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A Review on the Folate-Linked Prodrugs for Cancer Chemotherapy

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Abstract

During the last few decades, many methods have been developed in order to facilitate the drug design and discovery phases. Most of these methods were devoted to find new chemical entities that provide the most meaningful interaction with the desired receptors or enzymes with the potential to have minimal unwanted interaction. However, this strategy is time consuming, costly and requires screening of thousands of molecules for biological activity of which only one might enter the drug market. One of the most attractive and promising method is the prodrug approach, in which the active drug molecule is masked by a promoiety to alter its undesired properties.It is concluded that These FR-targeted technologies can also pave the way for inspiring further sophisticated drug conjugates, especially as this receptor is being targeted by use of several complementary technologies: small molecule, nanoparticle and protein-based thus providing broad and distinct knowledge in the area.

Keywords: Prodrug, Folate, Small molecule–drug conjugates, Light-triggered drug release, Nanotubes.

1. Introduction

1.1 Prodrug

Generally, a drug is characterized by its biological and physicochemical properties. Some of the used drugs have undesirable properties that result in an inefficient delivery and unwanted side effects. The physicochemical, biological and organoleptic properties of these drugs should be improved in order to increase their usefulness and their utilization in clinical practice (Stella, 2010;Karaman et al., 2013).

During the last few decades, many methods have been developed in order to facilitate the drug design and discovery phases.Most of these methods were devoted to find new chemical entities that provide the most meaningful interaction with the desired receptors or enzymes with the potential to have minimal unwanted interaction. However, this strategy is time consuming, costly and requires screening of thousands of molecules for biological activity of which only one might enter the drug market.

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One of the most attractive and promising method is the prodrug approach, in which the active drug molecule is masked by a promoiety to alter its undesired properties (Jana*et al.*, 2010;Venkatesh and Lipper, 2000).

The prodrug, and also called proagent, term was introduced for the first time by Albert as a pharmacologically inactive moiety which is converted to an active form within the body (Albert, 1958).This term has been successfully used to alter the physicochemical, pharmacokinetic properties, (absorption, distribution, excretion and metabolism) of drugs and to decrease their associated toxicity (Stella*etal.*, 2007).

A prodrug must undergo chemical and/or enzymatic biotransformation in a controlled or predictable manner prior to exert its therapeutic activity (Stella and Nti-Addae, 2007).Basically, the use of the term prodrug implies a covalent link between an active drug and a promoiety (Figure 1) (Rautio et al., 2008).

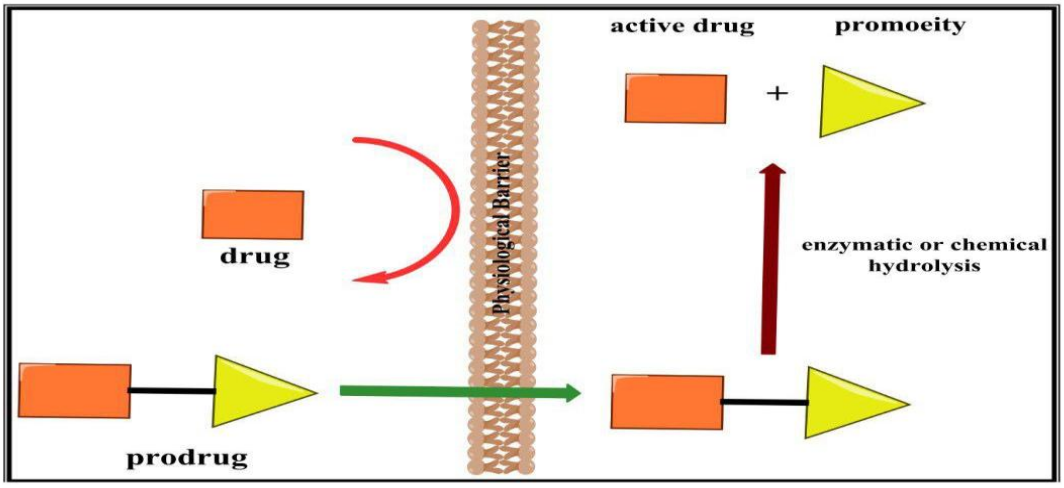

Figure 1. Schematic representation of a prodrug strategy.

This strategy is designed to overcome barriers through a chemical approach rather than a formulation approach (Müller,2009).In general, the imminent goal behind the use of prodrugs is to develop new entities that possess superior efficacy, selectivity, and reduced toxicity (Jana*et al.*, 2010).

An ideal prodrug should undergo biotransformation rapidly via chemical or enzymatic process to its active form and a non-toxic moiety within the body (Stella and Nti-Addae, 2007;Chipade *et a*l., 2012).

The prodrug must release the active drug and the promoiety prior to, during, or after absorption, or in a specific target tissue or organ, depending upon the purpose of which the prodrug has been designed(Stella*et al.*, 1985).

Nowadays, the prodrug approach is considered as one of the most promising site selective drug delivery strategies that utilize target cell- or tissue-specific endogenous enzymes and transporters (Han and Amidon, 2000).

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Earlier examples of compounds fulfill the classical criteria of prodrug were acetanilide and phenacetin, which exhibit their activities after being metabolized within the body (Albert,1958). Acetanilide is an antipyretic agent that was in use since 1886. It undergoes metabolism (aromatic hydroxylation) to paracetamol. This is similar to phenacetin which produces paracetamol via O-dealkylation (Figure 2)(Bertolini*et al.*, 2006).

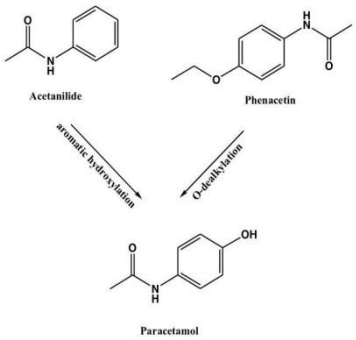

Figure 2: Phenacetin and acetanilide metabolism.

1.2Prodrugs classification

The conventional method used to classify prodrugs is based on derivatization and the type of carriers attached to the drug. This method classifies prodrugs into two sub-major classes:

(1) Carrier-linked prodrugs: in which the promoiety is covalently linked to the active drug but it can be easily cleaved by enzymes (such as an ester or labile amide) or non-enzymatically to provide the parent drug. Ideally, the group removed is pharmacologically inactive, nontoxic, and non-immunogenic, while the promoiety must be labile for in vivo efficient activation(Jana *et al.*, 2010; Stella,1975).

Carrier-linked prodrugs can be further subdivided into: **(a) bipartite** which is composed of one carrier (promoiety) attached directly to the drug, **(b)** t**ripartite** which utilizing a spacer or connect a group between the drug and a promoiety. In some cases bipartite prodrug may be unstable due to inherent nature of the drug-promoiety linkage. This can be solved by designing tripartite prodrug and (c**)mutual prodrugs**, which are consisting of two drugs linked together

(2) Bioprecursor prodrug, which are chemical entities that are metabolized into new compounds that may be active or further are metabolized to active metabolites (such as amine to aldehyde to carboxylic acid). In this prodrug type there is no carrier but the compound should be readily metabolized to induce the necessary functional groups(Stella *et al*., 2007; Müller, 2009; Roche,1977).

1.3Folates

Folate is an essential nutrition component (important B vitamin) in the human diet, involved in many metabolic pathways, mainly in carbon transfer reactions such as

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purine and pyrimidine biosynthesis and amino acid inter-conversions. Folates exist as vitamers (one carbon folate derivatives) that are ploy glutamated with varying oxidation states and substituents (Kariluoto *et al*.,2010).

Folates are important as they synthesize neurotransmitters by depleting excess homocysteine from the blood, thereby benefiting cardiovascular disease patients (Blom and Smulders, 2011).The major sources of folates are green leafy vegetables, liver, beans and legumes, egg yolk, wheat germ, yeast, and folate fortified breakfast cereal products.

Folates include naturally occurring folates and synthetic folic acid in supplements and fortified foods (Allen,2008; Iyer and Tomar, 2009). Natural folates exist in different forms that vary in both their oxidation state and the carbon group linked to the N5 and N10 positions of the pteridine ring (Serrano-Amatriain *et al.*, 2016).

Based on the literature, common natural folates are grouped into 5-methyl-tetrahydrofolate (5-CH_3-THF), formyl folates and unsubstituted folatesas depicted in (Figure 3).According to the oxidation states of the pteridine moiety, unsubstituted folates mainly consist of three types: fully oxidized folic acid (FA), reduced 7,8-dihydrofolate (DHF) and 5,6,7,8-tetrahydrofolate (THF) (Strandler *et al.*, 2015).

Formyl folates include 5-formyl-tetrahydrofolate (5-HCO-THF) and 10-formyl-tetrahydrofolate (10-HCO-THF) as well as their interconversion products such as 5,10-methenyl-tetrahydrofolate (5,10-CH_2-THF), 5,10-methylene-tetrahydrofolate (5,10-CH_2-THF), and 5-formimino-tetrahydrofolate (5-CHNH-THF).(Jagerstadand Jastrebova, 2013).

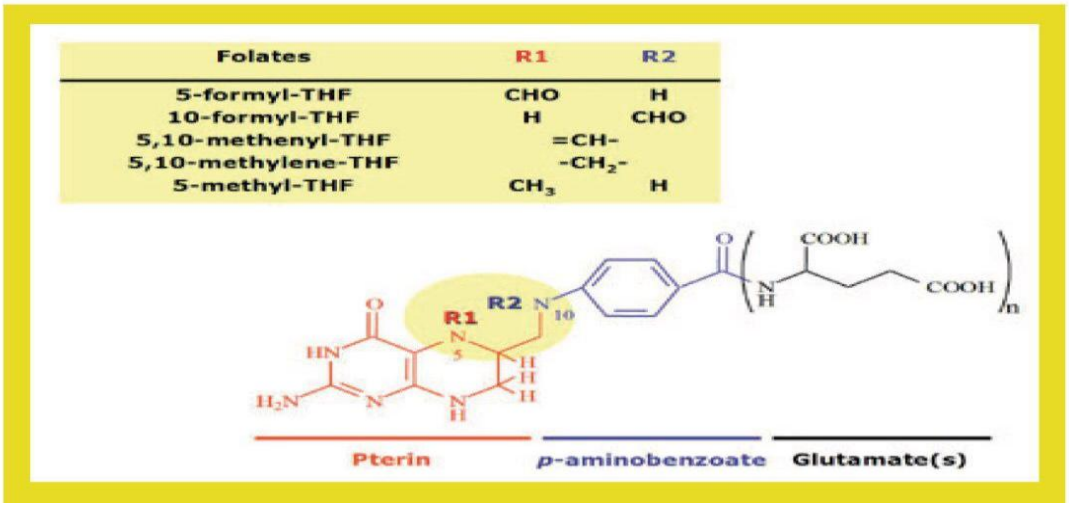

Figure 3. structure of natural folates (reduced one carbon substituents of polyglutamates) (Taiz and Zeiger, 2010).

Most naturally occurring folates are pteroylpolyglutamates, containing two to seven glutamates joined in amide linkages to the γ-carboxyl of glutamate. The principal intracellular folates are pteroylpentaglutamates, while the principal

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extracellularfolates are pteroylmonoglutamates. Pteroylpolyglutamates with up to 11 glutamic acid residues exist naturally. (LeBlanc *et a*l., 2007).

1.4 Folate receptor

It is a cell surface glycoprotein of molecular weight in the range of (35-40 kDa) known as the folate receptors (FRs) (Quici et al., 2015).It can be divided into three different isoforms: FRα, FRβ and FRγ. The α and β variants are attached to the cell membrane via glycosylphosphatidylinositol (GPI) anchors, whereas FRγ is found only in hematopoietic cells (Mironava et al., 2013), and lacks the GPI component, making it freely soluble(Quici et al., 2015; Ledermann et al., 2015).FRβ, which shares ~70% sequence homology with FRα, is most frequently found in a non-folate-binding isoform on normal granulocytes, possibly due to an alternative posttranslational modification(Vaughan et al., 2011).

The FR-α and -β transport folates into cells via receptor-mediated endocytosis. Although all FRs have been reported to have high binding affinity with folic acid, relative affinities of FR-α and -β for folate conjugates are significantly different, in the range of 2~100 fold (Wang *et al* ., 1992).

1.5Up-regulation of folate receptor in cancer chemotherapy

FRβ is upregulated on activated myeloid cells (primarily monocytes and macrophages) that participate in inflammatory and autoimmune diseases (Xia*et al.*, 2009; Puig-Kroger*et al*., 2009).The FRβ isoform has also been detected in tumor-associated macrophages (TAMs) of many cancers, including those of the liver, kidney, skin, lung, blood and soft tissue.(Kuraharа*et al*., 2012;Sun *et al*., 2014;Shen *et al.*, 2015).

These macrophages can penetrate solid tumors and promote their metastasis and growth by suppression of $CD8^+$ T cells and secretion of proangiogenic factors (Feng*et al.*, 2011).FRβ expression is regulated by retinoid receptors and can be upregulated by all-trans retinoic acid, particularly in combination with histone deacetylase inhibitors (Wang *et al.*, 2000).The FRβ isoform can consequently serve as a potential target for the selective delivery of cytotoxic agents in cancer treatment. (Pan *et al.*, 2000).

Notwithstanding FRβ's expression on some cancers, the FRα isoform has the most potential for targeted cancer therapy as it is the most widely expressed of all the FR isoforms (Chen*et al.*, 2013)and is overexpressed in a large number of cancers of epithelial origin, including breast (Patel *et al*., 2016),lung, kidney and ovarian cancers (Siwowska *et al*., 2017).

Cancer types such as endometrial, cervix, ovary, testicular choriocarcinoma, lung, colorectal, pediatric ependymomas, mesotheliomas, and renal cell carcinomas show FRα over-expression(Chancy *et al*., 2000; Garin-Chesa,1993). The FRα over-expression in these carcinomas are about 100–300 times higher than on healthy cells and in the order of 1–10 million receptor copies per cell. (Sun *et al.*, 2015; Vlahov and

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Leamon, 2012). It has also been shown that FRα has a low expression on the apical surface of most normal cells. This difference in expression makes FRα a very attractive therapeutic target for novel anticancer agents that would have limited toxicity on normal tissues (Lorusso *et al*., 2012; Bellati *et al*., 2011).

FRγ has been detected in normal and malignant hematopoietic cells, as well as in carcinomas of the ovary, endometrium, and cervix (Kelemen, 2006; Shen *et al*., 1995; Salazar and Ratnam, 2007).

1.6Examples of folate-linked prodrug

1.6.1Small molecule–drug conjugates (SMDCs)

This ability to attach chemical warheads to ligands that seek out FRα-expressing tumors confers excellent selectivity to the construct while preserving drug potency and this approach has led to the development of many small molecule–drug conjugates based on folic acid (FA–SMDCs).

1.6.1.1Vintafolide

The most successful FA–SMDC is vintafolide, (formerly EC145): a water-soluble conjugate that selectively delivers the drug desacetyl vinblastine monohydrazine (DAVLBH) to tumors that overexpress FRα.29 Preclinical studies have shown vintafolide to bind to FRα with high affinity, and therefore has very specific and potent activity against FRα positive tumor xenografts as opposed to the untargeted DAVLBH .

The four constituent modules of vintafolide consist of: (1) a folic acid moiety to target FRα, (2) a hydrophilic peptide spacer, (3) a self-immolative disulfide linker, and (4) a microtubule-destabilising drug DAVLBH (Figure 4). (Vlahov and Leamon, 2012).

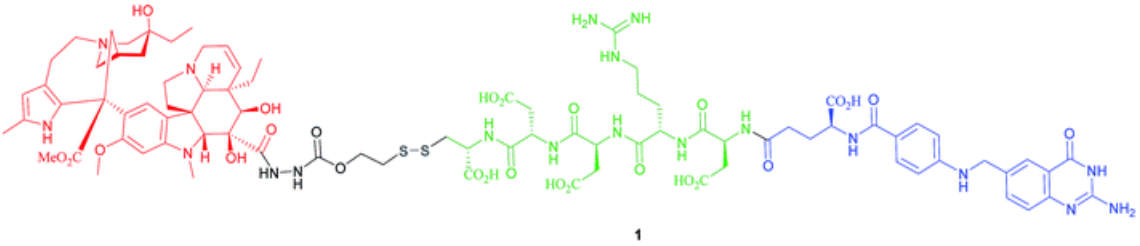

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Figure 4. Chemical structure of the folic acid-based SMDC vintafolide 1 is comprised of a folate targeting ligand (blue), a peptide spacer (green), a self-immolative disulfide linker (grey) and the potent cytotoxic drug DAVLBH (red)

Since folic acid is lipophilic, the spacer serves to ameliorate the overall water solubility of the drug conjugate and in so doing, eliminates non-specific diffusion across cell membranes and ensures cell internalization via receptor-mediated endocytosis (RME). Typical examples of spacers commonly employed in FA–SMDCs include polysaccharides, peptides and polyethylene glycol (PEG) chains(Srinivasarao*et al.*, 2015 ;Vlahov and Leamon,2012).

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An additional function provided by the spacer is to physically separate the drug cargo and targeting ligand, thereby minimizing steric interference between the two and ensuring the retention of receptor binding affinity for the ligand (Srinivasarao et al., 2015 ;Vlahov and Leamon, 2012) .However, spacer length should not be too great as long, flexible spacers can allow the drug moiety to loop back and interact with the targeting ligand, jeopardizing its affinity for the receptor (Srinivasarao et al., 2015).Small size (typically lower than 2000 Da) is critical for superior FA–SMDC tumor penetration and rapid systemic clearance. (Vlahov and Leamon,2012).

Possessing a molecular weight of 1917 Da, vintafolide fulfills this criterion and displays a distribution time of 6 minutes(Bailly, 2014).This short delivery time indicates rapid uptake of the drug conjugate by FR-positive tumor tissue, which is a desirable characteristic in minimizing circulation time, and thus precluding premature drug release. This FA–SMDC is also rapidly cleared from the body (elimination half-life of 26 min) via the kidneys and liver(Vergote and Leamon, 2015).

1.6.1.2Folate–taxoid conjugates

Seitz et al. have developed a highly potent next-generation folate–taxoid for use against drug-resistant and drug-sensitive cancer cell lines.(Seitz*et al*.,2015).This folate–taxoid conjugate incorporates a folic acid targeting moiety and a highly potent taxoid SB-T-1214, which is a derivative of the chemotherapeutic drug Taxol. Similar to vintafolide, this SMDC possesses a self-immolative disulfide linker, and a hydrophilic PEGylated dipeptide spacer (Figure 5). (Seitz *et al*.,2015).

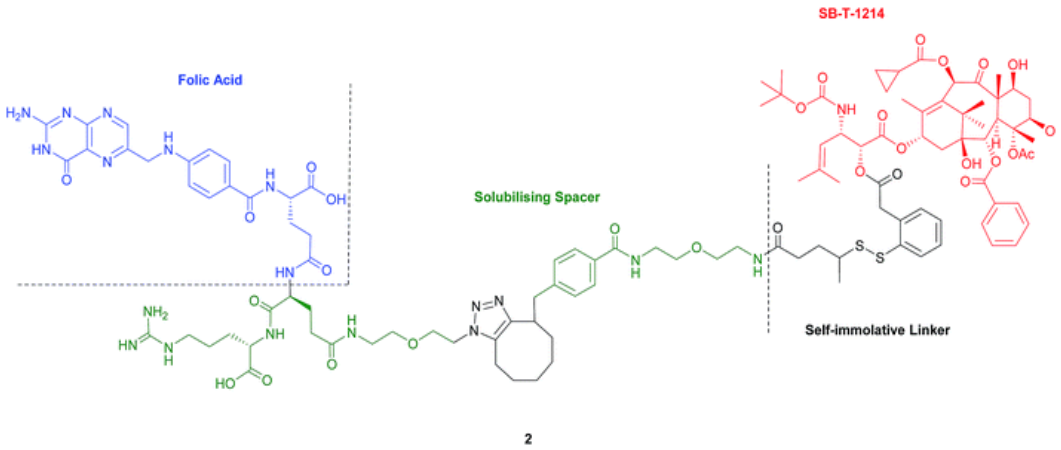

Figure 5. Structure of the folate–taxoid conjugate 2 developed by Seitz *et al*

In vitro analysis was carried out to compare the activity of the taxoid conjugate 2 and free SB-T-1214 in FRα-positive and FRα-negative cells. As expected, free SB-T-1214 was highly potent against all cell lines. Conversely, taxoid conjugate 2 exhibited appreciable cytotoxicity against the FRα-positive cell lines, displaying IC_{50} values more than three times smaller than those observed for the FRα-negative cells. This notable potency has been ascribed to the uptake of the folate–taxoid 2 occurring via RME, an internalisation pathway unaffected by the folic acid naturally present in the

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cell culture medium, which suggests that folic acid required for cell growth is principally shuttled into cells through folate transport proteins in lieu of RME. Further, taxoid conjugate 2 also exhibited an over 1000-fold decrease in toxicity against healthy cells compared to the free drug. As with vintafolide, the cytotoxic activity of 2 stems from intracellular GluSH-triggered reduction of the disulfide linker to release the free toxic drug SB-T-1214 (Seitz*et al*.,2015).

Ideally for maximum biological activity, the drug should be released in its unmodified form, as with conjugate 2, giving further weight to the aforementioned speculation that the failure of vintafolide analogues may be due to the liberation of a chemically altered payload(Khalil and Mustafa, 2020; Mohammed and Mustafa, 2020; Mustafa, Bashir, *et al.*, 2020; Mustafa, Mohammed, *et al.*, 2020; Oglah and Mustafa, 2020a, 2020b). Moreover, the efficient release of the chemical warheads is contingent on the GluSH levels present in the intracellular milieu, the concentration of which can vary in different cell lines(A.M. Nejres *et al.*, 2020; Aws Maseer Nejres *et al.*, 2020; Moath Kahtan Bashir *et al.*, 2020; Mustafa, Khalil, *et al.*, 2020; Mustafa, Oglah, *et al.*, 2020; Oglah and Mustafa, 2020b; Oglah, Mustafa, *et al.*, 2020). It is therefore important to consider this particular variation when selecting tumor cell lines to be targeted by SMDCs whose activity is dependent on the intracellular GluSH concentration(Mustafa, 2019; Aldewachi *et al.*, 2020; Moath Khtan Bashir *et al.*, 2020; Mustafa and Abdulaziz, 2020; Oglah, Bashir, *et al.*, 2020). Partly in view of this potential complication/limitation with certain cancer cells and serum stability questionability, FA–SMDCs have been developed where degradation to release free drug is not mediated by intracellular GluSH(Mahmood *et al.*, 2014; Mustafa, 2018; Mustafa *et al.*, 2018, 2021).

The above examples comprise a small, but representative, selection of FA–SMDCs from a vast field of conjugates that employ a disulfide linker for cytotoxic drug release. It is of particular relevance to highlight that folate conjugates to many other drugs via a disulfide linker, such as mitomycins(Reddy *et al*., 2006), tubulysins (Leamon et al.,2008) and camptothecins,(Henne*et al*., 2013) have been prepared and appraised.

1.6.1.3Dendritic β-galactosidase-responsive folate–monomethyl-auristatin E conjugate

There are a variety of free thiol-containing compounds present in the blood and as such, the disulfide bond in FA–SMDCs is susceptible to cleavage in circulation by these thiols, potentially giving rise to undesired premature drug release. Consequently, alternative approaches have been developed in which the FA–SMDCs do not possess disulfide linkers, a structural property which would ideally minimize off-target drug liberation in the bloodstream.

One such example developed by Alsarraf et al. is the β-galactosidase-responsive drug conjugate 3 that delivers the potent antineoplastic drug monomethylauristatin E

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(MMAE) to cancer cells (Alsarraf et al., 2015). This SMDC consists of a galactoside trigger, phenolic and aniline self-immolative linkers, a folic acid targeting ligand and two MMAE molecules centeredon a chemical amplifier, enabling a release of two drug molecules via a single internalization and activation pathway. The warhead release mechanism was studied by incubating folate-conjugate 3 with β-galactosidase at pH 7.2 and at 37 °C.

The cleavage mechanism begins with the enzyme-mediated hydrolysis of SMDC 3's glycosidic bond, generating a phenol intermediate 4 which undergoes 1,6-elimination and a successive decarboxylation to concomitantly yield quinone 5 and an aniline intermediate 6. Ensuing 1,6- and 1,4- elimination processes result in the release of two MMAE molecules (Figure6).

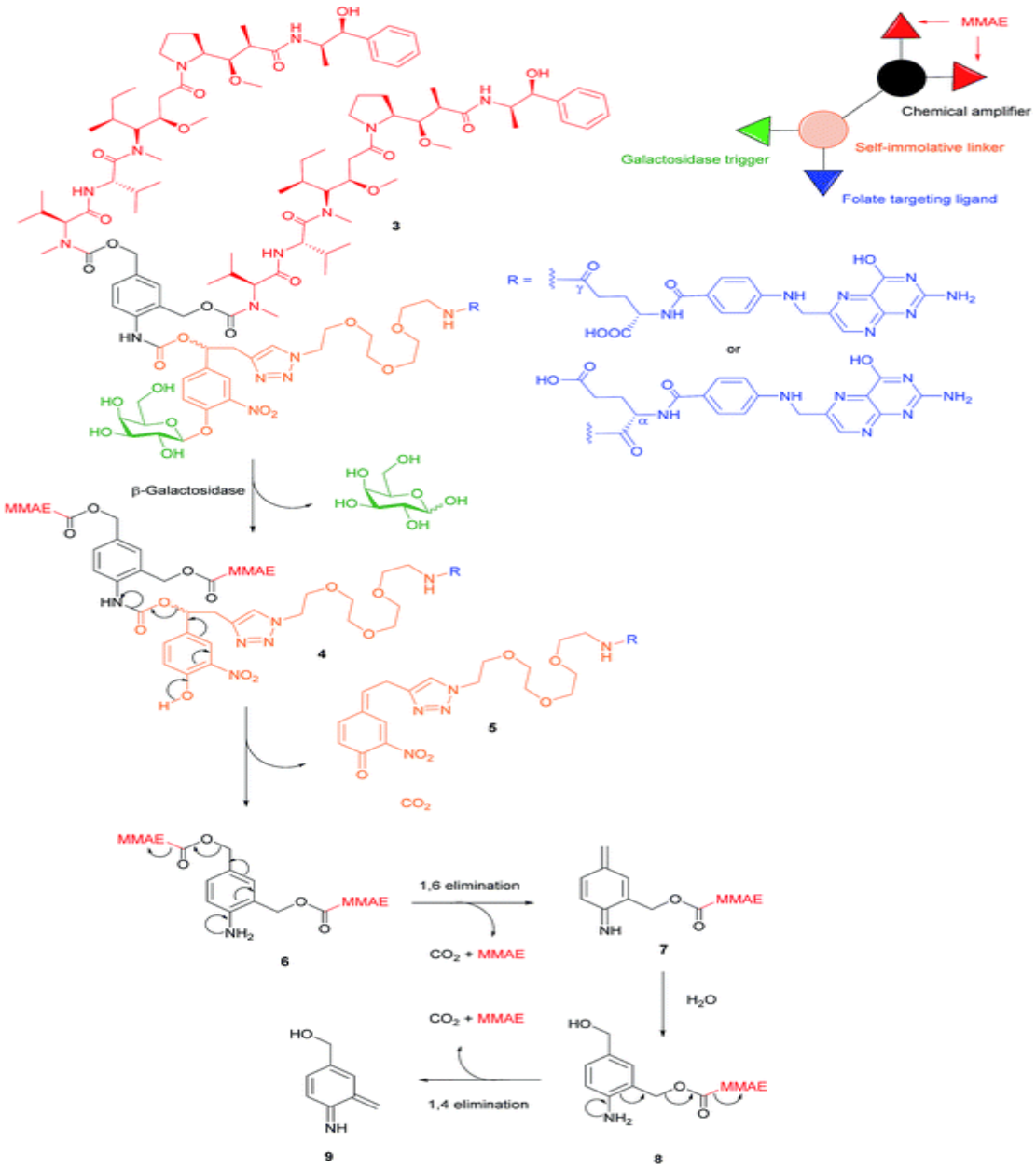

Figure 6. Enzyme-catalysed double drug release mechanism of β-galactosidase-responsive folate–MMAE conjugate 3

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A further example of an FA–SMDC that does not bear a disulfide linker and is cleaved by an enzyme is a folate–camptothecin conjugate degraded by the cathepsin B enzyme(Paranjpe et al.,2005)

In addition to FA–SMDCs that are cleaved by enzymes already present in the tumor milieu, folate–enzyme conjugates have also been developed to deliver an enzyme to the folate receptor of the tumor cell prior to the administration of a prodrug that is converted to the active form by this enzyme. An example of this therapy utilises penicillin-V amidase and a doxorubicin prodrug(Lu et al.,1999).

1.6.1.4Other linker platforms

1.6.1.4.1.Boron–nitrogen linker

In addition to the commonly employed disulfide and carbon-based linkers for drug release inside the cell, the covalent attachment of boronic acids to Schiff base ligands to yield boronate complexes can also be utilized as a platform to selectively deliver cytotoxic drugs to cancer cells. Gois et al. designed such a complex (10), which comprises the cytotoxic drug bortezomib, PEG chains and folate targeting units (Figure7).(Santos*et al.*,2017).

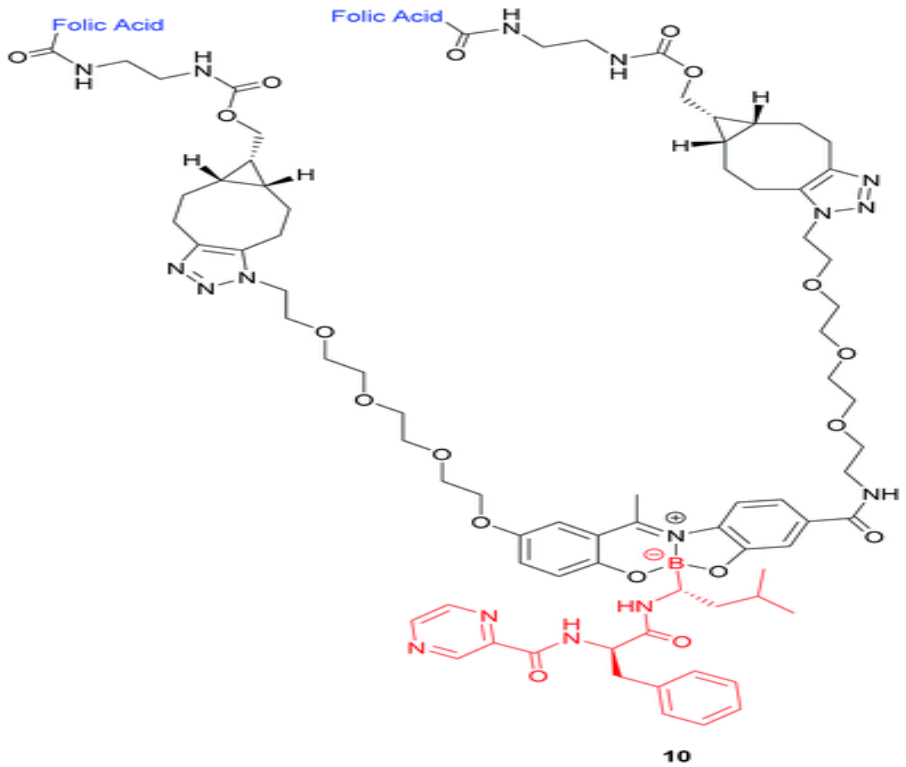

Figure 7. Structure of the boron complex 10 developed by Gois et al. consisting of (i) a folic acid targeting moiety (blue), (ii) PEG chains and (iii) the cytotoxic agent bortezomib (red).

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A bivalent folate targeting moiety was chosen to mimic the bivalent Fab regions present on immunoglobulin Gs (IgGs) that give rise to high affinity and specificity of antibodies for particular antigen epitopes(Santos *et al.*,2017).

Complex 10 exhibited an IC50 value of 62 nM against MDA-MB-231 cancer cells, lower than that of free bortezomib, but superior selectivity for these FRα-overexpressing cells as compared to the free drug. As GluSH is present in millimolar concentrations in the cell, Gois et al. investigated the GluSH-mediated cleavage mechanism by synthesizing complex 11, a less sterically hindered analogue of complex 10. The mechanism of drug release, as determined by HPLC, is thought to proceed via GluSH addition to the iminium carbon of the complex followed by opening of the five-membered ring and subsequent hydrolysis to promote release of drug 15 (Figure 9).

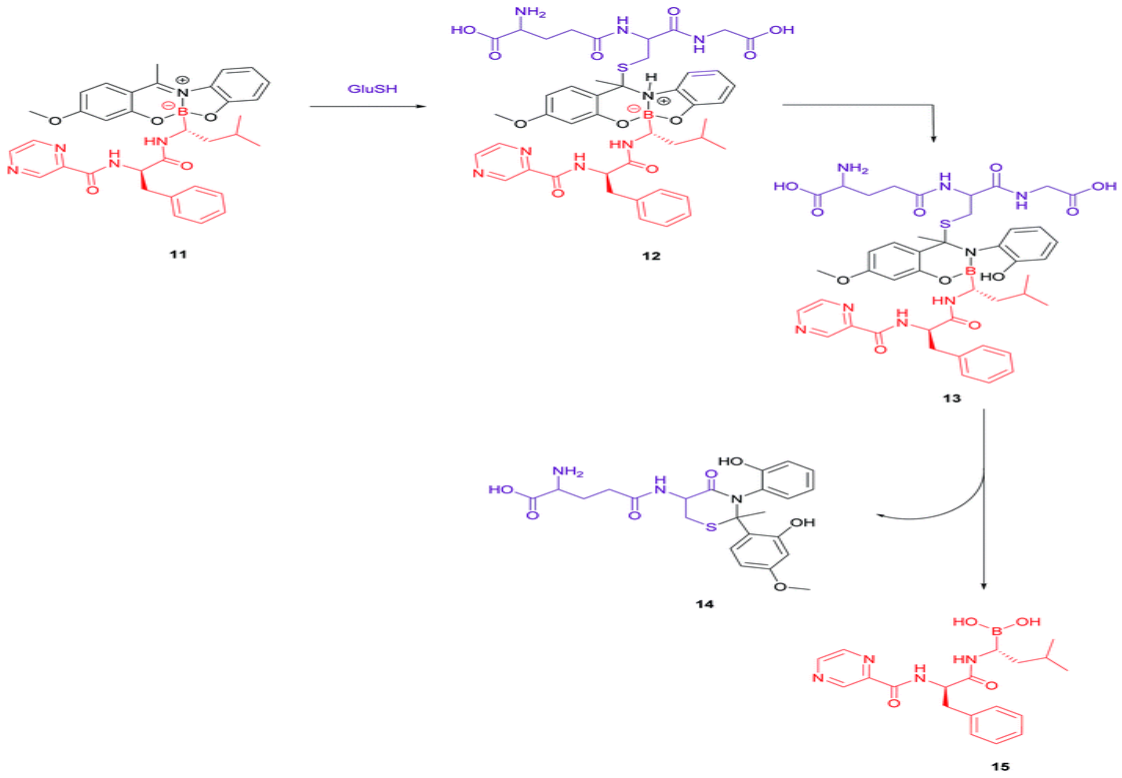

Figure 8. Proposed mechanism for GluSH-mediated release of bortezomib (15) from complex 11(Santos et al., 2017).

1.6.1.4.2. Light-triggered drug release

Methods to induce cytotoxicity with light, such as photodynamic therapy (PDT) have also attracted considerable interest for applications in cancer therapy. This technology involves light-mediated activation of a photosensitizer in the presence of oxygen and the subsequent generation of reactive oxygen species that neutralize the cells that have been exposed to the photosensitizer(Li*et al.*,2015). Moreover, the advantages of light-based techniques include non-invasive activation and added selectivity from the ease of this medium's spatial and temporal manipulation(Dcona *et al.*,2017).

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An example of a promising class of photosensitizers is boron dipyrromethene (BODIPY) derivatives that possess attractive optical and photophysical properties as well as displaying high stability in aqueous media.Ke et al. have developed two diiododistyryl folate-conjugated BODIPY-based photosensitisers (16a and 16b) with differing glycol linker lengths (Figure9) (Ke *et al*., 2013).

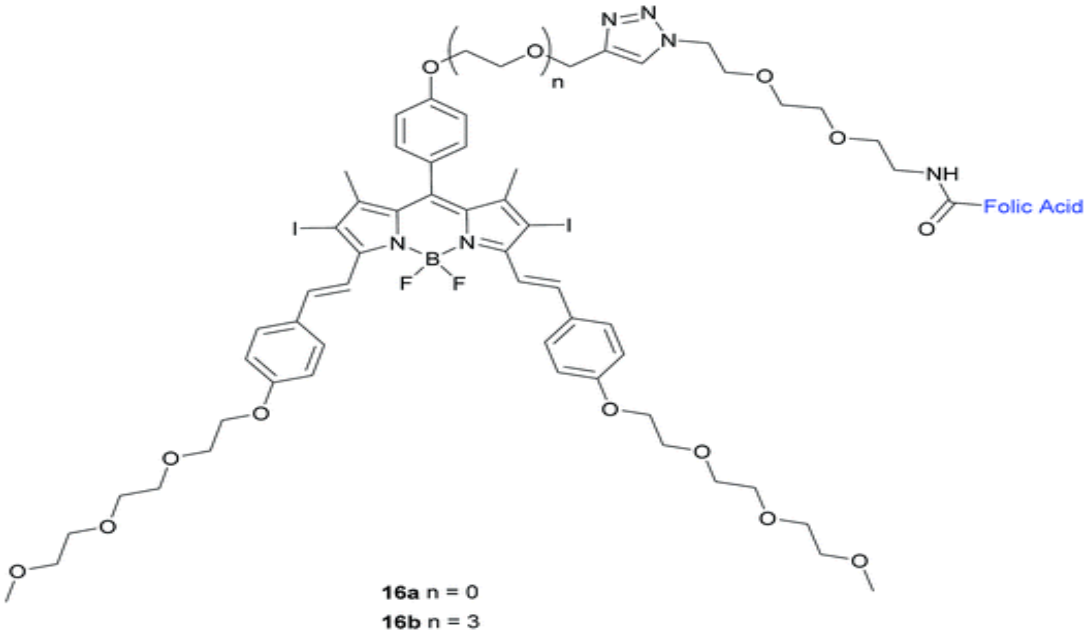

Figure 9. Chemical structure of folate-BODIPY conjugates.

The *in vitro*photosensitizing ability of 16a and 16b,present in the above figure, was investigated by incubation both with KB human nasopharyngeal carcinoma cells, which have high expression of FRα and with MCF-7 human breast adenocarcinoma cells, which have low expression of FRα**.**No cytotoxic activity was detected for either in the absence of light, whereas activity was observed upon the illumination with IR light. Conjugate 16a, with no triethylene glycol linker, displayed cytotoxic activity 3-fold higher (IC50 of 60 nM) than that of 16b (IC_{50} of 180 nM) (Ke *et al*., 2013).

The difference in cytotoxicity can be explained by the observation that 16b aggregates more in RPMI culture medium than 16a, probably due to the triethylene glycol linker of the former inducing dipole–dipole interactions in the neighboring oligoethylene glycol chains.Thus, conjugate 16a with the shorter linker is an attractive candidate for use as a photosensitizer against cancer cells in PDT(Ke *et al*., 2013).

As described above, FA–SMDCs represent a varied class of conjugates for targeted drug delivery. Whilst a large number of these platforms have been targeted to FRα overexpression applications, these platforms can readily be applied to FRβ overexpression scenarios (an emerging field) since folic acid binds to both these receptors. SMDCs are not the only group of treatments available for FR positive tumors, and the development of anti-folate antibodies that preferentially target FRα or FRβ with specificity and selectivity (as they do not possess an indiscriminate folic acid targeting moiety) represents an alternative strategy (Ledermann*et al*.,2015).

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1.6.2.FR-targeted monoclonal antibodies

1.6.2.1 IMGN853 (FRα targeted)

In addition to stand-alone therapeutic antibodies such as the aforementioned farletuzumab, antibody–drug conjugates (ADCs), where a cytotoxic agent is covalently linked to an antibody, are now being employed as vehicles for the selective delivery of drugs to tumors. This technology combines the exquisite binding selectivity of antibodies and the potent toxicity of a chemical warhead, whose cell-killing potential is distinct from antibody-dependent cytotoxicity, whilst also minimizing off-target toxicity(Chudasama et al.,2016).

This consequently enables the use of drugs that would otherwise be too toxic to be employed in conventional chemotherapeutic regimens. Moreover, the attachment of the cytotoxic agent magnifies the antibody's activity and has the potential to circumvent the rarely curative action of unconjugated antibodies(Senter, 2009).

As opposed to the short circulation half-life typical of SMDCs, antibodies' large size confers a substantially longer half-life to the ADCs in the bloodstream, which in turn augments the proportion of the administered dose reaching and penetrating the tumor.An example of such a FRα-targeting ADC is IMGN853, and it comprises three elements: (1) an anti-FRα antibody that targets the FRα-expressing cancer cells, (2) DM4, an antimitotic agent that inhibits tubulin polymerisation and microtubule assembly and (3) a disulfide-based linker that connects the drug to the antibody (Figure10) (Vergote and Leamon,2015).

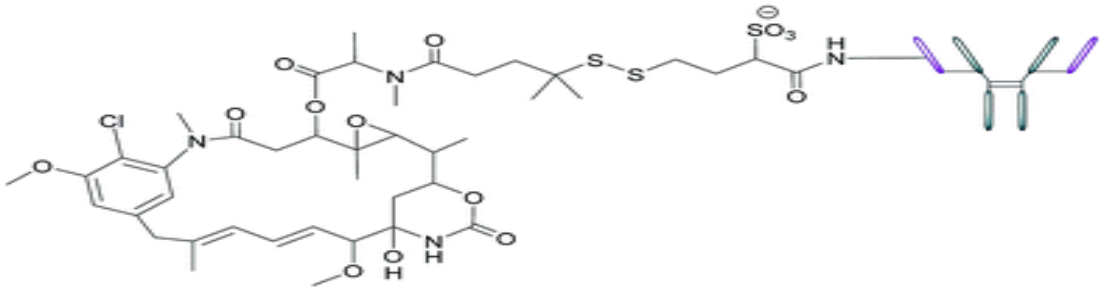

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Figure 10 Structure of IMGN853, the anti-FRα antibody is conjugated to the DM4 drug via a self-immolative disulfide linker.

As with the FA–SMDCs, IMGN853 binds to FRα, is internalised via RME, and ensuing enzymatic degradation of the antibody and linker releases the DM4 drug, which induces cell-cycle arrest and death by disrupting microtubule function. IMGN853 has demonstrated anti-tumor activity and is currently being assessed in phase II trials as a single agent and in combination regimens for patients with FRα-positive platinum-resistant ovarian cancer. This ADC represents a first generation construct of its type and there is plenty of scope to refine its chemistry should the clinical trials be unsuccessful(Kurkjian *et al.*, 2013 ; Moore *et al.*, 2014).

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1.6.3.Nanotechnology

1.6.3.1Nanoemulsions (FRα targeted)

As highlighted above, conventional chemotherapy is limited by a lack of selectivity, and the unwanted side effects caused by the non-specific cellular uptake of platinum-based regimens can be especially problematic. Nonetheless, due to its highly responsive nature, platinum-based therapy is still used as a leading chemotherapeutic agent in almost all stages of ovarian cancer.

However, the case for further support of this choice of therapy is waning. For instance, the high frequency of Pt-based treatment cycles often result in acquired drug resistance which can occur via the decreased cellular uptake of Pt, which limits the formation of cytotoxic Pt–DNA adducts. Additionally, intracellular GluSH mediates the detoxification of Pt and leads to the inactivation of Pt by the formation of cisplatin–thiol conjugates; thereby preventing cell death occurring after the formation of the lethal Pt–DNA adducts(Tapia and Díaz-Padilla, 2013).

In light of this, there is a critical need to modify the Pt therapeutic options currently available. To this effect, Patel et al. have reported the synthesis of NMI-350 Pt-theranostic nanoemulsions (NEs). The NMI-350 family is based on naturally occurring polyunsaturated fatty acid (PUFA) rich omega-3 and -6 fatty acid oils and gadolinium (Gd) labelled multicompartmental NEs. Their oily core can encapsulate the cytotoxic and hydrophobic difattyacid platins and C6-ceramide, and the NE surface can be employed for the attachment of imaging agents and folate ligands for targeting (Figure 11) (Patel *et al*., 2016).

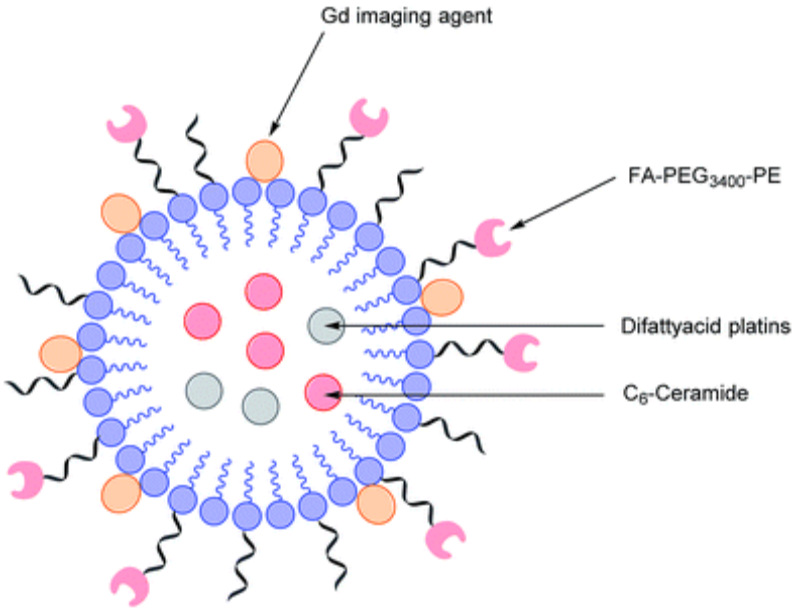

Figure 11: Schematic representation of a NMI-350 nanoemulsion. Difattyacid platins and C6-ceramide are encapsulated in the lipid core and lapidated gadolinium and folate are attached to the surface.

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Through the aforementioned architecture, these NEs allow the controlled delivery of combined chemotherapy and additionally lengthen the blood circulation half-life of Pt to maximise uptake of nanodrug conjugates in malignant cells over a prolonged period of time. Moreover, the synthesis of the di-fattyacid platinum construct has been greatly improved: Patel et al. have developed a synthesis which takes 24 h, as opposed to previously reported procedures requiring 21 days (Maeda *et al*., 1986).

Di-fattyacid platins of different chain lengths were synthesised using this more efficient method and folate was attached to the NE surface via a DSPE-PEG3400 spacer (Figure 12). The fully functionalised NEs displayed a particle size in the range 120–150 nm.

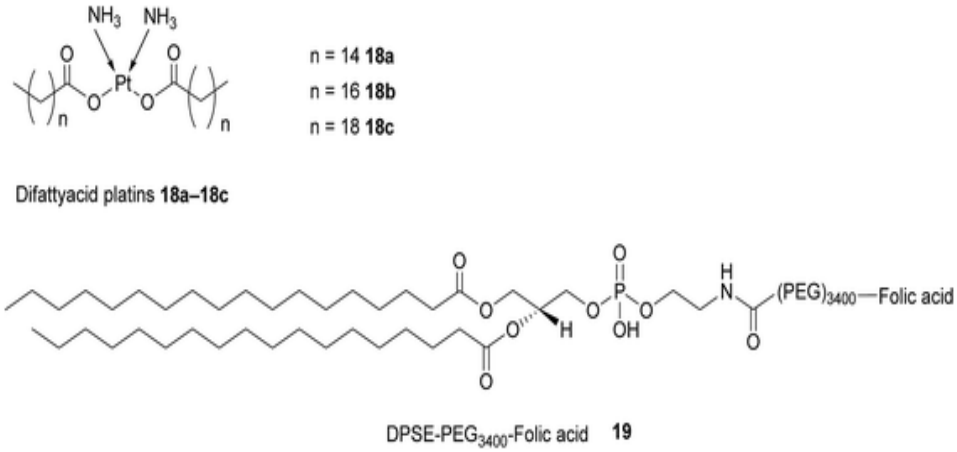

Figure 12: The FA spacer.

FRα-binding efficiency of the NEs was then tested on two FRα-rich cell lines, KB-WT (Pt-sensitive) and KBCR-1000 (Pt-resistant) cell lines and analyzed by flow cytometry. Both lines were treated with non-targeted rhodamine labeled NEs (NT-Rh-NE) and FA-targeted rhodamine labelled NEs (FA-Rh-NE), with the latter being functionalised with 100, 300, 1200 and 3600 FA molecules. As expected, cellular uptake in both the lines increased with higher levels of FA conjugation(Patel *et al.*, 2016).

The FA-Rh-NE labelled with 300 FA molecules was then selected for a cytotoxic assay due to being the most stable and cost effective relative to the other FA-Rh-NEs. This FA-Rh-NE was compared to cisplatin in a cytotoxic assay using the same Pt-sensitive and Pt-resistant cell lines, and this NE produced a ca. 30-fold increase in potency as compared to unconjugated cisplatin. This heightened cytotoxicity has the potential to reverse Pt-resistance and can be ascribed to the synergistic effect of the Pt and the exogenously added C6-ceramide.

After binding to FRα and ensuing internalization via RME, dissociation of the NE is promoted by the acidic environment of the endosome, permitting the diffusion of the free Pt and C6-ceramide across the endosome into the intracellular milieu, where they can exert their cytotoxic activity on chromosomal and mitochondrial DNA.

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Intracellular depletion of C6-ceramide constitutes a resistance mechanism that shifts the equilibrium away from apoptosis in tumor cells.

The addition of the ceramide to NEs serves to combat this resistance mechanism by shifting said equilibrium back towards apoptosis and encapsulation of the ceramide inside the NE shields it from metabolic degradation and inactivation.

The effect of the di-fattyacid cisplatin aliphatic linker length (C14, C16 and C18) was also evaluated and while the linkers had no effect on the stability of the NEs, the shortest chain 18a produced the most potent cytotoxic activity.

This observation can be rationalized by considering the shortest chain to be the best leaving group during Pt–O bond cleavage, resulting in quicker liberation of reactive Pt which can go then go on to form adducts with the tumor cell's DNA (Patel *et al*., 2016).

1.6.3.2.Nanotubes (FRα targeted)

Wang et al. have developed the first example of Ni–folate biomolecule-based coordination complex nanotubes (BMB-CCNTs) of an inner diameter of 5–8 nm and which incorporate FA as a targeting ligand, hydrazine as a linker, Ni as a connector and cisplatin as the cytotoxic agent (Wang *et al.*, 2015).

These nanotubes' sufficiently large cavity permits a high drug loading which overcomes the small deliverable payload dose associated with other folate conjugates. Moreover, these nanotubes evade the undesirable accumulation in the kidneys typical of smaller folate–drug conjugates(Wang*et al.*, 2015).

The initial stage of nanotube synthesis comprises the formation of a tape-like structure as the pteroic acid unit of FA can form hydrogen bonds with the pteroic acid moiety of other FA molecules. The glutamic acid portion of FA can then coordinate to Ni2+ without compromising the intermolecular hydrogen bonds and hydrazine serves as a bridging ligand between two Ni atoms, resulting in the formation of a nano-sheet. The high temperature of this reaction aggravates the relative intermolecular movement of the nano-sheets and thus stimulates curling in order to minimize the free surface energy. The high temperatures also promote nanotube formation by the breaking of partial initial bonds and the formation of new ones, with the hydrazine acting as a molecular string, tying the nano-sheets into nanotubes (Figure13)(Wang *et al.*, 2015).

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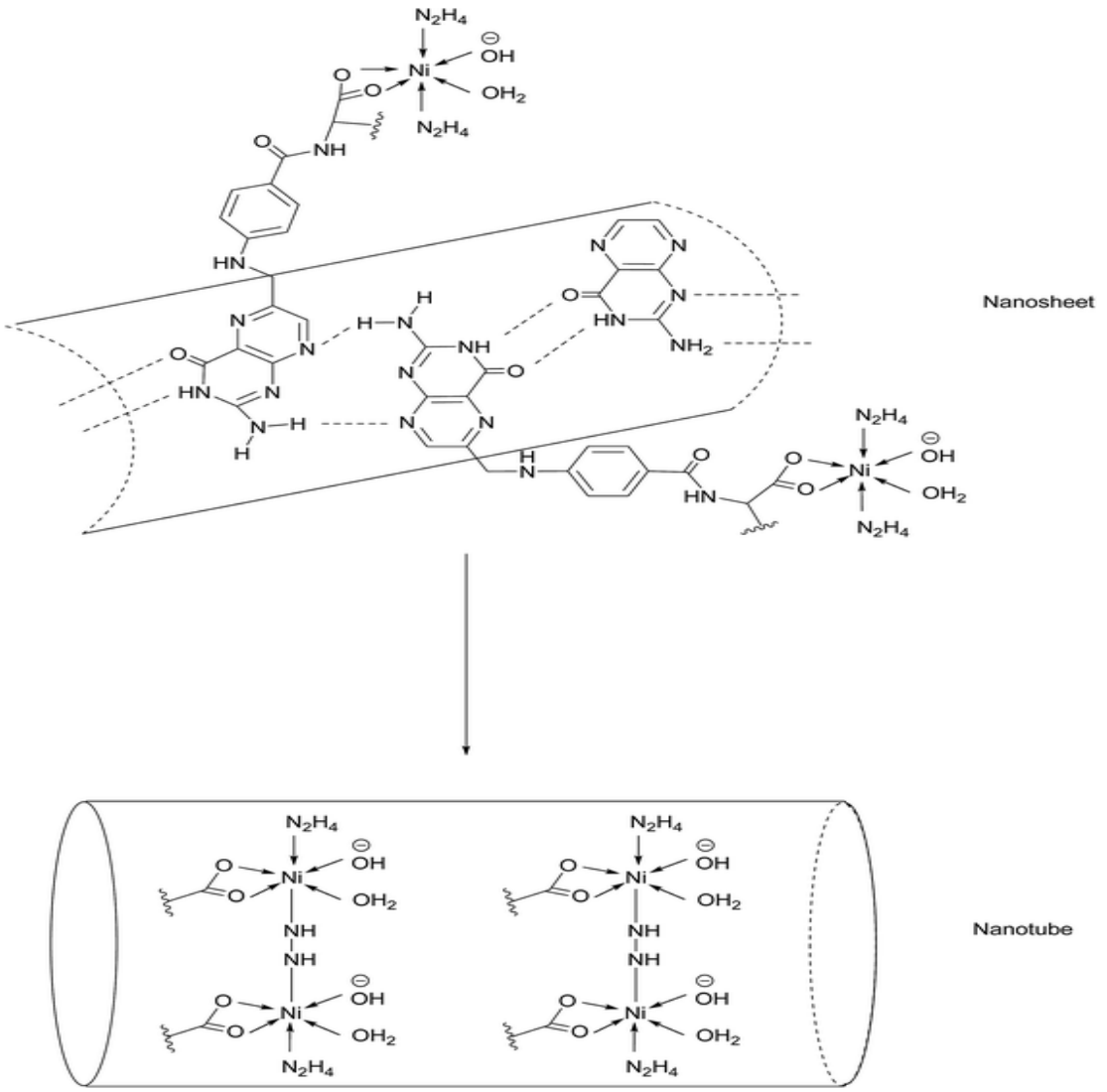

Figure 13. Nanotube formation from nanosheets

1.6.4. Imaging:

1.6.4.1 99mTc-etarfolatide (FRα targeted)

Appraisal of FRα expression can be a useful diagnostic tool, allowing the FRα status to be monitored throughout the duration of treatment, with several avenues having been explored for FRα detection. However, despite the high specificity and sensitivity of these methods, their clinical use usually requires invasive tissue biopsies, which are typically taken from a single lesion(Maurer*et al.*, 2014).

Furthermore, the heterogeneous nature of FRα expression on tumors and the changing characteristics of tumors with time makes it difficult to construct an accurate representation of a patient's FRα status, thus generating an incomplete picture. Whole-body imaging that utilises folate radioconjugates can overcome this limitation by providing realtime and non-invasive FRα appraisal for multiple lesions at several time points (Naumann*et al.*, 2013;Morris *et al.*, 2014).

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Etarfolatide (EC20) is one such example and is a folate-targeted radioimaging agent composed of 99mtechnetium (Tc) complexed to folic acid via a short non-cleavable peptide linker (Figure14). EC20's linker is non-degradable as the release of the 99mTc is not a requirement for radiofolate imaging (Ledermann *et al*., 2015).

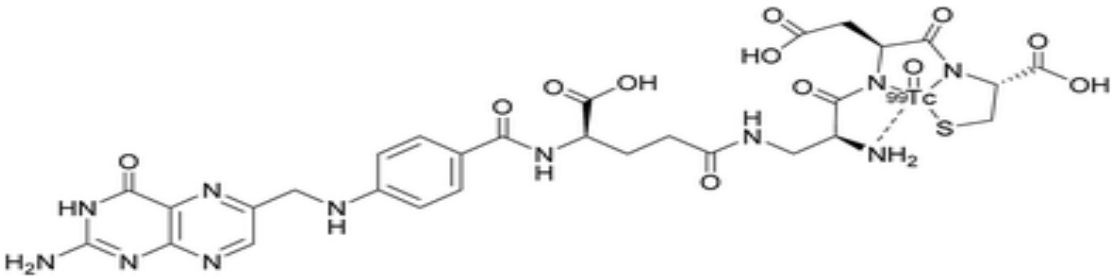

Figure 14. Chemical structure of 99mTc-etarfolatide.

99mTc is a frequently employed radiographic tracer, possessing a half-life of 6 h and whose principle form of radioactive decay is gamma emission(Ledermann *et al.*, 2015). Moreover, 99mTc-etarfolatide displays a strong binding affinity to FRα and tumors that overexpress FRα typically internalise a high proportion of the administered 99mTc-etarfolatide (∼17% ID g−1) (Leamon*et al.*, 2002).

Added benefits of this probe conjugate include rapid accumulation at the tumor target site and subsequent swift clearance from the bloodstream via the kidneys. This in turn diminishes the non-specific tumor uptake of 99mTc-etarfolatide and permits the quick generation of images(Ledermann *et al.*, 2015).

99mTc-etarfolatide makes use of Tc's optimal single-photon emission computed tomography (SPECT) imaging characteristics, namely, a half-life of 6 h and a photon energy of 140 keV. Consequently, this probe conjugate has been subject to evaluation in numerous clinical trials, including those involving vintafolide, with 99mTc-etarfolatide as a companion imaging agent(Morris*et al.*, 2014; Fisheret al., 2008).

Although no safety concerns have been established in this line of treatment, undesired adverse effects such as lower abdominal pain, nausea and vomiting, have all been identified as being 99mTc-etarfolatide-related, although these were only observed in <1% of patients (Maurer et al., 2014).

While several phase II trials have demonstrated that 99mTc-etarfolatide imaging can be utilised to determine patients most likely to respond to vintafolide therapy(Naumann *et al.*, 2013; Morris *et al.*, 2014). The imaging results and their interpretation can be influenced by physiological factors: principally the observation that 99mTc-etarfolatide is uptaken into the kidneys, bladder, and spleen and somewhat into bone marrow. This may interfere with the interpretation of receptor expression in lesions close to these organs and for this reason, small quantities of folic acid are injected prior to 99mTc-etarfolatide administration in order to partially saturate the FRαs (Maurer *et al.*, 2014).

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Another limitation of this probe conjugate stems from activated macrophages (that express FRβ) also internalizing 99mTc-etarfolatide, a phenomenon which can result in regions of inflammation or infection falsely appearing as FRα-positive tumortissue (Maurer et al., 2014).

Early studies on 99mTc-etarfolatide imaging were constrained by having to employ separate SPECT and computed tomography (CT) imaging, but contemporary SPECT/CT fusion imaging has greatly ameliorated spatial localization and is able to determine whether tumors are FRα-positive or FRα-negative. 99mTc-etarfolatide has proved to be valuable for the selection of patients likely to respond to treatments targeting the FRα. This probe conjugate has also shown promise for the staging and restaging of tumors, the assessment of disease prognosis and for the identification of patients who could benefit from intraoperative fluorescence FRα imaging to help reveal deep-seated tumors that can evade detection by intraoperative optical imaging due to limited signal penetration in human tissue (Maurer *et al.*, 2014).

99mTc-etarfolatide may also have future applications for the prognosis of FRα-positive ovarian and lung cancer(O'Shannessy*et al.*, 2012; Chen *et al.*, 2012).

2. Conclusion

For many years, prodrug strategy has been developed enormously to solve many unwanted drug properties. “Folate” is a generic term for forms of Vitamin B9 and their derivatives. Folates play a vital role in body functions like nucleic acid synthesis and RBC formation. Natural folates are preferable over synthetic forms since they have lesser side effects and are body-own forms; and also the metabolism of synthetic folic acid is very individual specific. Naturally occurring folates are found in foods and in metabolically active forms in the human body.These FR-targeted technologies can also pave the way for inspiring further sophisticated drug conjugates, especially as this receptor is being targeted by use of several complementary technologies: small molecule, nanoparticle and protein-based, thus providing broad and distinct knowledge in the area.

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