued

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<tr>
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<th>CD13</th>
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</thead>
<tbody>
<tr>
<td><strong>68</strong>G1-NOTA-G3-NGR2</td>
<td><strong>in</strong></td>
<td></td>
<td></td>
<td>preclinical xenograft HT-</td>
<td>excreted mainly and rapidly through kidneys, higher tumour</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1080</td>
<td>uptake and lower accumulation in vital organs, tumour uptake</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>blocked by unlabelled conjugates</td>
</tr>
<tr>
<td><strong>99m</strong>TcO-N3S-PEG2-Probestin</td>
<td><strong>in</strong></td>
<td></td>
<td></td>
<td>preclinical xenograft HT-</td>
<td>visible tumour uptake at 1 hour post-injection which was blocked</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>1080</td>
<td>by nonradioactive ReO-N3S-PEG2-Probestin</td>
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<tr>
<td><strong>Brain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>highest tumour-to-normal tissue contrast 24-48 hours post injection</td>
</tr>
<tr>
<td><strong>bivalent-IA-Cy5.5</strong></td>
<td><strong>Integrin</strong></td>
<td><strong>in</strong></td>
<td></td>
<td></td>
<td>(Li et al., 2010)</td>
</tr>
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<td></td>
<td></td>
<td>(Pathuri et al., 2012)</td>
</tr>
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<td></td>
<td></td>
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<td>(Shao et al., 2014)</td>
</tr>
</tbody>
</table>

* includes head and neck cancer (n=10) and lymphoma (n=7).

† conducted on benign ovarian tumours (n=8), ovarian carcinomas (n=7), breast carcinomas (n=6), and pituitary adenomas (n=6). 1 patient each for small cell lung carcinoma, lung carcinoma (type unspecified), colon, endometrial, thyroid carcinomas, non-Hodgkin’s lymphoma, sarcoma, and glioma.

18F-DCFBC, N-[N-[S]-1,3-dicarboxypropyl[carbamoyl]-4-[18F]fluorobenzyl-L-cysteine; 18F-3f, N-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-2-(2-[[18F]-fluoroethoxy)-5-iodo-3-methoxybenzamide; 4FMFES, 4-fluoro-11β-methoxy-16α-[18F]-fluoroestradiol; β-18F-FMOX, 17α-ethynyl-11β-methoxy-16β-[18F]-fluoroestradiol; 18F-FENP, 21-[18F]-fluoro-16α-ethyl-19-norprogesterone; 18F-FFNP, 21-Fluoro-16α,17α-[((R)-(1'-α-furylmethylidene)dioxy]-19-norpregn-4-ene-3,2O-dione; 18F-FPTP, 18F-fluoropropyl tanaproganet.
2.1.5 Other Impacts of Active Targeting

Active targeting tends to have some limitations because most of the targeting agents used to date are natural ligands and some of the targeted molecules are expressed on normal healthy cells, albeit at lower levels than in cancer cells. Other impacts of active targeting may include immune-modulation and non-targeted toxicity, which are mediated by the ligands.

2.1.5.1 Immune-modulation

Some ligands used for active tumour targeting were found to possess some immunomodulation properties. For instance, folate, which was classically used as a targeting ligand against folate receptor expressing tumours, was found capable of promoting the survival of the activated monocytes and lymphocytes that expressed folate receptors in rheumatoid arthritis (Nakashima-Matsushita et al., 1999; Paulos et al., 2004b). Estradiol on the other hand suppresses inflammatory cytokines such as tumour necrosis factor-α and interferon-γ (Matejuk et al., 2001) while promoting anti-inflammatory mediators such as interleukin-10 cytokine secretion and enhancing regulatory T cells function via up-regulation of programmed death-1 and FoxP3 expressions (Wang et al., 2009). Such immunomodulation properties may either assist the development or exacerbate the antitumour immunity in the host when these ligands are used as the targeting counterpart in an active-targeting ligand-drug conjugate (Amato et al., 2014; Siebels et al., 2011). Figure 2.4A shows some possible impacts induced by active targeting ligand on immune cells.
2.1.5.2 Non-targeted Toxicity

A certain receptors or cell surface proteins that are overexpressed on the tumour cells may be found expressed at a high level in some of the normal body tissue. For example, folate receptors that are often overexpressed in breast cancers may be abundantly found in kidney tissue. Hence, administration of ligand-drug conjugates that targets cell surface proteins may lead to collateral accumulation of the conjugates in the normal tissue, and thus induces toxicity (Fisher et al., 2008). Figure 2.4B shows the possible toxicity induced by active targeting agent on non-targeted tissues.

![Figure 2.4: Other targeting of ligand conjugates.](image)

**A. Immunomodulation** is one of the possible effects of active targeting. Targeted ligands generally possess similar abilities as the natural ligands and can transduce signals upon binding to receptors that are expressed on normal cells, for *e.g.* immune cells. The ligands are known to be able to modulate immune responses in two different ways, (I) direct binding on monocytes to promote or inhibit the release of cytokines, which can then alter the cytokine milieu systemically, thereby affecting the adaptive immune cell (T lymphocytes) differentiation, and (II) binding of ligand conjugates to
different subtypes of T-lymphocytes to regulate their survival and functions. In both ways, the effect may be either pro-tumour or anti-tumour depending on the ligand and receptor involved. In addition, the therapeutic cargo carried by the ligand conjugate may also modulate the immune response, for *e.g.* cyclophosphamide (an anticancer drug with immune modulation properties such as reduces T-suppressor cells). **B. Non-target tissue toxicity** is another possible effect of active targeting. Some receptors that are overexpressed in cancer cells are also highly expressed in the normal cells in healthy organs. Therefore, while killing cancer cells with overexpressed targeted molecules, mild or acute toxicity to non-targeted tissues might also happen in active targeted therapy.

2.2 Tropomyosin Receptor Kinase (Trk)

2.2.1 Receptor Biochemistry

Tropomyosin kinase (Trk) or neurotrophin receptors consist of three subtypes, namely TrkA, TrkB and TrkC. These receptors bind to their respective ligand neurotrophins, *i.e.* TrkA binds to neurotrophin growth factor (NGF), TrkB binds to brain-derived neurotrophin factor (BDNF) and neurotrophin-4 (NT-4), and TrkC binds to neurotrophin-3 (NT-3). Trk is transmembrane receptors containing an intracellular domain and an extracellular domain. Extracellular domain of Trk receptors include a combination of two cysteine clusters, a tandem array of leucine-rich motifs, and two immunoglobulin-like domain in the membrane proximal region (Schneider & Schweiger, 1991) (Figure 2.5). Specificity and affinity to neurotrophin ligands is dictated by the second immunoglobulin like domain (residues 266-381) of the receptor (Urfer et al., 1995), whereas the leucine rich motif serves as a binding site for neurotrophins (Windisch et al., 1995).
Figure 2.5: Schematic picture of tropomyosin receptor kinase.

Diagram obtained and modified from Marchetti et al., 2015.

Trk receptors are mainly found in neuronal populations, where TrkA is found in basal forebrain cholinergic, dorsal root ganglion and sympathetic neurons, whereas TrkB and TrkC are found predominantly in the central and peripheral nervous system (Longo & Massa, 2013). These receptors are known to promote neuron cell survival and differentiation upon neurotrophins binding (Huang & Reichardt, 2003). Other roles of Trk receptors and neurotrophins are in tumourogenesis (section 2.2.2) and immune response (section 2.2.3).

2.2.2 Trk Receptors and Tumourigenesis

Trk receptor was first identified as a product of colon derived oncogene. This oncogene (genomic DNA rearrangement or mutation) was generated from the fusion of
a tropomyosin gene with a tyrosine kinase-related locus (Trk proto-oncogene) (Martin-Zanca et al., 1986). Proto-oncogene of Trk was also reported to play a role in regulating invasiveness of cancer (Vaishnavi et al., 2015) in neuronal cancer such as neuroblastoma (Brodeur et al., 1997; Yamashiro et al., 1997) and medulloblastoma as well as non-neuronal melanoma (Xu et al., 2003), breast (Blasco-Gutierrez et al., 2007; Jin et al., 2010) and pancreatic (Sakamoto et al., 2001) cancer. TrkA-C receptors exist with their respective ligands to regulate the differentiation and survival of tumour cells, and conferring potency to invasion and metastasis. Among the Trk receptors, TrkC is the one that showed a high correlation with various cancer growth and became a biomarker for prognosis of tumour progression and invasion (Vaishnavi et al., 2015) in neuroblastoma (Brodeur et al., 1997; Yamashiro et al., 1997), glioblastoma (Kumar & de Vellis, 1996; Wang et al., 1998), thyroid cancer (McGregor et al., 1999), melanoma (Xu et al., 2003) and breast cancer (Blasco-Gutierrez et al., 2007; Jin et al., 2010).

Trk receptors mediated tumourigenesis depends on tumour types. For instance, in neuroblastoma, TrkA and NGR promote apoptosis of neuroblastoma cells, and hence aggressive behavior of neuroblastoma were found to have low TrkA expression, thus reduced TrkA expression was said to be a good prognosis for neuroblastoma. Conversely, neuroblastoma expresses high TrkB and BDNF to increase their survival (Chou et al., 2000). Thus, suggested that Trk receptors expression is depends on tumour types and metastasis level.

### 2.2.3 Trk Receptors, Neurotrophins and Immune System

Trk receptor expression is not only restricted to neuronal cell, but also reported to be expressed in immune cells such as monocytes, mast cells and lymphocytes, but at lower
levels compared to neurons (Vega et al., 2003). In the light of this, neurotrophins and Trk receptor may play a role in the regulation of inflammation and the development of antitumour immunity. Previously, neurotrophins have been reported to regulate hypersensitivity reaction by up-regulating the release of hypersensitive-associated mediators such as histamine and IL-5 from immune cells upon their binding to the Trk receptors (Bischoff & Dahinden, 1992). Neurotrophins also studied to stimulate T helper 2 (Th2) cell differentiations, and release the pro-inflammatory cytokine IL-6 from stromal cells (Rezaee et al., 2010; Sekimoto et al., 2003; Vega et al., 2003) which affects the systemic T-lymphocyte subtypes populations. In detail, a study reported that Th2 subtype expressed higher level of TrkC than other T-lymphocyte subtypes such as Th1. This suggests the possible role of neurotrophins in regulating the survival and function of Th2 cells, especially during the allergic and hypersensitivity responses. Moreover, transforming growth factor (TGF)-β cytokine is also regulated by TrkC; activated TrkC receptor will block the TGF-β receptor-mediated Smad2/3 phosphorylation and thus cease all the TGF-β production and its downstream signaling (Jin et al., 2007).

2.3 Photodynamic Therapy (PDT)

2.3.1 Background

Photodynamic therapy (PDT), a therapy using filtered light from a carbon-arc lamp was first reported by Danish physician, Niels Finsen in 1901 for the treatment of tubercular condition of skin (Lupus Vulgaris). In 1978, Dougherty and co-worker at Roswell Park cancer institute, Buffalo, New York, clinically tested photosensitiser hematoporphyrin derivative in treating cutaneous or subcutaneous malignant tumour,
and found 98% of tumours (21 of 24 patients) had complete or partial response (Dougherty et al., 1978).

Photofrin® (porfimer sodium), which is a hematoporphyrin derivative is the first FDA approved photosensitiser. To date, there are only a few other FDA approved anticancer photosensitising drugs including Foscan® (temoporfin, meta-tetrahydroxyphenylchlorin; Biolitec AG), Visudyne® (verteporfin, benzoporphyrin derivative monoacid ring A; Novartis Pharmaceuticals), Levlulan® (5-aminolevulinic acid; DUSA Pharmaceuticals, Inc.), and Metvix® (methyl aminolevulinate; PhotoCure ASA) (Voon et al., 2014). PDT has become one of the clinical treatment options for early stages of cancer such as nasopharyngeal, gastroenterological, brain and gynecological cancer (Dolmans et al., 2003; Huang, 2005).

2.3.2 Components of PDT

PDT is non-invasive. It consists of components which are non-toxic when exist individually. The components of PDT include (i) photosensitiser (PS), a light sensitive drug that is administrated systemically or locally. After systemic distribution and accumulation in the tumour, the tumour lesion is then focally irradiated with (ii) light at a specific wavelengths to activate the PS, and in the presence of (iii) oxygen molecules to generate singlet oxygen species through the photochemical reaction. (Dolmans et al., 2003; Dougherty et al., 1998). Ideal photosensitisers are generally activated by red light (630 – 700 nm), corresponding to light penetration depth of 0.5 – 1.5 cm (Salva, 2002). Figure 2.6 illustrates the procedures of cancer PDT.
PDT involves two procedures. Photosensitiser is first injected either to tumour region or intravenously. At few hours post administration, the tumour region will be irradiated with an appropriate wavelength by a laser light to activate the photosensitiser that accumulated in tumour. The reactive oxygen species generated will causes selective destruction at irradiated region while healthy cells (non-irradiated region) remain untouched.

For instance, a dye called boron-dipyrromethene (BODIPY) has been used as a photosensitiser in PDT and as a fluorescent probe in molecular imaging (Yogo et al. 2005). Extensive research has been conducted on BODIPY due to its ideal characteristic as photosensitiser, including high absorption extinction coefficients, high quantum efficiencies of fluorescence, insensitive to environment, resistance to photobleaching (Gorman et al. 2004) and relatively high light-dark toxicity ratio (Wainwright et al. 1997; Yogo et al. 2005). Meanwhile, the modification of core BODIPY with the addition of heavy atoms, especially iodine at 4-pyrollic position has further enhanced
the singlet oxygen production, and exhibited potent photo-induced cytotoxicity with low IC\textsubscript{50} concentration compared to core BODIPY (Lim et al. 2010). The absorption maximum and excitation wavelength of I\textsubscript{2}-BODIPY are 534 nm, and emission at 552 nm in methanol (Voon et al. 2016; Kamkaew et al. 2012), which is not suitable to treat the deeper tissue. In addition, the free photosensitiser has poor selectivity at the targeted sites, which leads to low therapeutic efficacy. Recently, a study on the designs of the nanoparticles-coated I\textsubscript{2}-BODIPY for passive delivery to the tumour region was conducted, which is an approachable strategy to improve the limitations of the BODIPY. This strategy was significantly improved the accumulation of I\textsubscript{2}-BODIPY to the targeted regions, and thus enhanced antitumour efficacy compared to free I\textsubscript{2}-BODIPY (Voon et al. 2016). Another approach to target the photosensitiser to the tumour sites by conjugating with an antibody or receptor-ligand by active targeting.

2.3.3 Mechanisms of PDT

Upon irradiation, the photosensitiser at ground singlet state is activated to highly energised and stable triplet state, which enables it to interact with surrounding molecules for generation of various cytotoxic species (\(\text{^{1}O_2}\)). There are two types of photochemical reactions to generate cytotoxic species, as illustrated in Figure 2.7. Type I reaction involves electron transfer reactions between the excited PS with organic substrate molecules in the cellular microenvironment to form highly reactive free radicals, which subsequently interact with oxygen molecules to produce singlet oxygen such as superoxide, hydroxyl radicals and hydrogen peroxide that are cytotoxic (Foote, 1991).
In type II reaction, the triple state PS transfers its energy to react with oxygen molecules to form cytotoxic singlet oxygen. The singlet oxygen generated has a short lifetime (<0.04 μs) and a very short radius of action (0.02 μm), hence can limits the migration from the site of formation without affecting adjacent cells or tissues (Foote, 1991). Singlet oxygen induces oxidative damage of tumour by directly disrupting cell membrane as well as shutting down the blood supply in the tumour microenvironment (Juarranz et al., 2008). Detailed impacts of PDT will be discussed in section 3.3.4.

![Schematic illustration of mechanism involved in PS activation and PDT.](image)

**Figure 2.7: Schematic illustration of mechanism involved in PS activation and PDT.**

The PS initially absorbs a photon that excites it to the first excited singlet state and this can relax to the more long lived triplet state. This triplet PS can interact with molecular oxygen in two pathways, type I and type II, leading to the formation of reactive oxygen species (ROS) and singlet oxygen respectively. Diagram obtained and modified from Dai et al., 2012.

### 2.3.4 Biological Target of PDT

Tumour destruction by PDT involves three mechanisms. Activated photosensitiser will generates cytolytic singlet oxygen, which has function of killing the tumour cells,
shut off the tumour vasculature and induces tumour specific antitumour immune responses. Figure 2.8 illustrate the three biological targets of PDT.

Figure 2.8: Three mechanism of PDT mediated tumour destruction.

Singlet oxygen generated destructs tumour by inducing **necrosis and apoptosis** on tumour cell, **shutting down the blood vessel** supply in tumour microenvironment, and **activation of innate and adaptive immune responses** locally and systematically. Diagram modified from both Castano et al., 2006 and photoimmune.org (photoimmune therapies).

2.3.4.1 Direct Cytotoxicity

PDT promotes tumour cell death either via apoptosis or necrosis. The mode of cell death depends on the subcellular location of the photosensitiser, photosensitiser concentration and irradiation conditions (Dougherty et al., 1998). In general, a low light
dose is associated with apoptosis, while a higher light dose results in necrosis. Another crucial factor in determining PDT-mediated cytotoxicity is the subcellular localisation of photosensitiser, which is determined by the chemical properties, formulation, route of delivery, concentration of the photosensitiser, and microenvironment of the lesion as well as phenotype of target cells (Huang et al., 2008). Organelles such as endoplasmic reticulum, Golgi apparatus, lysosomes, mitochondria, nucleus, and cell membrane have been identified as the subcellular targets for photosensitisers. Among the organelles, mitochondria are the primary subcellular target for most of the photosensitisers (Morgan & Oseroff, 2001). Mitochondria cytochrome-c release, photodamage to Bcl-2 (pro-survival proteins), and phospholipase activation (Agarwal et al., 1993) are example of the apoptosis pathways that have been reported for PDT.

The localisation of photosensitiser in plasma membrane has been studied leads to cell necrosis upon the photosensitisation process (Du et al., 2003; Mroz et al., 2011). Upon irradiation, singlet oxygen species damage the plasma membrane proteins, lipids and even cytoplasmic proteins. This leads to loss of membrane integrity and intracellular ATP supplies. In addition, studies showed that activation of photosensitiser stimulates autophagic cell death (Kessel & Reiners, 2007). The autophagy process in PDT has dual functions, either in promoting survival by degrading or recycling the damaged organelles, or by inducing cell death (Scherz-Shouval & Elazar, 2007), depending on the severity of the damages mediated by generated singlet oxygen. In general, autophagy cell death happens in tumour cells that lack apoptotic proteins (caspases-3, Bax, Bac) (Buytaert et al., 2006). Therefore, autophagy can be an alternative cell death pathway in cells deficient in apoptotic proteins.
2.3.4.2 Vascular Effect

Tumour microenvironment obtains their nutrients and oxygen primarily via the blood capillaries, which are generated by the tumour itself through angiogenesis. Abnormal neovascularisation in tumour lesions not only leads to enhanced supply of nutrients for tumour growth, but also constitute an important point in cancer progression (Folkman, 2002). Microvascular destruction is an important mechanism for cancer PDT (Siemann et al., 2005). Microvascular shutdown in tumour microenvironment upon PDT leads to formation of hypoxic environment and nutrient deprivation, and ultimately tumour regression (Henderson & Fingar, 1987).

Factors that affect the microvascular damage in PDT are the type of photosensitiser used and the duration of drug-light interval (DLI), which refers to the time between drug administration and irradiation. Photosensitisers that are suitable for use in vascular targeting are photofrin (Allison et al., 2006), talaporfin sodium (Juzeniene, 2009), verteporfin (Houle & Strong, 2002). They have been studied to be rapidly circulated and accumulated in the vascular system after injection. PDT-mediated microvascular damage regulated by DLI is depends on the pharmacokinetic and biodistribution of photosensitisers in different tissues at different time points. In general, short DLI (0.5 h) has better vascular targeting compared to long DLI (> 1 h) (Chen et al., 2002).

2.3.4.3 Immune Responses

Unlike conventional chemotherapy and radiotherapy, which are known to be immune-suppressive (Penn & Starzl, 1973), PDT has been studied to stimulate antigen specific immune responses, which can work synergistically with photosensitisers in
mediating short-term tumour killing, as well as long-term tumour immunity (Pizova et al., 2012; Korbelik, 1996; Korbelik and Dougherty, 1999).

Preclinical studies have shown that PDT damages the tumour cells and causes the release of damage-associated molecular pattern (DAMP) molecules which mediate an inflammation (Korbelik, 2006). The release of DAMPs attracts the recruitment of inflammatory cells such as neutrophils, mast cells and monocytes to the site of irradiation after PDT. Inflammatory cytokines such as IL-1β, IL-2, IL-6, tumour necrosis factor (TNF)-α, IL-10 and granulocytic-colony stimulatory factor (G-CSF) are also reported to be actively secreted by immune cells and tumour stroma post-PDT, which may regulate the systemic and tumour microenvironment immune responses (Gollnick et al., 2003). In addition, PDT was reported to increase the release of tumour antigens, which are then taken up by antigen presenting cells (APC), leading to the presentation of tumour antigens via MHC class I protein complex to lymphocytes. This initiates the onset of adaptive immune responses such as stimulation of tumour antigen specific CD8+ T-cells with the help of helper CD4+ T-cells. The activated T-cells will undergo proliferation and differentiation into different T-cell subtypes based on the surrounding cytokine milieu (Abdel-Hady et al., 2001). The detail of cancer immunology will be discussed in section 2.4.

Recent research has led to the introduction of PDT-generated tumour lysate as vaccines, which is a highly favourable method to stimulate and maximise the host antitumour immune responses (Korbelik, 2011).
2.4 Cancer Immunology

The immune system plays a critical role in protecting the host from various diseases, including cancer, through immune surveillance. Section 2.4.1 to 2.4.2 outlines the activation mechanisms of the tumour antigen specific immune responses, and explains the role played by an immune system to restrict tumour growth.

2.4.1 Tumour Antigen in Triggering Immune System

The benign tumour may turn to malignancy when they developed mechanisms to escape the host’s immune surveillance. Despite the immunosuppressive chemotherapy, there are some cases where the anti-tumour immune response can be elicited via the drug-mediated cell killing that releases the tumour antigen (Casares et al., 2005). Antigen presenting cells (APCs) including dendritic cells, macrophages and B cells are responsible for processing the engulfed tumour antigens into short peptides and presenting them to CD8 T-cells via major-histocompatibility complex (MHC) class-I molecules. Self-antigen (tumour antigen) present their peptides in the form of MHC class I molecules, whereas non-self-antigen present peptides in the form of MHC class-II molecules to CD4 T helper cells.

Another factor that can activate the immune responses is tumour immunogenicity, defined as the ability of tumour to elicit adaptive immune responses in vivo. Tumour immunogenicity varies among cancer types and different individuals (Blankenstein et al., 2012). There are three possible ways for self-antigens to become tumour antigens (Finn, 2008): First is mutation that causes failure of cells to repair DNA damage, resulting in abnormal peptide generation and presentation on the surface of tumour cells. Secondly, mutated tumour cells overproduce proteins and presented them on cell surface at highly abnormal levels. Thirdly, abnormal protein post-translation processes
such as splicing, glycosylation, phosphorylation or lipidation might result in presentation of abnormal peptides on tumour cell surface. These tumour-specific antigens presented on the tumour cell surface are recognised by immune cells and can elicit adaptive immune responses. The responses are mostly mediated by T-cells, and are considered as antigen-specific responses.

### 2.4.2 Cancer Immunoediting

The cancer immunosurveillance or cancer immunoediting is a continual process during tumourigenesis where the immune system resists tumour development. This process is composed of three distinct phases: tumour elimination, tumour equilibrium and tumour escape (Figure 2.9).
Figure 2.9: Three processes (Elimination, Equilibrium and Escape) of immuno-editing in cancer.

**Elimination** involves tumour suppression by both innate and adaptive immune cells such as NK cells, macrophage, dendritic cells, CD4+ and CD8+ T helper and NK T-cells, with high levels of inflammatory and cytolytic molecules including IFN-γ, IL-12, TRAIL, NKG2D, and perforin. **Equilibrium** proceeded when tumour cells started to undergo genetic instability and mutation to become new variants that are poor immunogenics and resist to immune attack, at the same time immune cells still act on highly immunogenic tumour cells. **Escape** occurs when poor immunogenic and immunosuppressive transformed new variant tumour cells become dominant. Tumour evades from immunosurveillance by expressing PD-L1, secretes immunosuppressive mediators including IDO, TGF-b and IL-10 to encourage recruitment and differentiation of immunosuppressive Treg cells to attenuate the effector T-cells function. Diagram modified from Vesely et al., 2011.

2.4.2.1 Tumour Elimination

Tumour elimination phase is an example of immunosurveillance, which involves the cytokines and cells of both innate and adaptive immunity to destroy the tumour cells before it become clinically apparent (Vesely et al., 2011). When the tumour cells grow invasively, they start to produce stromagenic and angiogenic proteins such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) to enhance the blood nutrient supply to their microenvironment (Hanahan & Folkman, 1996). The growth of the tumour cells cause the disruption of the tumour surrounding tissues and induce inflammatory signaling to recruit the infiltration of immune cells into tumour microenvironment. The major effector immune cells that infiltrate into tumour microenvironment are natural killer cells (NK), dendritic cells (DC), macrophages, neutrophils, eosinophils, basophils, mast cells, and cytotoxic T-lymphocytes. These immune cells will be stimulated to produce inflammatory cytokines including interferon-γ (IFN-γ), interleukin (IL)-12 and tumour necrosis factor (TNF) (Dranoff,
2004). The IFN-\(\gamma\) was said to be the pivotal cytokine in tumour elimination, as it can induce CXCL-9, CXCL-10 and CXCL-11 chemokines production to attract more T-lymphocytes infiltrate into the tumour site and generate antiangiogenic activity (Angiolillo et al., 1995; Coughlin et al., 1998). All these factors will kill the tumour cells by mechanisms including tumour necrosis factor-related apoptosis inducing ligands (TRAIL), perforin, reactive oxygen and nitrogen intermediates (Ikeda et al., 2002; Takeda et al., 2002; Trinchieri, 1995).

The process continues when dead tumour cells were engulfed by APCs, processed and presented to naive T-lymphocytes in lymph nodes to prime more tumour-specific CD4+ and CD8+ T-lymphocytes. Dendritic cell is APC that bridge between innate and adaptive immune response, and may stimulate different CD4+ T-lymphocytes mediated inflammatory responses such as T helper (Th) 1, Th2, Th17 and regulatory T-cells (Treg). In general, the tumour antigen primed CD4+ T-lymphocytes will differentiate to IFN-\(\gamma\) expressing Th1 to help the development and enhance the function of tumour-specific CD8+ cytotoxic T lymphocytes (CTL). CTLs kill the remaining tumour cells by secreting the cytolytic mediator perforin (Russell & Ley, 2002), and the surrounding IFN-\(\gamma\) increases the sensitivity of tumour cells to CTLs-secreted perforin. Studies have proved that IFN-\(\gamma\) and perforin are the two essential mediators for tumour elimination. IFN-\(\gamma\) and perforin knockout mice respectively have significant incidence of lung adenocarcinoma and lymphoma development compared to wild-type mice (Street et al., 2002), and less effective in regulating the initiation, growth and metastasis of solid tumour (Street et al., 2001; van den Broek et al., 1996). Other immune regulatory cytokines including IL-12, IL-18 and TNF are the other important cytokines in eliminating tumour (Street et al., 2002).
2.4.2.2 Tumour equilibrium

Those tumour cells that have survived the elimination process will enter into an equilibrium process. The equilibrium state is originally from study of transplantation of certain tumour cell lines into pre-immunised mice, where the immunity restrained the outgrowth of inoculated tumour cells (Weinhold et al., 1977). In this state, the tumour cells are considered “dormant”, as the elimination process continues and some of the tumour cells undergo mutation and transformation to form a new variant with resistance to immune attack (immune-escape). Some tumour cells in equilibrium are immunogenic and susceptible to be cleared by immune system, whereas those that have undergone transformation have attenuated immunogenicity and can escape immune surveillance.

Converse to the elimination process that requires both innate and adaptive immunity, the equilibrium process depends fully on adaptive immunity, which is the T-lymphocytes (Koebel et al., 2007). Koebel and colleagues reported that administration of anti-CD4, anti-CD8, anti-IFN-γ and anti-IL-12 (cytokine critical for IFN-γ production) promoted sarcoma outgrowth in mice which were in the equilibrium state. The equilibrium state was proposed because the mice injected with a single low dose of chemical carcinogen 3’-methylcholanthrene (MCA) displayed small stable masses at the site of MCA injection even up to 200-230 days post MCA inoculation. Under similar experiment condition, tumour outgrowth was not observed in mice that deplete innate immunity including NK cell recognition (NKG2D) or tumour necrosis factor-related apoptosis inducing ligands (TRAIL). In contrast, the mice with Rag2 knockout gene (gene that regulates adaptive T- and B-lymphocytes development, but possess innate immunity) had progressive tumour outgrowth compared to wild-type in equilibrium state.
Equilibrium state has the longest time periods among the three states where immunity controls the cancer growth. The cancer in equilibrium state can become functionally dormant and clinically unapparent to host (Vesely et al., 2011). Hence, cancer immunotherapy can be applied in this state to enhance the adaptive immunity to further improve tumour control.

2.4.2.3 Tumour escape

The new tumour variants developed during equilibrium state are less immunogenic, and thus can survive and enter the escape state. Due to their insensitivity to immunesurveillance, the new variant tumour cells begin to expand and growth in an uncontrollable manner and become malignant (Dunn et al., 2002). In addition, tumour evades from immunesurveillance by suppressing both the local and systemic immunities via the active production of immunosuppressive molecules such as transforming growth factor (TGF)-β, IL-10, soluble Fas ligand to induce CTL apoptosis, immunosuppressive enzyme indolamine-2,3-dioxygenase and programmed death-ligand 1 (PD-L1).

Studies have reported that tumour cells produce high level of TGF-β and tumour microenvironment has high population of immunosuppressive regulatory T-cells (Treg) compared to effector T-cells. The Treg cells actively produce immunosuppressive cytokines TGF-β and IL-10 to suppress the effector immune cells in both systemic and tumour microenvironment. Moreover, induced-Tregs (iTregs) are produced alongside increase in TGF-β, which is the main cytokine in priming naive or effector CD4+ T to Treg cells (Liu et al., 2007). Thus, tumour evades from immune-mediated destruction and become malignant. This was reported in many cancer types including lung, ovarian (Woo et al., 2001), head and neck (Chikamatsu et al., 2007), melanoma (Cesana et al.,
Indolamine-2,3-dioxygenase (IDO) is an enzyme responsible for tryptophan catabolism amino acid through the kynurenine pathway. It is an immunosuppressive enzyme as it induces tryptophan catabolism. This suppresses T-lymphocytes, NK cells and B-lymphocytes proliferation by reducing the supply of essential tryptophan (Frumento et al., 2002; Moffett & Namboodiri, 2003). Also, the tryptophan metabolite kynurenine and 3-hydroxyanthranilic acid (3HAA) are toxic to activated immune cells (Fallarino et al., 2003). In addition, IDO can trigger the generation of iTreg from naive T-cells via plasmacytoid dendritic cells (PDCs) which express high levels of IDO (Chen et al., 2008). Clinical studies reported that IDO was highly expressed in 56.7% of surgically resected ovarian cancer tissues and its expression was correlated with a reduced number of CD8+ tumour infiltrated lymphocytes (Inaba et al., 2009). Moreover, ovarian cancer that were positive for IDO staining was found to be correspondence with the primary lesion and metastatic site, suggested the increase of IDO had impaired survival in ovarian cancer patient (Takao et al., 2007). Another study involving transfections of IDO vector into non-IDO expressing human ovarian cancer cell line OMC-1 revealed that it could promote tumour growth, inhibits NK cells in tumour microenvironment and induce angiogenesis (Nonaka et al., 2011).

Another molecule overexpressed in cancer cells is programmed death ligand-1 (PD-L1) (Maine et al., 2014). PD-L1 binds to PD1 receptors that are expressed on activated T-lymphocytes, B-lymphocytes and myeloid cells (Agata et al., 1996). PD1 down-regulates activated immune cells to reduce autoimmunity and promotes self-tolerance. Many cancer types including ovarian (Maine et al., 2014), kidney (Thompson et al.,
2004), breast (Ghebeh et al., 2006), melanoma (Hino et al., 2010), esophageal (Ohigashi et al., 2005) and pancreatic (Nomi et al., 2007) express high PD-L1 and this is associated with poor prognosis in patient. The high PD-L1 is associated with low tumour infiltrated lymphocytes into tumour microenvironment (Hamanishi et al., 2007).

Other than Treg, myeloid derived suppressor cell (MDSC) is another immunosuppressive cell that negatively regulates immune responses in cancer. MDSC is a heterogeneous population of activated myeloid cells characterised by a mixture of granulocytic and monocytic cells lacking the surface markers associated with fully differentiated monocytes, macrophage or dendritic cells (Youn et al., 2008). MDSC is highly expanded in cancer due to the active secretion of expansion factors such as IL-6 (Bunt et al., 2007), prostaglandin (Sinha et al., 2007), TGF-β (Terabe et al., 2003; Yang et al., 2008), macrophage-colony stimulating factor (Menetrier-Caux et al., 1998) and VEGF (Gabrilovich et al., 1998) by tumour cells. MDSC functions by disrupting the binding of specific MHC peptide in APC to CD8+ T-lymphocytes (Nagaraj et al., 2007). Preclinical (Donkor et al., 2009; Younos et al., 2012) and clinical (Diaz-Montero et al., 2009; Porembka et al., 2012; Wang et al., 2013) studies have revealed that the number of MDSC in systemic circulation is correlated with poor prognosis, tumour angiogenesis and tumour escape from immunity.
CHAPTER 3: TROPOMYOSIN RECEPTOR KINASE-C (TRKC) LIGAND CONJUGATED DIODO-BORON DIPYRROMETHENE ERADICATES TRKC EXPRESSING TUMOUR IN PHOTODYNAMIC THERAPY (PDT)

3.1 Introduction

Active targeting ligand-drug conjugate in cancer refers to the specific delivery of therapeutic drugs or imaging probes that are conjugated with the targeting ligand to the tumour cells. This therapeutic approach is different from mechanistic or direct targeting, that uses cytotoxic agent intended to target surface molecules or intracellular pathways that are up-regulated in cancer cells (Agarwal et al., 2008; Minko et al., 2004). To date, targeting ligand such as folic acid and prostate specific membrane antigen (PSMA) inhibitor to target folate receptor and PSMA, respectively (Hilgenbrink & Low, 2005; Low & Kularatne, 2009; Lu & Low Philip, 2012; Xia & Low, 2010) are probably the most widely appreciated examples. These PSMA and folate receptor conjugates have been brought to clinic for therapeutic and imaging of solid tumours (Srinivasaraao et al., 2015). However, there are no clinically approved small molecule active targeting agents for delivering therapeutics to breast cancer (Meng & Li, 2013).

Other than targeted cancer therapy using active targeting ligand-drug conjugate, photodynamic therapy (PDT) is considered as another selective anticancer therapy. PDT requires administration of photosensitiser (PS), follow by the illumination of tumour at specific wavelengths to activate the PS that had been accumulated in the tumour to generate cytolytic singlet oxygen species to mediate tumour cell killing (Dolmans et al., 2003; Dougherty et al., 1998). PDT has minimal invasiveness and consider specific due to the only targeted region being illuminated, which can reduce the non-selective toxicity to normal cells that uptake the PS. However, the low to moderate accumulation
and localised of PS in tumours to fully eradicate irradiated tumour region is the limitation of PDT.

![IY-monomer](image)

**Figure 3.1: Structure of synthetic peptide Isoleucine-tYrosine (IY) to bind TrkC receptor.**

Tropomyosin receptor kinase C (TrkC) is a cell surface receptor that is overexpressed in breast cancer and plays an essential role in breast cancer growth and metastasis (Ivanov et al., 2013; Stephens et al., 2005). Studies have shown that suppression of TrkC expression in highly metastatic mammary carcinoma cells inhibited their growth *in vitro* and their ability to metastasis from the mammary gland to the lung *in vivo* (Jin et al., 2010). The high expression of TrkC has made it a good biomarker for therapeutic purposes in cancer. A novel molecular fragment IY (Figure 3.1), of which consist of amino acid Isoleucine (I) and tYrosine (Y) has been designed as a TrkC targeting ligand (Brahimi et al., 2009; Chen et al., 2009; Liu et al., 2010). A preliminary study had shown that two fragments of IY (IYIY)-ligand gave good adherent to TrkC receptor, whereas one fragment IY-ligand induces weak functional effects (Chen et al., 2009).
Following the successful targeting ability of the IYIY-ligand to TrkC expressing cancer (Chen et al., 2009), the ligand had then conjugated with photosensitiser diiodoboron dipyrromethene (I₂-BODIPY) as a cargo (Figure 3.2). Boron dipyrromethene (BODIPY) and derivatives have excellent attributes for PDT with high extinction coefficients, favorable light-to-dark toxicity ratios, high antitumour efficacies in vivo, and good body clearance (Awuah & You, 2012; Byrne et al., 2009; Kamkaew et al., 2012; Lim et al., 2010; Yogo et al., 2005). The conjugate, named IYIY-I₂-BODIPY (Figure 3.3, upper panel) was used to investigate the binding selectivity, toxicity profile, biodistribution and in vivo efficacy on TrkC expressing murine 4T1 breast cancer cells in PDT. In this study, non-TrkC targeted scrambled control, named YIYI-I₂-BODIPY (Figure 3.3, lower panel) and free drug I₂-BODIPY were used as negative controls.

**Figure 3.2: Structure of photosensitiser diiodinated BODIPY used in this study.**

![I₂-BODIPY](image-url)
Figure 3.3: Structure of synthetic TrkC targeted conjugate (IYIY-I$_2$-BODIPY) and non-TrkC targeted conjugate as scrambled control (YIYI-I$_2$-BODIPY).

Red represent the TrkC targeted agent, blue is the linker and green is the photosensitiser I$_2$-BODIPY.
3.2 Materials and Methods

3.2.1 Compounds and Cell Lines

Compounds IYIY-I₂-BODIPY, YIYI-I₂-BODIPY and I₂-BODIPY were a gift from Prof. Dr. Kevin Burgess from Texas A&M University, USA. 4T1 murine mammary carcinoma cell line was purchased from American Type Culture Collection, ATCC (Manassas, VA, USA) and cultured in RPMI media with 10% FBS. 67NR murine mammary carcinoma cell line was purchased from Barbara Ann Karmanos Cancer Institute, Detroit, MI, USA; and cultured in DMEM high glucose medium with 10% FBS. All cells were maintained at 37°C in a 5% CO₂ incubator.

3.2.2 In vitro Photocytotoxic Assay

4T1 and 67NR cell lines were respectively cultured in 96 wells plate at density of 4000 cells per well for 24 hours. Compounds IYIY-I₂-BODIPY, YIYI-I₂-BODIPY and I₂-BODIPY were dissolved in DMSO and diluted to the range of 0.1-10 μM. The cell lines were incubated respectively with compounds at prepared concentrations for 2, 4 and 6 hours. At the end of incubation, all cells were washed twice with PBS. Fresh media was added to the culture wells and irradiated with a light dose of 7.3 J/cm² from a halogen light source at the fluence rate of 12.2 mW/cm² (Kamkaew & Burgess, 2013), and further incubated for 24 hours. Briefly, 20 μL of MTT solutions (5 mg/mL) were added to each well and incubated for 4 hours in 37°C. The solutions were removed and 100 μL of DMSO was added to dissolve the formazan crystal formed. Plate was read at absorbance of 570 nm using SpectraMax® M4 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The viability of each group of treatment in different cell lines was calculated as percentage of dead cells = 100 – (OD treated / OD untreated control) × 100.
3.2.3 Animal Model

Female 8- to 10-week old wildtype BALB/c mice were purchased from Monash University, Malaysia campus (Sunway, Selangor, Malaysia) for in vivo studies. The mice were maintained under AAALAC accredited satellite animal facility in Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. All animal experiments were performed according to protocol approved by the Faculty of Medicine Institutional Animal Care and Use Committee, University of Malaya (FOM IACUC). (Ethics Approval No. 2013-05-07/PHAR/KLV, appendix A).

3.2.4 In vivo Toxicity Study

The toxicity profiles of all compounds were determined. Mice (n=2) were injected intravenously with IYIY-I₂-BODIPY, YIYI-I₂-BODIPY and I₂-BODIPY at 20-100 mg/kg via tail vein. Toxicity was observed based on the Berlin test of typical symptoms such as apathy, horrent fur, behavior changes and a loss of body weight for two weeks.

3.2.5 Biodistribution Study

4T1 tumour bearing female BALB/c mice at the tumour volume of 80 mm³ were divided into 2 groups, and intravenously injected with 10 mg/kg of IYIY-I₂-BODIPY and YYI-I₂-BODIPY respectively. Mice (n=3) were then sacrificed at different time points (0, 0.25, 1, 3, 6, 24, 48 and 72 hours) and major organs such as liver, spleen, lung, kidney, draining lymph nodes, skin, eye and tumour tissues were isolated. Organs and tissues were imaged using an In Vivo MS FX PRO imager (Carestream Molecular Imaging, Woodbridge, CT, USA) with an excitation filter at 530 nm and an emission filter at 550 nm. Mice with saline treatment were used as control. Fluorescent intensity of each organs and tissues were quantified using Carestream Molecular Imaging.
software 5.0 (CT, USA). The time point that gives a high accumulation in tumour tissues and large differences with the scrambled control was chosen for PDT.

3.2.6 Tumour cells inoculation and PDT in mouse

The fur of the mouse was first shaved. Murine 4T1 and 67NR at density of $5 \times 10^5$ tumour cells in 0.1 mL of medium was then orthotopically injected into the mammary fat pad of the mice (18-20 g, 8-10 weeks old) respectively. The mice were then randomly divided to groups for PDT at 8 day post tumour inoculation when the tumour size reaches approximately 60-80 mm$^3$. Compounds (IYI-I$_2$-BODIPY, YIYI-I$_2$-BODIPY and I$_2$-BODIPY) at 2-10 mg/kg body weight were dissolved in cocktail of 2.5% ethanol and 2.5% Cremophore EL. The mixture was then further dissolved using saline to volume of 0.2 mL and administered by intravenous tail vein injection to the mice (n=7). Mice were then kept in dark for 1 hour. Anesthetic with 90 mg/kg of ketamine and 10 mg/kg of xylazine cocktail was then given to mice. Thereafter, PDT was performed using Lumacare LC-122A fibre optic light delivery system (Lumacare Medical Group, Newport Beach, CA, USA) emitting light at 530 nm. Glass slide with thickness of 4mm was used as a barrier to avoid direct photo-thermal effect on tumour. Illuminating spot was positioned at the tumour region and the surrounding was covered using black cloth to avoid PDT effect on other part of body. PDT was conducted at 100 J/cm$^2$ with the fluence rate of 160 mW/cm$^2$. Mice were kept in dark and tumour sizes were monitored 3 times per week. Tumour volume changes were determined by caliper measurements with tumour volume, mm$^3 = (L \times W^2 / 2)$, where L is the longest dimension and W is the shortest dimension. The experimental mice were euthanised when either tumour diameter reached the 20 mm ethical limit approved by IACUC Guidelines for Maximum Tumour Bearing in Laboratory Rodents, 2012, or at the end of the 90 day of monitoring period for survivor mice.
3.2.7 Histology Sample Preparation

Survivor mice were sacrificed at 90 days post therapy, and major organs such as liver, kidney, spleen, draining lymph node, lung, and heart were isolated for histological analysis. The isolated specimens were fixed with 10% formalin solution for a minimum of 48 hours at room temperature, following which, tissues were trimmed into representative segments and then dehydrated using an ascending series of alcohol, cleared in xylene, and embedded in paraffin. Microtomy was performed using a Leica RM2255 microtome (Leica Microsystems, Germany). Sections were cut at 5 μm thickness and placed onto appropriately labeled microscope slides. The slides were then stained with haematoxylin and eosin (H&E) and coverslipped, and then evaluated by a board certified veterinary pathologist at the Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, in Singapore.

3.2.8 Statistical Analysis

*In vitro* and *in vivo* experiments were performed to compare the efficacy of three compounds. Results were analysed using one-way ANOVA with Dunnett’s Multiple Comparisons when comparing between three groups of compounds using SPSS with \( p < 0.05 \) is considered as significant. Student t-test was used to analyse between two groups, with \( p < 0.05 \) considered as significant.

3.3 Results

3.3.1 *In vitro* photocytotoxicity of ligated conjugates and unconjugated I2-BODIPY

TrkC targeted dipeptidomimetic ligand (IYTY) has been studied to transduce signals in promoting neuronal cell survival and differentiation in a similar pattern as TrkC
natural ligand NT-3 (Chen et al., 2009). In addition, IYIY-I$_2$-BODIPY, which is a conjugate of photosensitiser Diiodo Boron Dipyrrromethene (I$_2$-BODIPY) with IYIY ligand, has been studied to bind to TrkC receptor and internalised in lysosomes in a similar way as NT-3. Moreover, the IYIY-I$_2$-BODIPY competes with NT-3 in binding to TrkC, as demonstrated by co-treatment of different NT-3 dosages leaded to the dose-dependent reduction in photocytotoxicity in IYIY-I$_2$-BODIPY treated TrkC gene-transfected NIH-3T3 cell (Kamkaew & Burgess, 2013). Hence, the objectives of this study are to investigate the 	extit{in vitro} photocytotoxicity and 	extit{in vivo} antitumour efficacies of IYIY-I$_2$-BODIPY in TrkC positive murine breast cancer cell line (4T1) and TrkC negative murine breast cancer cell line (67NR) (Jin et al 2010). YIYI-I$_2$-BODIPY and I$_2$-BODIPY were used as a scrambled sequence ligand compound and a non-ligated free compound controls, respectively.
Figure 3.4: Tested compounds were not toxic to cells in non-irradiated condition. (A) 4T1 and (B) 67NR cells were cultured in 96-well plates. After 24 hours, IYIY-I$_2$-BODIPY, YIYI-I$_2$-BODIPY and I$_2$-BODIPY with indicated concentrations were added to cells and incubated for 2 hours. The media was removed and the cells were then washed with PBS before new media was added. MTT was carried out 24 hours after further incubation. Data represent mean ± SEM of three independent experiments in PDT.

The conjugates and free drug photosensitiser were first tested for dark cytotoxicity. As shown in Figure 3.4, all compounds tested were not toxic to 4T1 and 67NR cells in non-irradiated condition. When exposed to light, IYIY-I$_2$-BODIPY induced significant photocytotoxicity in 4T1 cell line (IC$_{50}$ = 0.325 µM) in a dose-dependent manner compared to control YIYI-I$_2$-BODIPY and I$_2$-BODIPY (indeterminable IC$_{50}$ values) (Figure 3.5A). Importantly, IYIY-I$_2$-BODIPY, YIYI-I$_2$-BODIPY as well as I$_2$-BODIPY showed no photocytotoxic effect in TrkC negative cell line 67NR (Figure 3.5B). These results suggest that IYIY-I$_2$-BODIPY induces selective photocytotoxicity in TrkC expressing cells via TrkC targeting.
Figure 3.5: IYIY-I$_2$-BODIPY was photocytotoxic only to TrkC positive cell lines.

(A) 4T1 and (B) 67NR cells were cultured in 96-well plates. After 24 hours, IYIY-I$_2$-BODIPY, YIYI-I$_2$-BODIPY and I$_2$-BODIPY with indicated concentrations were added to cells and incubated for 2 hours. The media was removed and the cells were then washed with PBS before new media was added. Cells were then irradiated with 7.3 J/cm$^2$ of light at a fluence rate of 12.2 mW/cm$^2$ (10 minutes). MTT was carried out 24 hours after irradiation. Data represent mean ± SEM of three independent experiments in PDT. *, $p < 0.05$; **, $p \leq 0.01$ vs controls using one-way ANOVA (Dunnett’s test).
3.3.2 In vitro photocytotoxicity of ligated conjugates at different incubation time in TrkC positive and TrkC negative cancer cells.

All the photocytotoxicity experiments described above involved adding the test compounds for 2 hours incubation, followed by illumination. The effects of prolonging the incubation with the cells on the cell viability post-illumination were also studied. Interestingly, the data revealed that IYIY-I$_2$-BODIPY mediated selective cell death was achieved for Trk positive 4T1 compared to TrkC negative 67NR at 2 hours incubation (Figure 3.6A), and the difference becomes less noticeable when 4- and 6-hours incubation was used. This observation leads to the conclusion that a shorter incubation time is optimal for selective photo-killing by the TrkC seeking conjugate. For a full comparison, the same time course experiments for the untargeted YIYI-I$_2$-BODIPY (Figure 3.6B) and the parent I$_2$-BODIPY was conducted (Figure 3.6C). As expected, both compounds increased photo-killing with increasing incubation time. However, the extent of cell death observed between 4T1 and 67NR cells were similar across the different time points for incubation, implying no selective binding to cell surface receptors.
Figure 3.6: Compounds induced non-selective photocytotoxicity in TrkC positive and TrkC negative cells when treated for prolonged periods.

4T1 and 67NR cells were cultured in 96-well plates. After 24 hours, (A) IYIY-I₂-BODIPY, (B) YIYI-I₂-BODIPY and (C) I₂-BODIPY with indicated concentrations were added to cells and incubated for 2, 4 and 6 hours. The media was removed and the cells were then washed with PBS before new media was added. Cells were then irradiated with 7.3 J/cm² of light at a fluence rate 12.2 mW/cm² (10 minutes). MTT was carried out at 24 hours after illumination. Data represent mean ± SEM of three independent experiments. * p < 0.05, ** p < 0.005 vs 2 hours treatment.
3.3.3 Toxicity profile of I$_2$-BODIPY and ligated conjugates in murine model

To test the toxicity of compounds in murine model, the ligated conjugates and free drug at 20, 30 and 100 mg/kg (equimolar dose) were administered to mice intravenously via the tail vein. The acute toxicity associated primarily with an apparent loss of body weight based on the Berlin test of typical symptoms such as apathy, horrent fur, diarrhea, behavior changes and a loss of body weight were recorded (Koudelka et al., 2010). Mice receiving IYIY-I$_2$-BODIPY at doses of 30 mg/kg and above were found to experience motility and balancing difficulties, and died at 1-3 hours post drug administration. However, an IYIY-I$_2$-BODIPY dose of 20 mg/kg was well tolerated by mice, with no signs of toxicity and death found along the 17 days monitoring period (Figure 3.7). On the other hand, no death or signs of toxicity was observed in mice receiving equivalent doses of YIYI-I$_2$-BODIPY and I$_2$-BODIPY. Current results indicated the potential toxicity of IYIY-I$_2$-BODIPY and suggested 20 mg/kg as the MTD. Hence, in vivo experimental dosage for IYIY-I$_2$-BODIPY is conducted at dosage of lower than 20 mg/kg.
Figure 3.7: IYIY-I₂-BODIPY was not toxic to mice at 20 mg/kg.
Healthy 7–8 weeks old BALB/c female mice (n=2/group) were administered intravenously via tail vein respectively with IYIY-I₂-BODIPY and YIYI-I₂-BODIPY at 20, 30, and 100 mg/kg (I₂-BODIPY content equivalent to 6.25, 10, and 33 mg/kg, respectively, i.e., corrected for MW), and the parent I₂-BODIPY (33 mg/kg). The mice were then kept in the dark and observed for 16 days. Data represent the average body weight (grams) of two mice/treatment group.

3.3.4 In vivo biodistribution study in 4T1 tumour bearing mouse

The biodistribution of IYIY-I₂-BODIPY and the isomeric YIYI-I₂-BODIPY were monitored in 4T1 tumour bearing mice (n = 3/ each time point, 0, 0.25, 1, 3, 6, 12, 24, 48, 72 hours). At 1 hour post administration, the fluorescence intensity of tumours in the mice treated with IYIY-I₂-BODIPY was the highest among the time points investigated, and significant higher (2.1-fold) than the corresponding intensities for tumours treated with the YIYI-I₂-BODIPY (416000 ± 43000). Among the organs and tissues investigated, tumour tissue was the third highest in IYIY-I₂-BODIPY accumulation after excretory organs liver and kidney. Interestingly, the accumulation of IYIY-I₂-BODIPY dye intensity in tumour tissue remained significant high compared with YIYI-I₂-BODIPY for up to 6 hours (p = 0.038). The dye started to diminish from tumour tissue at 24 hours onwards with no significant differences between IYIY-I₂-BODIPY and YIYI-I₂-BODIPY (Figure 3.8).

The accumulation of both ligated conjugates in non-targeted organs such as liver, kidney and lung within the first 3 hours post administration was observed in high intensity (Figure 3.8), but these accumulations dissipated swiftly in the subsequent monitoring period. Similar accumulation and clearance pattern of both conjugates in
these organs is typical of small molecular weight compounds and indicates that the accumulation in these organs was random and not due to TrkC receptor binding.

In addition, non-selective accumulation of ligated conjugates was also observed in lymphoid organs such as spleen and lymph node, but at a much lower level. This observation supported by other study on low to negligible TrkC expression for these organs (Levanti et al., 2001). Other organs including eye and skin have relatively low TrkC receptor expression (Cui et al., 2002), and TrkC for skin mainly concentrated at nerve bundle portions (Botchkarev et al., 1999) (Figure 3.8). The maximum accumulation of IYIY-I₂-BODIPY at 1 hour post administration led us to adopt a drug-to-light interval of 1 hour in determining in vivo antitumour efficacies in the subsequent studies.
A. Figure 3.8: IYIY-I\textsubscript{2}-BODIPY has high accumulation in tumour tissue at 1 hour and cleared from the body at 72 hours post-administration. 4T1-tumour bearing female BALB/c mice were treated at 10 mg/kg \textit{via} the tail vein. Mice (n = 3) were sacrificed at 0, 0.25, 1, 3, 6, 24, 48, 72 hours. (A) Organs and tissues (tumour, tumour draining lymph nodes, spleen, kidney, liver, lung, skin, and eye) were harvested, and (B) fluorescence intensities in each organ were imaged using an \textit{in vivo}...
3.3.5 In vivo PDT antitumour efficacy of IYIY-I₂-BODIPY in TrkC positive 4T1 tumour bearing mouse

The efficacies of compounds in eradicating the TrkC positive tumour in BALB/c mouse model were examined. Aggressive TrkC positive murine breast carcinoma 4T1 cells were subcutaneously injected to the mammary fat pad, followed by intravenous injection of compounds (2 mg/kg and 10 mg/kg) when the sizes reached 60-80 mm³. At 1 hour post compound administration, the mice will be anaesthetised and irradiated at 100 J/cm² (Figure 3.9A).

The data revealed that there was significant reduction of tumour volume in IYIY-I₂-BODIPY treated group at 4–6 days post illumination, and healed from eschar by day 13–15. In contrast, YIYI-I₂-BODIPY and I₂-BODIPY treated mice showed a reduction in tumour size post PDT, followed by tumour regrowth at day 13-15 at the peripheral of necrotic tumour tissues (Figure 3.9B). In details, there were 61% and 96% maximum tumour reduction in mice treated with 2 and 10 mg/kg of IYIY-I₂-BODIPY compared to pre-treatment tumour size at 4-6 days post PDT. Conversely, YIYI-I₂-BODIPY and I₂-BODIPY treatment induced only moderate tumour size reduction within the first 6 days after illumination (20% reduction in mice treated with 10 mg/kg of YIYI-I₂-BODIPY, and 11% reduction in mice treated with 3.3 mg/kg of I₂-BODIPY, equimolar dose). Most importantly, 1 out of 7 mice (14%), treated with 2 mg/kg, and 5 out of 7 mice (71%), treated with 10 mg/kg of IYIY-I₂-BODIPY showed no palpable tumour for up to 90 days post-treatment. Conversely, no tumour remission was found in both the YIYI-I₂-BODIPY and I₂-BODIPY treated groups (Figure 3.9C).
The role of TrkC and NT3 (TrkC natural ligand) had also been reported correlates with tumour progression and invasion (Brodeur et al., 1997; Jin et al., 2010; McGregor et al., 1999; Ivanov et al., 2013). Moreover, the preliminary study had revealed that IYIY-ligand was able to transduce signal similar to NT-3 to induce neuronal cell survival upon binds to TrkC receptor (Chen et al., 2009). Hence, the effect of IYIY-I₂-BODIPY in promoting tumourigenesis was examined. The data in the dark revealed that saline control, IYIY-I₂-BODIPY and YIYI-I₂-BODIPY recipient mice have comparable tumour volume, with no significant differences between groups (Figure 3.9D). This suggests that IYIY-I₂-BODIPY did not promote tumourigenesis in normal condition.
Figure 3.9: IYIY-I₂-BODIPY effectively suppressed the growth of TrkC positive 4T1 tumour.

(A) Schematic procedure of PDT treatment in 4T1 tumour bearing mice. (B) Pictures of tumour sizes before and after PDT treatment for three compounds at 4-6 days and 13-15 days post PDT. (C) Graph of 4T1 tumour volume (mean ± SEM, n = 7) post irradiation. (D) Graph of 4T1 tumour volume (mean ± SEM, n = 5) post compounds administration without irradiation (dark) * p < 0.05, ** p < 0.005 vs IYIY-I₂-BODIPY using one-way ANOVA (Dunnett’s test).
3.3.6 *In vivo* PDT antitumour efficacy of IYIY-I₂-BODIPY in TrkC negative 67NR tumour bearing mouse

To confirm the targeting selectivity of IYIY-I₂-BODIPY *in vivo*, the efficacy of conjugate in non-TrkC expressing 67NR tumour in BALB/c mice was examined. Experiment procedure was as shown in figure 3.8A. As expected, neither IYIY-I₂-BODIPY nor YIYI-I₂-BODIPY at 10 mg/kg showed full tumour eradication in mice (Figure 3.10). Tumour volume was first reduced at 4-6 days post irradiation and then regrew at day 9. This concluded that IYIY-I₂-BODIPY is a potent conjugate in targeting TrkC positive tumour cells and increases the therapeutic value of I₂-BODIPY as a photosensitiser.

![Figure 3.10: IYIY-I₂-BODIPY and YIYI-I₂-BODIPY has equal antitumour efficacy on TrkC negative 67NR tumour.](image)

67NR tumour bearing mice were treated with IYIY-I₂-BODIPY and YIYI-I₂-BODIPY, respectively. At 1 hour post compounds administration, the mice were irradiated at 100 J/cm² with fluence rate of 0.16W/cm². Diagram showed the tumour volume (mean ± SEM, n = 7) post irradiation.
3.3.7 Histopathological analysis of IYIY-L2-BODIPY treated survivor mice at 90 days post-PDT

Part of the mice received IYIY-L2-BODIPY treatment showed no palpable tumour up to 90 days of observation. They were physically active and assumed to be tumour free. In order to confirm this, the 10 mg/kg IYIY-L2-BODIPY treated survivor mice (90 days post treatment) were sacrificed and histologically examined for their tumour stage by a certified veterinary pathologist. The Hematoxylin and Eosin staining showed no sign of 4T1 tumour metastasis in all the examined organs including liver, lung, draining lymph node, spleen, kidney, and heart. The examined organs were similar to tumour free control mouse (Figure 3.11). Taken together, these results indicated that IYIY-L2-BODIPY was effective in eradicated TrkC expressing tumour and enhanced survivor rate in mice post PDT.

Figure 3.11: IYIY-L2-BODIPY treated survivor mice have no metastasis sign.
IYIY-L2-BODIPY treated mice that had been survived for 90 days post-irradiation was sacrificed for histopathological analysis on disease stage. The organs in survivor mice including lung, draining lymph nodes, liver and spleen were sent for histopathological analysis for comparison with healthy and tumour metastasis mice. Scale bar: 100 μm.
The current results had been verified by certified veterinary pathologist. Yellow arrows indicate the metastatic tumour cells.

### 3.4 Discussion

BODIPY and its derivatives have been studied to be effective in inducing photocytotoxicity in cancer cells and promoting vascular damage in the Chick Chorioallantoic Membrane (CAM) model upon irradiation (Lim et al., 2010). BODIPY derivative ADMP06 had been reported effective in eradicating breast tumour in xenograft mice following PDT (Byrne et al., 2009). These studies were using free photosensitizer BODIPY for therapeutic. In order to increase the therapeutic efficacy of BODIPY, a targeting model for photosensitiser I$_2$-BODIPY was constructed by conjugating it with TrkC ligand. The resultant conjugate was reported specific and effective in targeting TrkC+ cells with no effect on TrkC- cells (Kamkaew & Burgess, 2013). This study was concordant with the *in vitro* preliminary study, which shown a significant selection of TrkC positive cells. Moreover, the *in vivo* studies revealed that the conjugate has a good biodistribution profile (cleared from major organs at 24 hours post administration), high accumulation in tumour site with outstanding antitumour activity on TrkC positive cells. Altogether, suggested that selective binding of IYIY-I$_2$-BODIPY was correlated with natural levels of TrkC expression in breast cell lines.

The *in vitro* cell study was extended for a longer interval between compounds incubation and illumination (4 – 6 hours, relative to 2 hours). Data showed that longer incubation of cells with conjugates tend to decrease binding specificity, perhaps due to relatively slow and non-selective interaction with cells (Peer et al., 2007; Veenhuizen et al., 1997). In addition, I$_2$-BODIPY was observed to reduce the cell viability at longer
incubation periods, and at concentrations higher than 1 μM at 2 h. This was due to the uptake of the I₂-BODIPY by the cells in a non-specific manner that has been reported by Lim et al. 2010, as observed using confocal microscope; photocytotoxicity of I₂-BODIPY at high concentrations and long incubation periods is possible. Hence, a shorter incubation period is optimum for ligand-mediated targeting agent in order to reduce undesired photocytotoxicity, due to non-specific binding. The in vitro tumour cell viability disaggregated from the tumour region post-PDT was not conducted in this study. The low cell viability is predicted based on higher survival rate and delayed tumour growth post-PDT in IYIY-I₂-BODIPY treated mice.

The in vitro observation was concordant with the in vivo biodistribution study, in which non-targeted YIYI-I₂-BODIPY slowly accumulated in tumour tissues at longer time points (3 and 6 hours post administration). However, the low specificity for prolonged incubation cannot be compared with the in vivo antitumour efficacy. It is remarkable that IYIY-I₂-BODIPY at 10 mg/kg caused, on average, 96% tumour volume reduction in the mice bearing TrkC positive tumour at 6 days post-PDT. The fact that IYIY-I₂-BODIPY was ineffective for suppressing TrkC negative 67NR tumours in mice supports the assertion that this conjugate targets TrkC expressing tissue in vivo.

Toxicity issue relates to PDT is the photosensitivity. An intravenous administration of photosensitisers can result in accumulation in the different tissues, especially skin and eye, hence induces undesirable photosensitivities (Solban et al., 2006). This can be overcome by targeting PDT agents to tumour that express targeted molecules. Nevertheless, the TrkC targeted IYIY-I₂-BODIPY conjugate is toxic to mouse at dosage more than 20 mg/kg, causes motility and balancing difficulties in mouse at 15 minutes post IYIY-I₂-BODIPY injection. In this case, TrkC could be anticipated since NT-3 was
known to promote neuronal cell survival, differentiation and synapse transmission. A claim that high doses of neurotrophins (including NT3) promote relatively rapid excitotoxic necrosis of neurons (Koh et al., 1995) might be pertinent. However, IYIY-I₂-BODIPY requires further structural modifications because the light wavelength to excite this conjugate is optimally around 530 nm, which has low tissue penetration compared to longer wavelength (700 nm) which has deeper tissue penetration (Ballou et al., 2005; Frangioni, 2003; Rao et al., 2007; Sevick-Muraca et al., 2002).

Throughout this study, an assumption of similar function between targeting ligand (IYIY) itself and conjugate IYIY-I₂-BODIPY in the dark was proposed. This is because the photosensitiser is not active in the dark and thus has been excluded for the use of core IYIY- and YIYI- ligand as another control. Given the apparent role of TrkC in tumourigenesis of breast, neuroblastoma and pancreatic cancer, targeting the TrkC in PDT maybe benefit for cancer patient that overexpressed TrkC in tumour cells. This study features dosing with a targeted-PDT agent alone, but there is also the intriguing possibility of combination therapies featuring Trk inhibitors currently in trials as chemotherapeutic agents \textit{e.g.} Lestaurtinib (Chan et al., 2008; Iyer et al. 2010) and PLX7486.

3.5 Conclusion

Current chemotherapy drugs used in the clinic today are toxic in nature, which lead to the non-specific killing, undesirable side effects, morbidity and even death in cancer patients. In this study, the synthesised TrkC targeted agent is the first to develop to target TrkC expressing cancer using active targeting approach. The conjugated photosensitiser will become active once activated by the light and subsequently kills the cells \textit{via} generation of singlet oxygen. This active targeted PDT agent (IYIY-I₂-
BODIPY) can enhance the therapeutic indices by selectively accumulates in the tumours but not in healthy tissues, suggesting that IYIY-I$_2$-BODIPY can become clinically important for the treatment of TrkC expressing cancer.
CHAPTER 4: IMMUNE RESPONSES INDUCTION AND ANTITUMOUR ACTIVITY OF TROPOMYOSIN RECEPTOR KINASE-C (TRKC) RECEPTOR TARGETED PHOTODYNAMIC THERAPY (PDT) CONJUGATE

4.1 Introduction

Conventional chemotherapy of cancer is nearly always associated with limitations that include non-selective toxicity, treatment resistance and immune response silencing (Hirsch, 2006; Weir et al., 2011). These restrictions generally lessen the effectiveness of chemotherapy. Actively targeted cancer therapies guide the agents to cell surface molecules (proteins, sugar or lipids), and thus increasing their cellular uptake through the endocytic internalisation to improve selectivity and counter resistance (Byrne et al., 2008). Many ligand-drug conjugates had been designed to selectively bind overexpressed cell surface molecules (generally survival or metastasis biomarker in cancer) such as biotin, folate, sigma-2, carbonic anhydrase IX, glucose receptors and others (Srinivasarao et al., 2015).

This paper is focus on the TrkC receptor due to its tumourigenesis characteristic and limited drugs to treat TrkC positive cancer. These receptors are found in neurons where they regulate the neuronal cell survival and growth, proliferation, differentiation and synaptic strength and plasticity (Huang & Reichardt, 2003), but also in neuroblastoma (Brodeur et al., 1997; Yamashiro et al., 1997), glioblastoma (Kumar & de Vellis, 1996; Wang et al., 1998), thyroid cancer (McGregor et al., 1999), melanoma (Xu et al., 2003) and breast cancer (Blasco-Gutierrez et al., 2007; Jin et al., 2010) where they impact malignancy. The expression and function of Trk subtypes are dependent on the tumour type. For instance, in neuroblastoma, TrkC expression correlates with good prognosis, but in breast, prostate and pancreatic cancers, the expression of the same Trk subtype is
associated with cancer progression and metastasis (Jin et al., 2010; Miknyoczki et al., 2002). Furthermore, ligands binding Trk receptors activate downstream intracellular signalling pathways that enhance tumour cell mitogenicity and survival (Brodeur et al., 1997; Yamashiro et al., 1997). Inhibition of Trk signaling significantly reduced tumourigenicity and invasive capability of tumour cells in vitro and in vivo xenograft models (Iyer et al., 2010; Jin et al., 2010) (Miknyoczki et al., 2002). Several Trk receptors targeted chemotherapeutic drugs which are inhibitors of all TrkA/B/C receptors, are currently in clinical trials for treatment of solid tumours (Vaishnavi et al., 2015).

Trk receptors and their ligands have been reported to modulate the immune system. Trk receptors are expressed in small quantities in monocytes and lymphocytes. The natural ligands of Trk receptors, neurotrophins, which include neurotrophin-3/-4 (NT-3/NT-4), brain-derived neurotrophin factor (BDNF) and neurotrophin growth factors (NGF), can function as non-cytokine mediators to modulate both innate and adaptive immune responses. Such modulations include increasing the pluripotent cytokine interleukin (IL) -6 secretion in bone marrow stromal cells (Marshall et al., 1999; Rezaee et al., 2010). In addition, neurotrophins have been reported to enhance differentiation of granulocytes (e.g. eosinophils, mast cells and basophils) during haematopoiesis (Matsuda et al., 1988). In T-lymphocytes, neurotrophins regulate T-cell subtypes balancing upon binding to TrkC expressing T helper (Th) 2 cell by promoting IL-4 release, which in turn blocks Th1 subtype and IFN-γ production (Sekimoto et al., 2003). Other than neurotrophins, TrkC was also reported to suppress TGF-β signaling by directly binding to type II TGF-β receptor and to reduce TGF-β mediated downstream Smad2/3 phosphorylation in TrkC expressing cells (Jin et al., 2007). Despite evidence that links Trk receptors to modulation of the immune system, there are currently no
reports that explore Trk receptors in the context of possible strategies for immune therapy.

TrkC receptor targeted ligand conjugated with photosensitiser diodo-boron dipyrrromethene (I$_2$-BODIPY), termed as IYIY-I$_2$-BODIPY (Kamkaew & Burgess, 2013) is designed for use in PDT of cancer. The IYIY-ligand induces some biological properties that are similar to neurotrophin-3 including internalisation into lysosomes, and transduction of signals that regulate neuronal cell survival and differentiation (Chen et al., 2009). Based on this and the previously reported immunological effects of TrkC and NT-3, this study investigated the systemic immunological impacts seen on the administration of the IYIY-I$_2$-BODIPY conjugate in a preclinical model. Specifically, the systemic modulation of cytokine levels, characterisation of myeloid innate immune responses and adaptive immune T-cell subtypes populations upon compounds administration under the non-irradiated conditions (dark) will be examined. As PDT agents are not active in the dark, studying the conjugate without photoirradiation can help to elucidate the immune-regulatory function of this TrkC ligand itself. This may be important, especially for PDT agents that have long drug-light interval (DLI) since the ligand in the conjugate may already begin to exert its therapeutic effect even before photoirradiation. This is the first study on the immunomodulation properties and anti-tumour activity of a TrkC ligand. In addition, the combination effect of conjugates and PDT will be examined as PDT has been known to contribute to the development of systemic antitumour immune responses (Nowis et al., 2005; Reginato et al., 2014).
4.2 Materials and Methods

4.2.1 Compounds

TrkC targeted IYIY-I$_2$-BODIPY (Isoleucin-Tyrosine-Isoleucin-Tyrosine, IYIY conjugated I$_2$-BODIPY), non-TrkC targeted scrambled control YIYI-I$_2$-BODIPY (Tyrosine-Isoleucin-Tyrosine-Isoleucin, YIYI conjugated I$_2$-BODIPY), free photosensitiser I$_2$-BODIPY and TrkC targeted ligand IYIY-TEG (Isoleucin-Tyrosine-Isoleucin-Tyrosine conjugated to triethylene glycol (TEG) without I$_2$-BODIPY) were a gift from Prof. Kevin Burgess (Kamkaew & Burgess, 2013).

4.2.2 Animal Model

Female 8-10 week old, wild type BALB/c mice were purchased from Taconic and InVivos Pte Ltd, Singapore and maintained in the AAALAC accredited satellite animal facility at the Department of Pharmacology, Faculty OF Medicine, University of Malaya. All animal experiments were performed according to protocols approved by the Faculty of Medicine Institutional Animal Care and Use Committee, University of Malaya (FOM IACUC). Ethics approval numbers: 20150303/PHAR/R/KCS (Appendix B).

4.2.3 Tumour Model Development

The fur of the BALB/C mouse was shaved and murine breast carcinoma 4T1 cell line (ATCC) at a density of $5 \times 10^5$ cells in 0.1 mL of RPMI medium was orthotopically injected into the mammary fat pad of the mice. The mice were monitored for tumour development every day and were then randomly divided into groups for compounds administration when the size reached around 60-80 mm$^3$. 
4.2.4 Compounds Administration

IYIY-I$_2$-BODIPY, YIYI-I$_2$-BODIPY at 10 mg/kg of body weight (consist 3.3 mg equivalent/kg of I$_2$-BODIPY and 6.7 mg equivalent/kg of IYIY-TEG) and 3.3 mg/kg of I$_2$-BODIPY were dissolved respectively in a cocktail of 2.5% ethanol and 2.5% CremophoreEL in saline. The mixture was then further dissolved using saline to a volume of 0.2 mL and intravenously administrated to tumour bearing mice *via* tail vein. The mice were then kept in an environment away from bright light for 2 hours and 24 hours (n = 5 per treatment group for every time point).

4.2.5 Blood sampling

At 2 hours (represented early innate immune response) and 24 hours (represented late or onset of adaptive immune response) time points post compounds administration, mice from each group (n = 5 per time point) were anaesthetised with anaesthesia cocktail (90 mg/kg of ketamine and 10 mg/kg of xylazine). 0.5 mL of blood was withdrawn *via* cardiac puncture after the onset of anaesthesia.

4.2.6 Flow Cytometry Quantification of Plasma Cytokines

The withdrawn blood was centrifuged at 5000 rpm for 10 minutes to separate the plasma and peripheral blood mononuclear cells. Cytokine levels in blood plasma were then determined using a BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA) according to manufacturer’s instructions. Cytokines IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-17A and IL-10 were quantified using FACSCanto II (BD Biosciences) and analysed using FCAP array software (BD Biosciences). TGF-β cytokine in blood plasma was quantified using
Human/Mouse TGF-β1 sandwich ELISA (eBiosciences, San Diego, CA, USA) based on manufacturer’s instructions.

4.2.7 Cell Isolation from Lymphoid Organs and Tumour Tissues

After the blood sampling at each indicated time points, mice (n = 5 per time point) were sacrificed and tumour draining lymph nodes (TDLN), spleen and tumour tissues were harvested. Single cell suspensions from TDLN and spleen were obtained by mincing the organs using frosted glass slides, followed by red blood cell lysis using red blood cell lysis buffer. Single cell suspensions from tumour tissues were obtained by mincing tumour tissue with frosted glass slides, followed by digestion with 0.5 mg/mL of collagenase type XI (Sigma Aldrich, St Louis, MO) for 30 minutes in a 37 °C shaker at 200 rpm. The cell suspensions were then filtered to eliminate the debris. All the single cell suspensions from TDLN and tumour tissues were stimulated for 4 hours with 50 ng/mL of Phorbol 12-Myristate 13-Acetate (PMA), 1 µg/mL of Ionomycin (Sigma Aldrich, St Louis, MO, USA) and 3 µg/mL of brefeldin A (BD Bioscience) (Kue et al., 2012).

4.2.8 Staining and Flow Cytometry Quantification of Immune Cells

The stimulated single cell suspensions from tumour tissues, TDLN and spleen were washed with PBS, and resuspended (1 x 10⁶ cells) in FACS washing buffer (0.5% FBS and 0.05% sodium azide in PBS). The cells were then stained with a panel of fluorochromes conjugated monoclonal antibodies purchased from BD Pharmingen to detect specific surface antigen that are FITC-anti mouse CD11b (553310), PE-anti mouse Ly6G (551461), APC-anti mouse F4/80 (17-4801), FITC-anti mouse CD4 (553729), PE-anti mouse CD25 (553075), FITC anti mouse CD8 (553031). The cells were fixed and permeabilised using Cytofix/Cytoperm kit (BD Biosciences) according
to the manufacturer’s instructions. The surface antigens stained cells were then labelled for intracellular cytokine using PE-anti-mouse IFN-γ (554412), PerCP-Cy5.5-anti-mouse IL-4 (560700), APC-anti-mouse IL-17 (17-7177), and APC-anti-mouse FoxP3 (17-5773) with different fluorochrome combination to avoid overlapping. We quantified the cells of our interest based on surface markers, Th1 (CD4^+IFN-γ^+), Th2 (CD4^+IL-4^+), Th17 (CD4^+IL-17^+), Treg (CD4^+CD25^+FoxP3^+), CTL (CD8^+IFN-γ^+), Tc-17 (CD8^+IL-17^+), granulocytic-MDSC (CD11b^+Ly6G^+), neutrophils (CD11b^+Ly6G^-F4/80^-). Effector T-cells were stained using PE-Cy5 rat anti-mouse CD44 (553135) for CD4^+CD44^high and CD8^+CD44^high populations. A total of 10,000 cells were analysed for every experiment. The stained cells was analysed in a FACS Canto-II Flow Cytometry (BD Biosciences). The data were analysed using Cell Quest software (BD Biosciences).

4.2.9 In vivo TrkC blocking studies

For TrkC blocking study, the tumour bearing mice (n = 4 per group) were treated with IYIY-I2-BODIPY (10 mg/kg) with or without mouse TrkC antibody (R&D systems, Minneapolis, MN, USA) at a dosage of 10 µg/mouse via tail vein and kept away from bright light as a non-irradiated study. Tumour bearing mice receiving isotype control Immunoglobulin G (R&D systems) at dosage of 10 µg/mouse (n = 4) was used as a antibody control in this study. At 2 hours post IYIY-I2-BODIPY and antibodies administration, the mice were sacrificed and tumour tissues, spleen and TDLN were harvested, stained with respective fluorescence conjugates antibodies and phenotyped for granulocytic-MDSC, neutrophils, CD4^+ and CD8^+ T-cell subtypes using flow cytometry.
4.2.10 Photodynamic therapy (PDT) in Mice

For irradiation, IYIY-I$_2$-BODIPY (10 mg/kg), YIYI-I$_2$-BODIPY (10 mg/kg), I$_2$-BODIPY (3.3 mg/kg) and saline were given respectively to the mice ($n = 5$ per group). The mice were kept away from bright light post compound administration for 1 hour. Thereafter, anaesthesia cocktail of 90 mg/kg of ketamine and 10 mg/kg of xylazine was given to the mice. Upon the onset of anaesthesia, PDT was performed using a Lumacare LC-122A fiber optic light delivery system (standard fiber optic probe model LUM V, 400–700 nm, Lumacare Medical Group, Newport Beach, CA, USA, with a 500/585 nm bandpass filter from Omega Optical) to emit light at 530 nm. A 4 mm thick glass slide was used as a barrier to avoid direct photothermal effect on tumour. The illuminating spot was positioned at the tumour and the surrounding was covered using a black cloth to avoid PDT effect on non-tumour region of body. PDT was conducted at 100 J/cm$^2$ with a fluence rate of 160 mW/cm$^2$, for 10 minutes. The irradiated mice were then sacrificed for blood and lymphoid organ isolation at 2 hours and 24 hours post-PDT. The methods of samples processing in irradiated mice were same as methods described above (blood sampling, flow cytometry quantification of plasma cytokines, cell isolation from lymphoid organs and tumour tissues, staining and flow cytometry quantification of immune cells).

4.2.11 Adoptive Transfer for Antitumour Immunity

For adoptive transfer studies, spleen and TDLN from healthy mice ($n = 5$) and the survivor mice (tumour free 60 days post PDT) in IYIY-I$_2$-BODIPY treated group of mice ($n = 5$) were harvested. Splenocytes at a density of $2 \times 10^7$ cells/0.2 mL/mouse and lymphocytes from TDLN at a density of $1.5 \times 10^7$ cells/0.2 mL/mouse from each mouse were injected into different recipients of healthy syngeneic mice ($n = 5$) via the tail vein. After 2 days of adoptive transfer, the recipient mice were inoculated subcutaneously.
with 4T1 tumour cells at a density of $5 \times 10^5$ cells/0.1 mL/mouse. The tumour growth in the mice that received survivor splenocytes and lymphocytes was monitored and compared with the mice that received splenocytes and lymphocytes from the healthy mice.

4.2.12 Statistical Analysis

The Student’s $t$-test and one-way ANOVA (Dunnett’s test) were used to determine the statistical differences between various experimental and control groups. A $p$ value of $< 0.05$ was considered significant.

4.3 Results

4.3.1 Th1/Th2/Th17/Treg cytokines level in blood plasma of TrkC positive 4T1 tumour bearing mice post compounds administration

In the initiation phase of the adaptive immune response, antigen-specific lymphocytes (T and B cells) are activated when they are exposed to antigens presentation. Such activation of T-helper (Th) CD4$^+$ lymphocytes causes them to differentiate into four different subtypes (Th1, Th2, Th17, regulatory T-cells) depending on the availability of the different types of differentiation factors (cytokines) in the environment. The differentiated subtypes of T-cells then secrete various specific cytokines. For instance, Th1 secretes interleukin (IL)-2, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, Th2 secretes IL-4, IL-6, IL-10, Th17 secretes IL-17 and regulatory T-cells (Treg) secretes transforming growth factor (TGF)-β. The released cytokines by differentiated T-cell subtypes will further stimulate CD8$^+$ T-cells such as cytotoxic T lymphocytes (CTL), Tc17 cells function. The following experiments were conducted to examine if IYIY-I$_2$-BODIPY induces the same effects.
First, the cytokine levels in TrkC positive murine 4T1 breast cancer model (Jin et al., 2010) that has been treated with compound IYIY-I\textsubscript{2}-BODIPY, YIYI-I\textsubscript{2}-BODIPY or I\textsubscript{2}-BODIPY, the latter two as controls were examined. Blood plasma samples were collected from 4T1 tumour bearing mice via cardiac puncture at 2 hours and 24 hours post compound administration with no exposure to light (hereafter referred to as dark treatment) to examine early and late responses respectively. All treatment groups had slightly increased levels of Th1 cytokine IL-2 at 2 hours and 24 hours (0.7 – 1.9 pg/mL) compared to saline (0 – 0.3 pg/mL; \( p = 0.05 \) for IYIY-I\textsubscript{2}-BODIPY and YIYI-I\textsubscript{2}-BODIPY; Figure 4.1A). Since IL-2 is usually produced to promote activation and proliferation of T-cells, elevation of that interleukin suggests that IYIY-I\textsubscript{2}-BODIPY in the dark induced T-cell responses. Increases in levels of other Th1 cytokines such as IFN-\( \gamma \) (Figure 4.1B) and TNF-\( \alpha \) (Figure 4.1C) were hardly significant in all treatment groups compared to the saline control, suggesting that Th1-mediated inflammation was mild.

Th2 cytokines such as IL-4, was increased (1 to 2-fold) in all mice treated with the IYIY-I\textsubscript{2}-BODIPY, YIYI-I\textsubscript{2}-BODIPY and I\textsubscript{2}-BODIPY at 2 hours only compared to mice treated with saline control (Figure 4.1D), in a non-compound specific manner. IL-6 is a set of pluripotent pro-inflammatory cytokines that regulate differentiation and functions of T lymphocytes as well as expansion of myeloid cells (Meyer et al., 2011; Mundy-Bosse et al., 2011). In this work, levels of IL-6 were significantly and selectively elevated in IYIY-I\textsubscript{2}-BODIPY treated mice (approximately 7.5-, 2.6- and 4.6-fold compared to mice treated with saline control, I\textsubscript{2}-BODIPY and YIYI-I\textsubscript{2}-BODIPY respectively at 2 hours; \( p = 0.000 \)). The increase of IL-6 level did not persist, as the level at 24 hours was almost as low as the other groups at 24 hours (Figure 4.1E). The increase of IL-6 was the same as other studies where neurotrophins binding to Trk
receptor increased IL-6 secretion in bone marrow stromal cells (Marshall et al., 1999; Rezaee et al., 2010). Levels of the Th2 immunosuppressive cytokine IL-10 induced by the compounds was comparable in these experiments (Figure 4.1G). Together, the data indicate that the Th2 cytokines (IL-4, IL-6) were transiently elevated, whereas the Th1 responses (very weak IFN-γ and TNF-α) were very mild.

Another proinflammatory cytokine IL-17A which is specifically secreted by IL-17+ T-cells was mild elevated (1.3-fold) only in IYIY-I2-BODIPY treated group at 24 hours compared to other treatment and control groups. There was no significant change in the IL-17A levels at 2 hours post compound administration when compared to I2-BODIPY (Figure 4.1F). Increased IL-17 in the IYIY-I2-BODIPY treated group is the same as the selective increase in the IL-6 level in the same group, probably because IL-6 is one of the essential cytokines for differentiating naive T-cells to IL-17+ T-cells. Based on the non-persistent increases of IL-2 and IL-6, suggest that ligated conjugate treated mice selectively provoke transient inflammation. Among the compounds investigated, IYIY-I2-BODIPY had the highest immune modulation activity.

In addition to the above findings, levels of immunosuppressive cytokine TGF-β was significantly suppressed in both IYIY-I2-BODIPY and YIYI-I2-BODIPY treated groups at 2 hours (4.0-fold and 3.0-fold respectively, Figure 4.1H) compared to I2-BODIPY. Previous study had shown that YIYI-I2-BODIPY possessed positive but lower targeting selectivity on TrkC positive cells compared to IYIY-I2-BODIPY when cells exposed to compound in prolong period (Figure 3.6), so it is conceivable that the YIYI-ligand could elicit similar but weaker immune responses compared to IYIY as reflected by the levels of IL-2, IL-4 and TGF-β. I2-BODIPY alone in the dark only caused mild immune responses (as reflected by the lack of significant changes in cytokine levels post
treatment). This further indicated that the immunomodulation properties of the TrkC ligand-photosensitiser conjugate was mainly contributed by the TrkC ligand counterpart. In addition, the data was also in agreement with the previous study showing that YIYI-ligand had weak binding affinity on TrkC receptor (Kamkaew & Burgess, 2013).

Figure 4.1: IYIY-I$_2$-BODIPY conjugate increases IL-2, IL-6, IL-17 and decreases TGF-β cytokine levels.

4T1 tumour bearing mice were randomly divided into four treatment groups (saline, I$_2$-BODIPY, YIYI-I$_2$-BODIPY and IYIY-I$_2$-BODIPY) and compounds were administrated via tail vein respectively. Mice were then sacrificed at 2 hours and 24 hours post compound administration. The blood plasma was extracted from these mice via cardiac puncture for cytokines analysis. Levels of cytokines (A) IL-2, (B) IFN-γ, (C) TNF-α, (D)
IL-4, (E) IL-6, (F) IL-17A, (G) IL-10 and (H) TGF-β are shown. Data represent mean ± SEM with minimum of four mice per group. * $p < 0.05$, ** $p < 0.005$ vs I2-BODIPY using one-way ANOVA (Dunnett’s test).

4.3.2 Myeloid cell subtypes quantification in compounds treated mice

Cancer growth is normally accompanied by expansion of a heterogeneous group of immunosuppressive cells collectively known as myeloid derived suppressor cells (MDSCs). In particular, the granulocytic-MDSC (G-MDSC) subtype promotes tumour relapse by impairing the effectiveness of host immunity through inhibition of T-cell activation when primed by the tumour antigen (Khaled et al., 2013; Nagaraj et al., 2007). The expansion and differentiation of MDSCs is known to be mainly regulated by cytokines IL-6 (Meyer et al., 2011; Mundy-Bosse et al., 2011). Following the increase of the levels of these cytokines in IYIY-I2-BODIPY treated mice, the changes in G-MDSCs cell populations in these animals was then examined. The data revealed that IYIY-I2-BODIPY treated mice had significant lower level of G-MDSCs (CD11b\(^{+}\)Ly6G\(^{+}\)) in spleen (4.9% ± 0.5%; $p = 0.032$) (Figure 4.2A) and tumour microenvironment (TM) (1.1% ± 0.3%; $p = 0.035$) at 2 hours (Figure 4.2B), at approximately 2-fold and 4-fold lower respectively compared to the other three control groups. Comparing the two ligated conjugates, YIYI-I2-BODIPY treated mice showed low G-MDSC population only at 24 hours, whereas IYIY-I2-BODIPY treated group had low G-MDSC population at 2 hours and remained low up to 24 hours (Figure 4.2). This data suggests that conjugates selectively reduce the populations of immunosuppressive G-MDSC that is usually highly up-regulated in cancer, and could serve as a cancer immunotherapeutic agent.
Figure 4.2: Lymphoid organs and tumour microenvironment has lowered populations of G-MDSC in IYIY-I$_2$-BODIPY treated mice.
4T1 tumour bearing mice were randomly divided into four treatment groups (saline, I$_2$-BODIPY, YIY-I$_2$-BODIPY and IYIY-I$_2$-BODIPY) and compounds were administrated via tail vein respectively. Mice were then sacrificed at 2 hours and 24 hours post compound administration. At scheduled time points post compound administration, mice were sacrificed, followed by isolation of TDLN, spleen and tumour tissues. Immuno-phenotyping of G-MDSC was conducted using surface staining of fluorescence conjugated antibodies and quantified using flow cytometry. (A) G-MDSC in spleen and (B) tumour tissues. Data represent mean ± SEM with minimum of four mice per group. * $p < 0.05$, *** $p < 0.001$ vs I$_2$-BODIPY using one-way ANOVA (Dunnett’s test).

4.3.3 Inflammatory cell (neutrophils) population quantification in compounds treated mice

Neutrophil, which is another subtype of myeloid cell, was examined to confirm the suppressive activity of IYIY-I$_2$-BODIPY on myeloid cell subsets. Neutrophils are defined by the expression of surface antigens CD11b and Ly6G, with the absence of the macrophage marker F4/80 (Rose et al., 2012). Similar to G-MDSC, the neutrophil population at 2 hours was reduced. The reduction was 50% in spleen ($p = 0.02$) and 24% in TM (statistically insignificant) of IYIY-I$_2$-BODIPY treated mice compared to I$_2$-BODIPY treated mice (Figure 4.3). In YIYI-I$_2$-BODIPY treated mice, the neutrophil population was reduced by 13% at 2 hours compared to I$_2$-BODIPY and at 24 hours, the reduction was approximately 44% in spleen. This suggests that the ligand-conjugates had direct impact on myeloid cells, rather than through IL-6 regulation.
Figure 4.3: IYIY-I₂-BODIPY treated mice have lowered neutrophils population. 4T1 tumour bearing mice were randomly divided into four treatment groups (saline, I₂-BODIPY, YIYI-I₂-BODIPY and IYIY-I₂-BODIPY) and compounds were administrated via tail vein respectively. Mice were then sacrificed at 2 hours and 24 hours post compound administration. At desired time points post compound administration, mice were sacrificed, followed by isolation of TDLN, spleen and tumour tissues. Immuno-phenotyping of neutrophil was conducted using surface staining of fluorescence conjugated antibodies and quantified using flow cytometry. (A) Neutrophil in spleen and (B) tumour tissues were shown. Data represent mean ± SEM with minimum of four mice per group. * \( \ p < 0.05 \) vs I₂-BODIPY using one-way ANOVA (Dunnett’s test).

4.3.4 CD4⁺ T-helper cell subtypes (Th1/Th2/Th17/Treg) quantifications in compounds treated mice

Different T-lymphocyte subtypes secrete cytokines into systemic circulation, and these cytokines especially IL-2 can affect the differentiation, expansion and survival of T-lymphocytes via autocrine or paracrine systems (Zhu et al., 2010). In addition, T helper lymphocytes are able to differentiate into four different subtypes, which are Th1, Th2, Th17 and regulatory T-cells (Treg) based on type of diseases and cytokines in the environment. Consequently, the impact of ligand-drug conjugates on T-lymphocyte subtypes populations was examined. Populations of CD4⁺ T helper (Th) cell subsets and antigen specific CD8⁺ T-cell subsets were determined in the tumour draining lymph node (TDLN) and tumour microenvironment (TM). Th1 cell populations (CD4⁺IFN-\( \gamma \)⁺) in IYIY-I₂-BODIPY treated mice were 2-fold higher in TDLN at 24 hours and in TM at 2 hours (\( \ p = 0.045 \)), compared to I₂-BODIPY treated mice (Figure 4.4A). In the YIYI-I₂-BODIPY treated group, the Th1 subset was moderately increased only at 24 hours in both TDLN (1.7-fold) and TM (1.3-fold) compared to I₂-BODIPY (Figure 4.4A). Th2 (CD4⁺IL-4⁺) populations in TDLN and TM were comparable among the treatment and control groups and saline control at 2 hours. There was a moderate increase in TDLN at
24 hours in IYIY-I₂-BODIPY and YIYI-I₂-BODIPY-treated mice compared to mice treated with I₂-BODIPY and saline. Unlike TDLN, TM had lower Th2 populations in both IYIY-I₂-BODIPY and YIYI-I₂-BODIPY-treated mice at 24 h compared to mice treated with I₂-BODIPY and saline (Figure 4.4B).

The Th17 cell population was increased compared to I₂-BODIPY by 2.4- and 2.0-fold at 24 hours in TDLN ($p = 0.024$) and TM respectively (Figure 4.4C). This observation coincided with the increase of IL-17 cytokine. Unlike Th1, the increase in Th17 cell was selective only in IYIY-I₂-BODIPY treated group. This is similar for IL-6 level which was also only elevated in IYIY-I₂-BODIPY treated group, supporting that IL-6 mainly skewed the differentiation of naive T-cells to the Th17 subset. Taken together, the T-helper population was concordant with the cytokines profiles in Figure 4.1, and in agreement with the conclusion that ligated conjugates increased Th1 and Th17 subsets of T-cells population.

Immunosuppressive regulatory T cell (Treg) was known to suppress antitumour T-cell responses as a method of escaping immune-surveillance during cancer development. In the case of Treg cells, the population in IYIY-I₂-BODIPY mice was found to decrease by approximately 1.4-fold in TDLN at 24 hours ($p = 0.027$) and 2-fold in TM at 24 hours ($p = 0.045$) (Figure 4.4D). Similar observations were made in experiments featuring YIYI-I₂-BODIPY treated mice only at 24 hours. This decrease in Treg concurred with the depressed levels of the immunosuppressive cytokine TGF-β in both groups. Together, the data suggest that IYIY-I₂-BODIPY was more rapid in reducing the populations of immunosuppressive Treg compared to YIYI-I₂-BODIPY, which was low only at the later time point (24 hours).
Figure 4.4: IYIY-I$_2$-BODIPY increases CD4$^+$ T-lymphocytes expressing IFN-$\gamma$ and IL-17, and decreases regulatory T-cells.

4T1 tumour bearing mice were randomly divided into four treatment groups (saline, I$_2$-BODIPY, YIY-I$_2$-BODIPY and IYIY-I$_2$-BODIPY) and compounds were administrated via tail vein respectively. Mice were then sacrificed at 2 hours and 24 hours post compound administration. TDLN and tumour tissues were isolated to generate single cells suspension. The suspension cells were activated using PMA/Ionomycin/golgi plug as described in Methods and Materials. Upon activation, CD4 marker was stained, followed by fixation and permeabilisation for intracellular staining. (A) Th1 cell was stained with fluorescence conjugated anti-IFN-$\gamma$, (B) Th2 with anti-IL-4, (C) Th17 with anti-IL-17 and (D) regulatory T-cells with additional surface marker CD25, and transcription factor FoxP3. Each group of cells was then quantified using flow cytometry. Data represent mean ± SEM with minimum of four mice per group. * $p < 0.05$ vs I$_2$-BODIPY using one-way ANOVA (Dunnett’s test).
4.3.5 CD8+ T-cells quantification in compounds treated mice

Successful cancer elimination is highly depending on antigen-specific CD8+ T-cells secreting IFN-γ (Cytotoxic T lymphocytes, CTL). Interestingly, CTL was significantly increased in IYIY-I2-BODIPY treated mice (approximately 2 fold in TDLN at 24 hours and in TM at 2 hours; \( p = 0.043 \)) compared to all control groups (Figure 4.5A). For CD8+IL17+ T-cells (Tc17), the population in TDLN was almost comparable among the three control groups. Unlike TDLN, TM has higher Tc17 populations in IYIY-I2-BODIPY treated mice at 24 hours (7.3% ± 1.3%; \( p = 0.035 \)) and for YIYI-I2-BODIPY treated mice (approximately 3.8%, \( p > 0.05 \)) compared to mice treated with I2-BODIPY (1.3% ± 0.05%; Figure 4.5B). This demonstrates that more effector T-cells were generated following IYIY-I2-BODIPY administration compared to I2-BODIPY, which then infiltrated to a high concentration at the TM (Figure 4.4 and 4.5). The elevation of effector T-cells such as IFN-γ+ and IL-17+ phenotypes is important for cytolytic activity and inflammation at tumour site.

Figure 4.5: IYIY-I2-BODIPY increases CD8+ T-lymphocytes expressing IFN-γ and IL-17.
4T1 tumour bearing mice were randomly divided into four treatment groups (saline, I\textsubscript{2}-BODIPY, YIY-I\textsubscript{2}-BODIPY and IYIY-I\textsubscript{2}-BODIPY) and compounds were administrated via tail vein respectively. Mice were then sacrificed at 2 hours and 24 hours post compound administration. TDLN and tumour tissues were isolated to generate single cells suspension. The suspension cells were activated using PMA/IONOMYCIN/golgi plug as described in Methods and Materials. Upon activation, CD8 marker was stained, followed by fixation and permeabilisation for intracellular staining. (A) CTL was stained with fluorescence conjugated anti-IFN-\(\gamma\), (B) Tc17 with anti-IL-17. Each group of cells was then quantified using flow cytometry. Data represent mean ± SEM with minimum of four mice per group. * \(p < 0.05\) vs I\textsubscript{2}-BODIPY using one-way ANOVA (Dunnett’s test).

4.3.6 Confirmation of immunomodulatory effects by using IYIY-TEG (ligand without photosensitiser)

In order to confirm the immunomodulatory activity of conjugate is derived from the TrkC ligand but not from the photosensitiser I\textsubscript{2}-BODIPY, a study of using another control, IYIY-TEG (ligand without photosensitiser) was performed. Data showed that IYIY-TEG reduced immunosuppressive G-MDSC and Treg cells (Figure 4.6A), and increased CD4\(^+\) (Figure 4.6B) and CD8\(^+\) (Figure 4.6C) expressing IFN-\(\gamma\) and IL-17 in lymphoid organs at 2 hours and 24 hours dark. These effects mirror those produced by both ligated conjugates, especially for IYIY-I\textsubscript{2}-BODIPY. Based on this and the lack of immune activity from free I\textsubscript{2}-BODIPY as observed in Figure 4.1-4.5, suggests that IYIY ligand itself (i) reduces immunosuppressive mediators TGF-\(\beta\), G-MDSC and Treg; and, (ii) elevates an adaptive immune response with IFN-\(\gamma\) and IL-17 phenotypes.
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Figure 4.6: Control TrkC ligand (IYIY-TEG) has similar immunomodulatory activities as IYIY-I\textsubscript{2}-BODIPY.

4T1 tumour bearing mice were administrated with IYIY-TEG via tail vein (equivalent dose to IYIY-I\textsubscript{2}-BODIPY) and mice were sacrificed at 2 hours and 24 hours post administration. Lymphoid organs were harvested and populations of (A) G-MDSCs and Treg cells (B) Th1 (CD4\textsuperscript{+}IFN-\gamma\textsuperscript{+}), Th17 (CD4\textsuperscript{+}IL-17\textsuperscript{+}) and (C) CTLs (CD8\textsuperscript{+}IFN-\gamma\textsuperscript{+}), Tc17 (CD8\textsuperscript{+}IL-17\textsuperscript{+}) were quantified using flow cytometry after labeling with fluorescence conjugated antibodies. Data represent mean ± SEM with minimum of four mice per group. * \textit{p} < 0.05 vs saline control using student t-test.

4.3.7 IYIY-I\textsubscript{2}-BODIPY mediated immune modulations upon TrkC receptor blocking

To address whether ligated conjugates induced immune modulation were TrkC dependent \textit{in vivo}, an experiment to co-inject either TrkC polyclonal antibodies or isotype control Immunoglobulin-G (IgG) with IYIY-I\textsubscript{2}-BODIPY via tail vein was performed. Due to the high immunomodulation activity of IYIY-I\textsubscript{2}-BODIPY, it was
selected to be co-administered with antibodies. Co-injection with 10 µg/mouse of TrkC blocking antibodies reversed the IYIY-I2-BODIPY-mediated G-MDSC ($p = 0.016$) and neutrophil suppression ($p = 0.034$) in TM to levels similar as control saline, but this reversed activity was not observed to the same extent in the mice that received the isotype IgG control (Figure 4.7). Reversal was not observed in the spleen G-MDSC and neutrophil levels, suggesting that the blocking effect of antibodies was local and not systemic. The other possibility might due to abundant TrkC expression in TM, which attracts more anti-TrkC antibody to the TM, and thus increases the blocking activity. The exact reason of no reversal in spleen is unknown, but the overall result from the blocking experiment suggests that IYIY-I2-BODIPY mediated immunomodulation occurred in a TrkC dependent manner.

\[ Figure\ 4.7:\ IYIY-I_2\text{-}BODIPY\ mediated\ myeloid\ cells\ reduction\ is\ TrkC\ dependent.\]

4T1 tumour bearing mouse were randomly divided into four groups (saline, IYIY-I2-BODIPY, IYIY-I2-BODIPY + 10 µg/mouse isotype control IgG, IYIY-I2-BODIPY + 10 µg/mouse anti-TrkC blocking polyclonal antibodies). Compound, alone or in combination with antibodies as described was administrated into mice via tail vein, respectively. Mice were sacrificed 2 hours after compounds administration. Percentages of MDSCs and neutrophils in spleen and TM were quantified using flow cytometry after
cell surface staining. Data represent mean ± SEM of four mice. *p < 0.05 vs IYIY-I₂-BODIPY was analysed using student t-test.

4.3.8 Effect of conjugates administration in the dark on TrkC positive 4T1 tumour growth

Up to now, the data have shown that the ligated conjugates inhibited immuno-suppressive mediators and increased adaptive immune responses in breast tumour bearing mice. Next, the effect of conjugates to cause the delay in tumour growth in the dark was carried out. The experiment involved single bolus injections of 10 mg/kg of all three compounds via tail vein, and recordings of tumour volume three times per week. The data shown in Figure 4.8 was quantified in comparison to the initial volume of the respective tumours to eliminate tumour-to-tumour variation. As illustrated in Figure 4.8A, single bolus injection had no effect on delaying tumour growth, as the tumour sizes were comparable with saline control in all treated groups. However, multiple injections of three compounds with a regime of 10 mg/kg every day for five consecutive days resulted in marginal delay in tumour growth only after IYIY-I₂-BODIPY administration at the first three days following treatment (days 7-9 after tumour cells inoculation), and significant delay at days 10 (∼ 16%) and 11 (∼ 20%) post tumour cells injection compared to all controls (Figure 4.8B). Unfortunately, the growth delay did not persist, as the tumour sizes became comparable when the administration of IYIY-I₂-BODIPY was stopped. Even though the delay in tumour growth was statistically significant, the effect may not be strong or prolonged enough for clinical relevance. In addition, only IYIY-I₂-BODIPY, but not YIYI-I₂-BODIPY transiently delayed tumour growth, as well as elicited strong immune responses, thus demonstrated that tumour control was contributed by immune modulation. Previous study by Jin et al observed that suppression of TGF-β by ligand-activated TrkC promoted tumourigenesis (Jin et al., University of Malaya).
which is different from this study which showed conjugate-activated TrkC administered in dark inhibited TGF-β production and delayed tumourigenesis. The discrepancy may be due to the dual function of TGF-β both as a pro- and an anti-tumour mediator (Massague, 2008; Wrzesinski et al., 2007).

Figure 4.8: Multiple bolus i.v. administration of IYIY-I₂-BODIPY transiently delays tumour growth.

At 7 days post 4T1 inoculation, the mice were randomly divided into 4 groups. (A) For single bolus i.v. administration, the mice were injected with compounds (saline, I₂-BODIPY, YIYI-I₂-BODIPY and IYIY-I₂-BODIPY) at dosages as shown in figure 4.8. (B) For multiple bolus i.v. administration, mice were injected with compounds at a regime of once a day for 5 days consecutively. Tumour growth was monitored and data represent mean percentage of initial volume ± SEM of n=6. *p < 0.05 vs control saline, **p < 0.05 vs all three control groups using one-way ANOVA (Dunnett’s test).

4.3.9 Combination effect of conjugates and PDT in cytokine productions

Adaptive immunity is highly specific to the antigens presented to the immune cells and generally can provide long lasting protection. In cancer, adaptive immune cells such as CD4⁺ T helper cells and CD8⁺ T-cells are activated through presentation of tumour antigen in Major Histocompatibility Complex (MHC)-Class II and Class I by antigen presenting cells, respectively. Activated T-lymphocytes will undergo differentiation to
various subtypes depending on the cytokines milieu present. In PDT, antigen specific adaptive immune responses are activated by release of high quantities of tumour antigens by the damaged tumour tissues (Nowis et al., 2005; Reginato et al., 2014).

Data obtained until this point suggest that the IYIY-ligand had immuno-stimulatory properties, hence we hypothesized that photo-activation of the I₂-BODIPY photosensitizer counterpart of IYIY-I₂-BODIPY and YIYI-I₂-BODIPY (hereafter referred to as light treatment) following the administration of the conjugates into the mice may induce stronger adaptive immune responses. To explore this idea, tumour bearing mice were administrated with the IYIY-I₂-BODIPY, YIYI-I₂-BODIPY and I₂-BODIPY (10mg/kg equivalent dose) and illuminated with 100 J/cm² of light after a drug-light interval of 1 hour. Mice were then sacrificed 2 hours and 24 hours after PDT.

The levels of cytokines IL-2 (Figure 4.9A), IFN-γ (Figure 4.9B), TNF-α (Figure 4.9C), IL-4 (Figure 4.9D) and IL-10 (Figure 4.9G) post-PDT were found to be higher compared to in dark, but similar among the groups including saline control, indicating the presence of some light-induced immune effects. The level of IL-6 in plasma of IYIY-I₂-BODIPY treated mice was significantly high, at 2.4-fold higher at 2 hours ($p = 0.039$) and the level was elevated further at 24 hours to 5.2-fold higher compared to I₂-BODIPY treated mice. Conversely, the YIYI-I₂-BODIPY and I₂-BODIPY treated groups showed increased IL-6 level (3.6- and 2-fold respectively) compared to saline control only at 2 hours but not at 24 hours (Figure 4.9E). The increase of IL-6 in all treatment groups following PDT is concordant with literatures (Du et al., 2006; Gollnick et al., 2003). As before, the moderately high IL-6 level in YIYI-I₂-BODIPY treated group compared to I₂-BODIPY treated group is probably due to the weak binding of the conjugate to TrkC receptor.
Similar to trends in the dark experiments (Figure 4.1F), the level of IL-17A level rose from low (4.8% ± 0.4%) at 2 hours to 2-fold higher at 24 hours (Figure 4.9F) solely in IYIY-I₂-BODIPY treated group. Similar levels of TGF-β inhibition by both IYIY-I₂-BODIPY and YIYI-I₂-BODIPY were observed in the light (Figure 4.9H) compared to in the dark at 2 hours (Figure 4.1H), suggesting that the ligand-mediated inhibition effect was not intensified by light irradiation.

For ease of comparison, the data for IYIY-I₂-BODIPY (highest immunomodulatory activity) treated mice has been re-tabulated in terms of fold changes in the blood cytokines in light treatment compared to those in dark, at 2 and 24 hours post-PDT. Among the cytokines investigated, the biggest fold changes were: (i) 5.5-fold down-regulation of TGF-β and (ii) 13-fold up-regulation of IL-6, both at 24 hours. High TGF-β suppression is similar to the previously reported effect of ligand-activated TrkC receptor in inhibiting TGF-β signaling (Jin et al. 2007), whereas the elevation of IL-6 might be due to the effect of ligand in promoting TrkC-induced IL-6 secretion (Rezaee et al. 2010; Marshall et al. 1999). IL-17A was 2.8-fold increased in the light compared to in the dark at 24 hours post-PDT, further suggesting that the high IL-6 level induced by IYIY-I₂-BODIPY had facilitated IL-17⁺ T-cell differentiation and IL-17 secretion. The rest of the cytokines investigated had fold changes ranging from 1.25 to 2.4 (Figure 4.9I).
Figure 4.9: IYIY-I₂-BODIPY conjugate increases IL-6, IL-17 and decreases TGF-β cytokine levels post-PDT.

4T1 tumour bearing mice were treated with respective compounds via tail vain. Mice were kept in dark for 1 hour, anaesthetised and irradiated with 100 J/cm² of light at a fluence rate of 160 mW/cm². Mice were then sacrificed at 2 hours and 24 hours post-PDT. Cytokines and immune cells quantification were examined based on the methods explained in M&M. Levels of cytokines (A) IL-2, (B) IFN-γ, (C) TNF-α, (D) IL-4, (E) IL-6, (F) IL-17A, (G) IL-10 and (H) TGF-β are shown. Data represent mean ± SEM with minimum of four mice per group. (I) Fold changes for each sample in the IYIY-I₂-
BODIPY treated group were obtained by dividing the percentage in the light over the percentage in the dark. * $p < 0.05$, *** $p < 0.001$ vs I$_2$-BODIPY using one-way ANOVA (Dunnett’s test).

### 4.3.10 Effect of IYIY-I$_2$-BODIPY and irradiation on neutrophil populations

In the TM sample from the light experiment, the population of myeloid precursor cells including G-MDSC and neutrophils were examined. Data revealed that G-MDSC populations decreased as much as those in the dark experiment, which demonstrated that irradiation did not further affect ligand-mediated suppression (Figure 4.10A). In PDT, acute inflammation at the treatment site occurs as a result of production of pro-inflammatory cytokines and the presence of neutrophils (Cecic et al., 2001; Cecic et al., 2006; de Vree et al., 1996). Hence the neutrophil population in TM post-PDT was examined. Interestingly, IYIY-I$_2$-BODIPY, but not other control compounds, significantly increased the neutrophils in TM ($p = 0.035$) compared to saline, YIYI-I$_2$-BODIPY and I$_2$-BODIPY (Figure 4.10B). This suggests that IYIY-I$_2$-BODIPY recruits more neutrophils to TM post-PDT. This can be explained by the good antitumour efficacy of IYIY-I$_2$-BODIPY in causing tumour damage as observed in figure 3.8 and 3.10, leading to the local accumulation of inflammatory neutrophils. This is concordant with de Vree et al. 1996 who proposed that high PDT efficacy is associated with increased population of neutrophils in systemic circulation.
Figure 4.10: IYIY-I$_2$-BODIPY enhances neutrophils population post-PDT.

4T1 tumour bearing mice were treated with respective compounds via tail vein. Mice were kept in dark for 1 hour, anaesthetised and irradiated with 100 J/cm$^2$ of light at a fluence rate of 160 mW/cm$^2$. Mice were then sacrificed at 2 hours and 24 hours post-PDT. Tumour tissues were isolated and examined for (A) G-MDSC and (B) neutrophils. Data represent mean ± SEM with minimum of four mice per group. * $p < 0.05$ vs I$_2$-BODIPY using one-way ANOVA (Dunnett’s test).

4.3.11 Combination effect of conjugates and PDT in adaptive immune responses

Adaptive immunity is highly specific to the antigens presented to the immune cells and generally can provide long lasting protection. In cancer, adaptive immune cells such as CD4$^+$ T helper cells and CD8$^+$ T-cells are activated through presentation of tumour antigen in Major Histocompatibility Complex (MHC)-Class II and Class I by antigen presenting cells, respectively. Activated T-lymphocytes will undergo differentiation to various subtypes depending on the cytokines milieu present. In PDT, antigen specific adaptive immune responses are activated by release of high quantities of tumour antigens by damaged tumour tissues (Nowis et al., 2005; Reginato et al., 2014). Hence,
the populations of CD4$^+$ and CD8$^+$ T lymphocytes and subtypes in conjugates treated mice post-irradiation were investigated.

In tumour microenvironment, the number of Th1 was significantly more in IYIY-I$_2$-BODIPY treated mice at 2 hours post-PDT in TM compared to I$_2$-BODIPY treated mice (3.4-fold; $p = 0.024$). At 24 hours, the Th1 populations dropped to become comparable among the groups. Similar but weaker increase in Th1 population was observed in YIYI-I$_2$-BODIPY treated mice. For CD8$^+$ T-cells, IYIY-I$_2$-BODIPY treated mice had higher populations of CTL, which was 2.1-fold ($p = 0.033$) higher than I$_2$-BODIPY. (Figure 4.11A). YIYI-I$_2$-BODIPY treated mice have 1.6-fold higher CTL population compared to I$_2$-BODIPY treated mice. The increase of CTL might be due to the PDT effect, as CTL was not increased in YIYI-I$_2$-BODIPY treated mice in the dark. Similar in the dark, IYIY-I$_2$-BODIPY treated mice had higher Th17 and Tc17 cells, which were 3.4-fold and 2.9-fold higher compared to I$_2$-BODIPY treated mice at 24 hours, respectively (Figure 4.11B). Regulatory T-cell that was reported decreases post IYIY-I$_2$-BODIPY treatment was found to be decreased as well post-irradiation, suggest that PDT did not affect the ligand-mediated Treg inhibition (Figure 4.11C). Th2 cell population was reduced in the light at 2 hours post-PDT compared to in the dark (Figure 4.11D). The decrease of the Th2 cells post-PDT and increase of the Th1 cell population are in line with the known subtype balancing of Th1/Th2 cells, where activation of either cell type can down-regulate the other (Kidd P, 2003). The fold changes in immune cell populations in tumour microenvironment in the light treatment compared to those in the dark was tabulated as shown in Figure 4.11E. The Th1, Th17, CTL and Tc17 populations investigated in IYIY-I$_2$-BODIPY treated group were in increasing trend at 2 h and 24 h post-PDT compared to in the dark, ranging from 1.4- to 1.9-fold in Th1 and Th17 and 1.2- to 1.8-fold in CTL and Tc17. Altogether suggests the combine between
ligand and PDT. This data may also explain the superior antitumour activity that was observed for IYIY-I₂-BODIPY with PDT as shown in Figure 3.8.

Figure 4.11: IYIY-I₂-BODIPY combined with PDT induces stronger adaptive immunity.

4T1 tumour bearing mice were treated with respective compounds via tail vein. Mice were kept in dark for 1 hour, anaesthetised and irradiated with 100 J/cm² of light at a
fluence rate of 160 mW/cm². Mice were then sacrificed at 2 hours and 24 hours post-PDT. Immune cells in tumour microenvironment were quantification based on the methods explained in M&M. Data of (A) Th1, CTL; (B) Th17, Tc17; (C) Treg cell and (D) Th2 cell was quantified using flow cytometry. Data represent mean ± SEM of minimum four mice in each group. (E) Fold changes for each cell type in the IYIY-I₂-BODIPY treated group were obtained by dividing the percentage of population in the light over the percentage of population in the dark. * p < 0.05 vs I₂-BODIPY using one-way ANOVA.

4.3.12 Effector T cells quantification in compounds treated mice at 20 days post-PDT

Effective PDT of cancer would induce antitumour immunity through inflammation and adaptive immune responses (Cecic et al., 2001; de Vree et al., 1996), which could further lead to the development of immune effector against the tumour (Reginato et al., 2014). As in figure 3.8, 71% of IYIY-I₂-BODIPY treated mice were cured from tumour post PDT. Thus, an experiment was performed to study whether IYIY-I₂-BODIPY treated mice have increased CD4⁺ and CD8⁺ effector T-cell populations post PDT. Compounds treated mice underwent PDT and at 20 days post-PDT, TDLN and spleen was isolated and quantified for effector T-cells using CD44 as a surface marker. As expected, IYIY-I₂-BODIPY treated mice had significant high CD4⁺ (Figure 4.12A) and CD8⁺ (Figure 4.12B) effector T-cells in TDLN and spleen compared to the other three controls. However, no increase in effector T cells was observed in YIYI-I₂-BODIPY treated mice. This implies that IYIY-I₂-BODIPY was a more effective PDT agent in inducing antitumour effector T-cells than the YIYI conjugate.
Figure 4.12: CD4$^+$ and CD8$^+$ effector T-cells in TDLN and spleen at 20 days post-PDT.

PDT was conducted at 100 J/cm$^2$ based on four groups as indicated above. At 20 days post-PDT, mice were sacrificed for quantification of (A) CD4$^+$ and (B) CD8$^+$ effector T-cells in TDLN and spleen that expressed CD44 surface antigen using flow cytometry. Dot plot represents one of the four independent experiments and bar chart represents mean ± SEM of three to four mice for each group. $^* p < 0.05$; $^{**} p < 0.005$ vs three control groups using one-way ANOVA (Dunnett’s test).
4.3.13 Antitumour immunity in IYIY-I2-BODIPY treated survivor mice post-PDT

Lastly, the presence of long term immunity against aggressive 4T1 tumour cells in IYIY-I2-BODIPY treated survivor mice post-PDT was examined. The splenocytes and lymphocytes (suspension cells from TDLN) of healthy and survivor mice were isolated and adoptively transferred into syngeneic healthy recipient mice via tail vein. Recipient mice were challenged by subcutaneous injection of 4T1 tumour cells at 2 days post adoptive transfer as described in the schematic plan in figure 4.13A. Interestingly, growth delay was observed in the mice receiving splenocytes and lymphocytes from the survivor mice, compared to controls (splenocytes and lymphocytes from healthy mice). In brief, the delay in tumour growth was transient at days one to four post tumour inoculation and became significant (~ 50%) in both splenocytes and lymphocytes recipient mice at 7 days post inoculation (Figure 4.13B). The delay in tumour growth continued, with approximately 40% smaller tumours sizes in mice receiving immune cells from survivor mice at both 9 and 11 days post inoculation compared to mice with cells from healthy donors. Unfortunately, there was no full immunity in the mice after adoptive transfer, probably due to the highly aggressive and metastatic nature of 4T1 cells. However, the delayed tumour growth was significant as compared to controls, suggesting the presence of anti-tumour immune effector T cells. Taken together, the higher survivor rate previously observed in IYIY-I2-BODIPY PDT treated mice compared to the other controls (Figure 3.8) might have been due to the enhanced immune effector T cells caused by the combined TrkC and PDT therapy.
4.4 Discussion

The immunological impacts of a TrkC-targeted PDT agent upon compound administration in a TrkC positive 4T1 breast tumour model were examined. The findings showed that the targeted PDT agent in the dark selectively increased pro-inflammatory cytokines IL-2, IL-6 and IL-17, and inhibited immunosuppressive mediators such as TGF-β cytokine, G-MDSC and Treg cells. In addition, there is an increase in the CD4$^+$ and CD8$^+$ antitumour responses with IFN-γ and IL-17 phenotypes,
and growth delay of aggressive 4T1 tumours in mice. While IYIY-BODIPY in the dark activated the immune system and decreased tumour growth to some extent, the assault by reactive oxygen species upon PDT accentuated these effects, probably because the sensitivity of the immune system was increased due to formation of damaged tissue. Upon PDT, this conjugate also enhanced the population of effector T-cells and delayed the tumour growth through adoptive transfer of immune cells. This is the first report of antitumour immune responses elicited by a targeted ligand designed primarily to actively direct a therapeutic agent to cancer.

Throughout the study, the scrambled control YIYI-I$_2$-BODIPY showed similar, but weaker immune-modulation properties than IYIY-I$_2$-BODIPY. This might be due to the isomeric characteristics of scrambled ligands (reversed order of amino acids), leading to similar, albeit weaker binding affinity to the targeted receptor compared to the unscrambled ligands (Koolpe et al., 2002). Another possibility is the presence of multiple bioactive conformations in peptide ligands that can be recognised by more than one receptor types (Pedragosa-Badia et al., 2013). It is therefore possible that the YIYI-I$_2$-BODIPY scrambled control adopts a bioactive conformation that binds to other unknown receptors to modulate immune response.

IYIY-I$_2$-BODIPY induced IL-6 secretion and reduced G-MDSCs and neutrophils both in dark and light treatments. The concurrent reduction in G-MDSCs and neutrophils however is in contrast as in other studies that reported correlative increases in IL-6 with MDSC expansion and inflammation (Bunt et al., 2007; Meyer et al., 2011; Mundy-Bosse et al., 2011). It is possible that the TrkC ligand mediated MDSC suppression was independent of IL-6 cytokine, but through direct binding to myeloid cells, since blocking with TrkC antibodies could reverse the IYIY-I$_2$-BODIPY-mediated
MDSC suppression. Another explanation for the mechanism of MDSC suppression may involve transcription factor STAT-3 which is an essential mediator for regulating myeloid progenitor proliferations (Zhang et al., 2010), as well as a downstream mediator of Trk signaling (Ng et al., 2006). In tumour bearing hosts with inflammation, MDSC is considered a subpopulation of neutrophils that have acquired suppressive phenotype (Pillay et al. 2013). Thus, suggests that the conjugates mediated MDSC and neutrophils suppression is concurrent.

Studies have revealed that the balance of Treg and Th17 differentiation is regulated by the relative abundance of differentiation factors TGF-β and IL-6. A high level of TGF-β generally promotes Treg differentiation and inhibits Th17 (Zhou et al., 2008), whereas a high IL-6 level stimulates Th17 differentiation and antagonises Treg formation (Zhou et al., 2007). The increased of Th17 upon IYIY-I₂-BODIPY administration both in dark and light treatments were in concordance with the latter scenario. Th17 cells secrete IL-17 cytokine that is known to possess both protumour and antitumour functions (Murugaiyan & Saha, 2009). The IYIY-BODIPY mediated IL-17⁺ cells in this study probably functioned in an antitumour way based on the observed delay in tumour growth post IYIY-I₂-BODIPY administration, the reduction of TGF-β which is known to block the production of vascular endothelial growth factor (VEGF), a potent angiogenic factor (Huang & Lee, 2003; Jeon et al., 2007) and, increased in Th1 and CTL cell populations which have been reported in the presence of IL-17 (Benchetrit et al., 2002; Muranski et al., 2008).

TGF-β signaling has dual functions in cancer: (i) as a tumour suppressor to mediate growth arrest and apoptosis in cancer cells (antitumour) (Derynck et al., 2001; Pardali & Moustakas, 2007), and (ii) as a potent immunosuppressive agent to suppress both innate
and adaptive immune responses comprising of CD4\(^+\) effector T-cells (Th1 and Th2), CD8\(^+\) cytotoxic T cells (CTLs), NK cells and antigen presenting cells, as well as to stimulate the generation of regulatory T-cells which inhibit effector T-cell functions (protumour) (Massague, 2008). Jin and co-workers reported TrkC and its tyrosine kinase activity mediate tumourigenesis \(\text{via}\) suppression of the TGF-\(\beta\) signaling (Jin et al., 2007), suggesting that TrkC acts as a pro-tumour agent by inhibiting tumour suppressor TGF-\(\beta\). Contrary to this, our data suggests that TrkC and TGF-\(\beta\) played an anti-tumour role. To explain, the binding of TrkC by the IYIY conjugate might have activated the tyrosine kinase activity of TrkC and subsequently blocked the TGF-\(\beta\) signalling and inhibited TGF-\(\beta\) production at an early time point, which in turn reduced the Treg cells in a late time point to overall delay the tumour growth.

TrkC has also been reported to play a role in regulating the systemic balance of T helper subtypes. A study from Sekimoto and colleagues revealed that neurotrophin-3 (TrkC natural ligand) increased the secretion of IL-4 by Th2 subtype, but did not affect the Th1 population IFN-\(\gamma\), as Th1 did not express TrkC but Th2 did expressed (Sekimoto et al., 2003). Other studies reported that neurotrophins increase IL-6 secretion \(\text{via}\) Trk receptors (Marshall et al., 1999; Rezaee et al., 2010). Hence, the increase in IL-4 and IL-6 levels observed upon IYIY-I\(_2\)-BODIPY administration is in agreement with what has been reported in literature. However, this increase in IL-4 and Th2 cells was also observed in I\(_2\)-BODIPY and YIYI-I\(_2\)-BODIPY control, suggesting that the increased in control groups might due to other unknown factors mediated by ligands or I\(_2\)-BODIPY itself.

PDT can lead to systemic induction of IL-6 (Du et al., 2006; Gollnick et al., 2003), and this cytokine has been studied to possess dual functions, either in enhancing PDT
efficacy (Barathan et al., 2013; Usuda et al., 2001; Wei et al., 2007) or inhibiting PDT mediated antitumour immunity (Brackett et al., 2011) via regulation of apoptotic proteins. The results in this study suggest that IL-6 was an antitumour factor, likely by enhancing PDT efficacy via upregulation of Th17 and Tc17 cells. This is contrary to the findings by Brackett et al. 2011, where they reported IL-6 attenuated PDT mediated antitumour immune memory in an IL-6 knockout mouse model implanted with 4T1 breast mammary tumour. The differences in findings might due to the different drugs administered (the ligand in IYIY-I2-BODIPY has additional immunomodulation properties) or the extreme difference in total systemic IL-6 level in different genetic background of mice (IL-6 knockout vs normal). The role of IL-6 in PDT mediated immune responses may depend on the severity of inflammation and its action in mediating the balance between differentiation of Th17 and Treg cells.

As shown in figure 4.8, single bolus administration has no antitumour effect, whereas multiple bolus administration only induced transient effect, and diminished when IYIY-I2-BODIPY was stopped giving to the mice. Thus, predicted that adoptively transfer of the immune cells from IYIY-I2-BODIPY treated donor mice without irradiation has no antitumour effect in recipient mice. Although the adoptive transferred of immune cells from survivor donor mice only induced delay in tumour growth, but not full immunity in recipient mice. However, this is sufficient to demonstrate the presence of effector T cells. Perhaps the antitumour effect would be further enhanced via in vitro expansion of the isolated T cells, which is a recognised adoptive immune cell therapy for cancer treatment.
4.5 Conclusion

Stimulation of inflammatory IL-6, IL-17 cytokines and inhibition of immunosuppressive mediators are potential contributors of immune stimulatory effects in IYIY-ligand, as summarised in figure 4.14. Hence, the conjugate has promise as a therapeutic agent for cancer treatment to selectively destroy the tumour cells in PDT and simultaneously stimulates antitumour immunity in the host.

![Diagram](image)

**Figure 4.14:** Summary findings of TrkC targeted PDT agent on immune responses.
5.1 Overall Conclusion

Enhanced delivery of anticancer drugs to the tumour tissue through the conjugation of the drugs to small molecule ligands that target the molecules overexpressed on cancer cell surface is one among the increasingly favorable approaches in anticancer therapy research. Active targeting of ligand-drug conjugates can be used to target cancer cells that are resistant to the free drug so as to enhance the anticancer effects (Yang et al., 2009; Zhang et al., 2008).

Previously, IYIY, a synthetic TrkC ligand has been shown to possess good selectivity in binding to cancer cells over-expressing TrkC receptor in vitro (Kamkaew and Burgess, 2013). These studies further proved that IYIY retained its selectivity in tumour cell binding upon conjugation to a model photodynamic therapy (PDT) agent I$_2$-BODIPY. The formed ligand-drug conjugate, i.e. IYIY-I$_2$-BODIPY has shown selective cell binding and cytotoxicity to TrkC expressing cancer cells, and have selectively accumulated in TrkC expressing tumour in vivo, with maximum accumulation at 1 hour post compound administration and at a significantly higher level (2.1-fold) compared to a scrambled control. In addition, among the major organs investigated, tumour tissue is the third highest in IYIY-I$_2$-BODIPY accumulation after excretory organs liver and kidney. In contrast, accumulation of YIYI-I$_2$-BODIPY in tumour was lower than excretory organs, lymph nodes and lung. This suggests the selectivity of IYIY-I$_2$-BODIPY in TrkC positive cells. In antitumour evaluation using TrkC expressing tumour model, the IYIY-I$_2$-BODIPY possesses superior antitumour activity with 71% mice showing no sign of primary tumour regrowth and metastasis for up to 90 days of
observation, and was confirmed by histopathological evaluation. The selectivity was further confirmed when moderate antitumor activity was observed in non-TrkC expressing tumour model.

As the IYIY was able to mimic the TrkC natural ligand NT-3 in activating signal transduction that regulate neuronal cell survival and differentiation (Chen et al., 2009); NT-3 can function as non-cytokine mediators to modulate both innate and adaptive immune responses (Matsuda et al., 1988; Marshall et al., 1999; Sekimoto et al., 2003; Rezaee et al., 2010). Hence, further investigation was conducted to access the immunomodulation capability of the IYIY-I\textsubscript{2}-BODIPY. This study shown that TrkC targeted ligand-drug conjugates (IYIY-I\textsubscript{2}-BODIPY and YIYI-I\textsubscript{2}-BODIPY) modulates immune responses through (i) the inhibition of immunosuppressive mediators (TGF-β, granulocytic-MDSC and Treg cells) and (ii) the stimulation of antitumour immune responses (Th1, CTL, Th17, Tc17). Moreover, IYIY-I\textsubscript{2}-BODIPY treated mice have higher effector T-cells post-light irradiation compared to all control groups. Adoptive transfer of immune cells from the IYIY-I\textsubscript{2}-BODIPY treated tumour survivor mice to other tumour bearing mice significantly delayed the tumour growth in these mice compared to mice receiving immune cells from healthy donors (Figure 5.1).
5.2 Future Perspectives

Currently, Trk expressing cancers including pancreatic, neuroblastoma and lymphoblastic leukemia are treated with Trk inhibitors such as Lestaurtinib and PLX7486 both in animal (Iyer et al. 2010) and clinical trial (Chan et al. 2008; Clinical Trials Identifier number: NCT01804530, NCT00557193). This TrkC targeted ligand-drug conjugate study resembles the first to report on the use of synthetic peptidomimetic ligand to target photosensitisier to TrkC expressing tumours in PDT.

The results obtained suggest that conjugation of the IYIY, or any other TrkC targeting ligand to FDA-approved anticancer drugs may be a potential approach to
improve the selectivity and efficacy profiles of the anticancer therapeutics in the treatment of TrkC expressing cancer. In addition, studies reported that small molecule Trk inhibitors were able to suppress the activities of TrkA/B/C kinases mediated survival pathway in cancer and subsequently inhibited tumour growth (Iyer et al., 2010; Minturn et al., 2011). Thus, combination therapy by using IYIY-I$_2$-BODIPY with Trk inhibitors (Lestaurtinib, PLX7486) is possible in order to increase the therapeutic efficacy on TrkC expressing cancer. Besides, mice treated with the synthetic TrkC targeted IYIY-I$_2$-BODIPY were found to develop significant antitumor immune responses post-PDT. This further suggests the possible use of the IYIY-ligand as a systemic photoimmunotherapeutic agent when combined with therapies such as photothermal therapy and sonodynamic therapy that can provoke the abundant release of tumour antigens. The shortcoming of this study is lacking of biodistribution data on brain organ. This is important to verify, as TrkC receptor is found predominantly in brain and neuronal cells. The ability of conjugates across the blood brain barrier to the brain is interesting to explore, perhaps the IYIY-ligand can be used to treat glioblastoma, in which the treatment remains one of the most challenging tasks in clinical oncology.

TrkC ligand can also be conjugated with imaging probes for theranostic purposes such as tracing biodistribution of drugs and detection of local and metastatic TrkC expressing tumours in a more precise way. This could be very useful in clinical for monitoring the status of TrkC in cancer patient. Despite of neurotoxicity characteristics of the IYIY-I$_2$-BODIPY prototype (probably via binding to Trk receptors of neuronal cells), the antitumor efficacy on TrkC+ tumour was superior at half-maximum tolerated dose. The toxicity problem can be reduced by conjugating IYIY ligand (active targeting) on nano-carrier (passive targeting). Through a combination of active and passive targeting approach, the ligand-nano-carrier complexes tend to accumulate more in the
tumour region via enhanced permeation and retention (EPR) effect compared to non-targeted neuron cells. Moreover, the amount of IYIY-ligand used as active targeting in this approach can be reduced, and hence reduced the undesired toxicity. This study showed that IYIY-ligand is very potent against TrkC+ tumour cells, targeting TrkC using the IYIY-ligand maybe benefit for TrkC expressing human cancer. Perhaps combination effect of IYIY-ligand-drug conjugate with Trk inhibitors such as lestaurtinib and PLX7486 (Chan et al., 2008; Iyer et al. 2010) can enhance the antitumour effect. In term of therapeutic purposes in PDT, further modification of IYIY-conjugate such as using a longer wavelength (600 nm and above) photosensitiser for deeper tissue penetration (Salva, 2002; Ballou et al., 2005; Frangioni, 2003; Rao et al., 2007; Sevick-Muraca et al., 2002) would be ideal.

Among the available anticancer therapies, immunotherapy has been recognised as an effective approach for anticancer therapy (American Association of Cancer Research). Recently, a study was conducted by Spiegel’s group on the use of antibody-recruiting small molecule (ARM-U2) as an immunotherapeutic agent for treatment of urokinase-type plasminogen activator receptor (uPAR) expressing cancer. The ARM-U2 act as a targeting agent to bind uPAR expressed on cancer cells, whereas the antibody will attract immune cells to ARM-U2/uPAR complex in cancer cells and mediates antibody-dependent cellular phagocytosis (Rullo et al. 2016). Perhaps this approach can be applied in the treatment of TrkC expressing cancer by conjugating similar antibody to TrkC targeting-ligand for effective therapeutic outcomes, particularly when newer TrkC ligand that are more selective in receptor targeting is developed.
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LIST OF PUBLICATIONS AND PAPERS PRESENTED

A) Research Presentations

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TrkC Ligand Conjugated Boron-Dipyromethene (BODIPY) Improves Efficacy and Eradicates TrkC Expressing Tumour in Photodynamic Therapy

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