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RESEARCH ARTICLE

FLUOROPHORE-DRUG CONJUGATES AS IMAGING BASED TARGETED DELIVERY AND THERAPY SYSTEMS IN COMBATING CANCER

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ABSTRACT

Cancer is one of the dreadful diseases having the highest mortality rate throughout the year worldwide. The treatments with conventional chemotherapeutics face the problems of drug resistance, insolubility, non-selectivity, toxicity and biological barriers that weaken the patient to cure effectively. To overcome these barriers, fluorophore-drug conjugates as imaging based targeted delivery and therapy systems have emerged as attractive platform against cancer to maximize their therapeutic efficacies to the specific site/s of interest, reducing the potential side effects. Anticancer drugs and other ligands anchored to fluorescein based chemosensors by various linkers have been investigated to monitor the real time fluorescent imaging in drug delivery followed by stimuli responsive therapies. The drug liberation triggered by bio and chemo hydrolytic environments is visualized based on 'switch on' fluorescence of the fluorophore-moiety of fluorescein-drug conjugates for non-invasive cancer imaging. The conjugation of free phenolic hydroxyl or carboxylic groups of fluorescein chemosensor to the hydroxyl group of drugs or specific carriers are employed by biodegradable amino and oxy acrylate linkers for targeted drug delivery applications. This review mainly demonstrates the biomedical applications of small molecule-based non-cleavable and cleavable fluorophore-drug conjugates as delivery systems through fluorescent monitoring of drug liberation, accompanied with chemo, photothermal and photodynamic therapies to avail higher targeted therapeutic efficiencies against cancer.

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INTRODUCTION

Since the 20th century, the usages of chemical dyes have opened the doors of cancer treatment, while the therapeutics of conjugated dyes, based on principle, should selectively kill the targeted pathogens without damaging healthy cells (Strebhardt and Ullrich, 2008). However, conventional cytotoxic agents, owing to their cytotoxicity, non-selectivity, insolubility, bio-instability, emergence of drug-resistance and biological barriers, clonal heterogeneity or stemness properties, are not effective enough against cancer cells, cancer or tumor to eliminate or remove them completely, and prevent further relapses in biological system. To overcome these obstacles, targeted drug delivery with monitoring systems, comprised of drugs/prodrugs attached to target-specific carriers such as peptides, antibodies, extracellular matrix compounds or nanoparticles, and equipped with switchable fluorescent dyes anchored to the drugs/prodrugs via linker molecules, have attracted attention to facilitate drug delivery and release into the specific site/s of interest to treat abnormal cells/tissues/organs as image-guided therapies (Alley *et al*., 2010; Hoppenz *et al*., 2020; Yazdi *et al*., 2020; Majumdar and Siahaan, 2012; Rozovsky *et al*., 2019; Dunuweera *et al*., 2019; Guo *et al*., 2020; Cherkasov *et al*., 2020; Yang *et al*., 2019; Ebaston *et al*., 2019; Chin *et al*., 2014; Fu *et al*., 2016; Hwang *et al*., 2020; Ozturk-Atar *et al*., 2018; Pattni and Torchilin, 2015; Patsenker and Gellerman, 2020; Zheng *et al*., 2019). To investigate drug delivery followed by drug release in biological systems, molecular imagings through spatial and temporal integrated resolutions-based positron emission tomography (PET) and computed tomography (CT), and to study dual-signal ratiometric drug delivery, Forster/fluorescence resonance energy transfer (FRET)-based fluorescence and confocal laser scanning microscopies have been employed as main fluorescence-based approaches due to their non-invasiveness, high resolution, real time detection, quantitative/qualitative ability in different biological systems such as tissue-based single cells, samples and animal models (Eter, 2009; Li *et al*., 2020; Bouchaala *et al*., 2016; Mandal, 2023).

The small molecule fluorescent dyes, having low-molecular weights, are attractive for bio-imaging to monitor drug delivery and distribution more accurately owing to their suitable structures via chemical synthesis, and for covering a wide range of wavelength and brightness (Choi *et al*., 2019). The fluorophore-drug conjugates, while the fluorophore parts also called fluorescent reporters, may be divided into switchable (turn on/activatable/fluorogenic) and non-switchable (always on) versions. In comparison with non-switchable, the switchable one is more attractive as the fluorescent changes occur upon the detection leading to better signal-to-noise ratio, lesser detection limit and greater sensitivity. Moreover, fluorophore-drug conjugates (Figure 1) may be classified into two types: I. Non-cleavable conjugates, while fluorophores are designed to combine directly with drug molecules via covalent bonding and used to track drug accumulation. II. Cleavable conjugates, while the more labile cleavable linkers (hydrazine, carbonate, ester, and disulfide bonds) tether the fluorophores with drugs, and are utilized to monitor real time drug delivery and sustained drug release under well-defined biological conditions or other stimuli. Currently, more advances have been employed for allowing cleavage of the tether to induct changes in fluorescent characteristics (turn-on/fluorogenic systems) or restore the pro-drug activity under physiological environment (Chan *et al*., 2012; Bildstein *et al*., 2011; Lee *et al*., 2018). In addition, tumor-destined ligands such as biotin, folates, antibodies and RGD (Arg-Gly-Asp) peptides have been attached to these conjugates for forming the targeted drug delivery systems to reduce drugs' cytotoxicity and improve selectivity for the treatment of cancer and their surrounding biological adverse environment. This review mainly demonstrates the role of the small molecules-based cleavable and non-cleavable fluorophore-drug conjugates for monitoring drug delivery and release therapeutic efficacies in the specific site/s of interest against cancer.

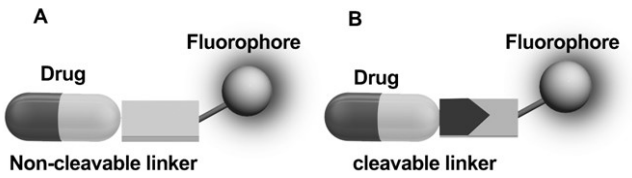

Figure 1. Schematic presentation of two types of small molecule-based drug-fluorophore conjugates

Non-cleavable conjugates with biomedical applications: To visualize drug delivery, fluorophores are tagged at suitable positions on the bioactive drug molecules for forming the stable and non-degradable linkages (Figures 2 and 3). A few NIR small molecule fluorophores such as BODIPY, Rhodamine Green, SiR-COOH, Oregon Green, tetraphenylethane and naphtalamide conjugated with different agents such as suldinac, ibrutinib, tamoxifen, camptothecin, GABA, coumarin, gemcitabine and doxorubicin have been utilized for cancer /cells imaging and treatment (Kim *et al*., 2015; Zhang *et al*., 2019; Zhao *et al*., 2016; Qian and Tang, 2017; Yuan and Liu, 2017; Yamura *et al*., 2016; Sakamoto *et al*., 2019; Wang *et al*., 2019; Xu *et al*., 2020). An acedan fluorophore tagged with SP600125 and bioorthogonal reporter has been utilized to get fluorogenic imaging of cellular enzymic activities as multifunctional conjugates to get more insightful information in drug therapy (Qian *et al*., 2019; Devaraj and Weissleder, 2011; Pan *et al*., 2018; Lang *et al*,. 2019; Ying *et al*., 2019). NIR heptamethine carbo/cyanine dyes (HMCDs) such as IR-780, IR-783, IR-786, MHI-148 and DZ-1conjugated with various agents such as docetaxel, gemcitabine, clorgiline, methotrexate, erlotinib, genistetin, isoniazid, crizotinib, dasatinib, rucaparib, cisplatin and simvastatin have been used for image-guided different cancer/cells types, and for targeting and accumulation in tumors mitochondria, lysosomes, and tumor and xenograft models, while tumor-uptakes are mediated by organic anion transporting polypeptides (OATPs), endocytosis, and / or albumin-mediated transports (Mrdenovic *et al*., 2019; Yang *et al*., 2013; Wu *et al*., 2014; Shao *et al*., 2014; Zhao *et al*., 2016; Cooper *et al*., 2021).

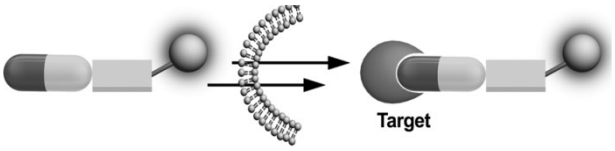

Figure 2. Design principle of permanent conjugates

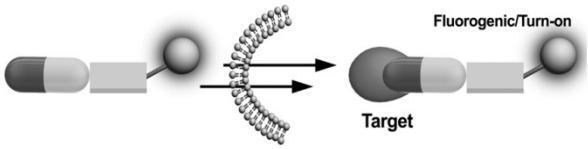

Figure 3. Design principle of fluorogenic permanent conjugates

Fluorophores based on excitation and emission in near infrared spectral region (~700-950 nm, I-region; ~1000-1700 nm, II-region) are very effective for bio-imaging in a non-invasive approach owing to their excellent tissue penetration and minimum interference from biological auto-fluorescence (Hilderbrand and Weissleder, 2010; Owens *et al*., 2016). By availing that advantage, NHS ester of FNIR-774 and IR-800 conjugated to monoclonal antibodies (panitumumab and cetuximab respectively), have been utilized to get higher tumor targeting efficiency (Sato *et al*., 2015). A kinase inhibitor (dasatinib) conjugated with a NIR heptamethine cyanine (Cy7) dye has been used for *in vivo* optical imaging and higher growth inhibition of HepG1 cancer cells (Usama *et al*., 2019). NIR fluorophore-based therapies (photothermal and photodynamic) have been utilized to visualize module and release of drug and production of reactive oxygen species under light irradiation (Shen *et al*., 2020; Liu *et al*., 2019). Heptamethine cyanine dye IR-822 conjugated with N^1-(pyridine-4-ylmethyl) ethane-1,2-diamine as a pH-sensitive receptor has been utilized in acidic tumor microenvironment, while light irradiation for several minutes by a low-powered NIR laser (808 nm, 0.4 w/cm^2) has enhanced tumor temperature to 57◦C to get their photothermal cellular killing (Meng *et al*., 2017).

Cleavable conjugates with biomedical applications: As the drug-modification sites are crucial to drug-target interactions and the sizes of fluorophores are sometimes quite bulky, and their hydrophobicity and solubility may affect the binding capability of the drugs to the deliberated cellular targets, the another conjugation type between organic dye and drug anchored via synthetically stable but high labile and specific cleavable link (biodegradable endogenous or external stimuli-triggered) has been attractive for drug delivery and therapy owing to the specific tumor microenvironment or target cells through controlled degradation of conjugate and

release of drug molecules with simultaneous generation of easy-to-monitor fluorescent signals (Yan *et al*., 2018; Zhang *et al*., 2020) (Figure 4; Table1). Endogenous stimuli, relied on cancer cells-over-expressed abnormal enzymes (matrix metalloproteinases, esterases, histone deacetylase, β-galactosidase, NAD(P)H:quinine oxidoreductase-1 (NQO1)), intracellular acidic pH at lysosomes and endosomes, up-regulated endogenous glutathione, hypoxia i.e. low oxygen levels owing to enhanced metabolic rates in tumor cells, or elevated level of ROS, and exterior stimuli such as light and temperature, have been utilized for achieving better disease and tumor specific selected therapy (Jang *et al*., 2016; Shin *et al*., 2016; Yao *et al*., 2018; Kolodych *et al*., 2017; Shahrijari *et al*., 2019; Leriche *et al*., 2012; Lee *et al*., 2018). The chemical structures of cleavable linkages chiefly include amides/peptides, esters, carbonates, carbamates, hydrazines, azos, disulfides and self-immolative moieties (Leriche *et al*., 2012; Gnaim and Shabat, 2019).

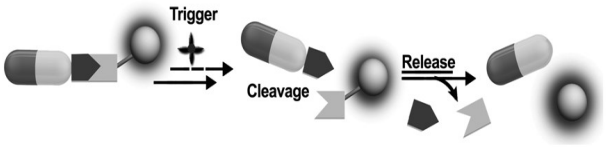

Figure 4. Design principle of fluorogenic and liberation-based cleavable conjugates

Table 1. A list of few trigger conditions with cleavable linkers

Triggers	Groups
Thiols (GSH, Trx)	Disulfides
Enzymes	Acetyls, amides, glycosidic bonds, etc.
pH	Hydrazines, etc.
ROS	Boronic acids, etc.
Hypoxia	Azos, nitro-appended aromatics
Photolysis	2-nitrobenzyls, etc.

Endogenous thiol molecules such as glutathione, hydrogen sulfide, cysteine (Cys), thioredoxin (Trx), and other thiol/cysteine containing peptides are over-expressed mostly in cancer cells. The disulfide bonds, stable in the blood stream and extracellular environment, undergo cleavage intracellularly via the chemical reduction process triggered by millimolar concentrated cytoplasmic thiols (Lee *et al*., 2015). Various disulfide-based fluorescent drug conjugates (such as naphthalimide-camptothecin/doxorubicin/gemcitabine) have been utilized for tumor-targeted bioimaging and drug delivery through internal charge transfer (ICT) processes leading to intramolecular cyclization for providing fluorescent signal increment and the subsequent active drug-release (Lee *et al*., 2012). Moreover, a cyclic RGD peptide, installed at the head of the fluorescent reporter (disulfide-based conjugate), has been used for U87 cancer cells-targeting, imaging and release of free drug within the cellular endoplasmic reticulum (Kong *et al*., 2017; Ye *et al*., 2016).

In hypoxic tumor microenvironment (lowered oxygen concentration ~0.02-2 % in comparison to ~2-9 % in normal cells), a few enzymes such as flavoproteins and oxidoreductases are activated. The conjugates containing azo-bonds, nitro-aromatic heterocyclics (nitrofuran, nitrobenzyl, etc.), indole-quinones and trimethyl-locked quinine systems dubbed as hypoxia-sensitive linkers have been used for selectively targeted tumor cells (Sharma *et al*., 2019; Kumar *et al*., 2016; Zhou *et al*., 2018). A hypoxia-responsive N,N-bis(2-chloroethyl)-1,4-benzene-diamine prodrug with a rhodamine-derived azobenzene scaffold tagged with a triphenyl phosphonium group has been utilized for mitochondrial targeting in a hypoxic cellular environment to get inhibitory efficacy against DU145 and MDA-MB-231 cancer cells and xenografted tumor mouse models through azo-reduction leading to the release of fluorescent rhodamine and prodrug, and subsequent "switch-on" fluorescence imaging signal increment (Verwilst *et al*., 2017). A new NIR (670/705 nm) azo-based prodrug has also been applied for the real-time visualization of drug delivery and release therapy to get inhibitory therapeutic efficacy against HepG2 and 4T1 cancer cells/bearing mice (Ding *et al*., 2019).

pH dysregulation (intra and extra cellular pH in normal tissue ~7.2, while lower ~6.6-7.1 in an extracellular tumor microenvironment) is highly related to cancer progression (Webb *et al*., 2011). Hydrazone-linkage having acid-labile response, is stable at physiological pH 7.4 but degrades quickly through hydrolysis in the acidic tumor microenvironment after entering cells owing to the exposure to late endosomes and lysosomes (pH~6.5-5.5) (Kong *et al*., 2018; Fernandez *et al*., 2017). A acid-sensitive conjugate consisting of pH-responsive N-acylhydrazone tagged with BODIPY and doxorubicin has been used to get dose-dependent release of cytotoxic doxorubicin with detectable green fluorescence imaging in acidic phagosomes of lipopolysaccharide-induced pro-inflammatory M1 macrophages in a zebrafish model (Fernandez *et al*., 2017). ROS (hydrogen peroxide, hypochlorous acid and hydroxyl radical), overproduced in tumor cells, have been utilized as potential triggers for targeted drug delivery and therapy (Tao *et al*., 2017; Gao *et al*., 2018; Redy-Keisar *et al*., 2015). A hydrogen peroxide-responsive conjugate composed of quinone cyanine-7 as the fluorophore and the central self-immolative phenyl boronic ester linkage as the hydrogen peroxide-triggering substrate and camptothecin as drug has been applied to remove triggering substrate by hydrogen peroxide-generated phenolate intermediate with subsequent 1,4-elimination to liberate drug at the tumor site and "turn-on" NIR fluorescence signal in tumor region in a U-87 MG tumor model (Redy-Keisar *et al*., 2015).

Many enzymes such as glycosidases and matrix metalloproteases having different expression profiles in pathological inflammation and cancer have been utilized for enzyme-responsive drug liberation. β-galactosidase (β-gal) exhibits increased enzymatic activities in primary ovarian cancer (Asanuma *et al*., 2015). The β-gal-sensitive NIR conjugate containing D-galactose component as an enzyme provoke, PEG linker for improving *in vivo* bio-distribution, and NIR chromophore for providing fluorescent and photo-acoustic imaging, has been utilized as multi-modal imaging-guided drug release therapy accompanied with NIR-irradiated generation of photothermal signal for further suppression of cancer growth (Zhen *et al*., 2018). Light, the external stimulus, serves as the effective activator to control and provide non-invasive or / and spatial-temporal drug-activation quantitatively through enabling its biorthogonal triggered liberation (Ai *et al*., 2016; Jang *et al*., 2019). The sequential photo-activatable prodrug gemicitabine-coumarin-NO_2 composed of photo-activated group and hypoxia-sensitive moiety 4-nitrobenzyl group has been utilized to get cytotoxic inhibitory efficacy against MCF-7 cancer cells, while upon hypoxic nitro reduction, the intermediate is eliminated through 1,6-rearrangement, and produced

hydroxyl group at the orthoposition of trans-cinnamic ester. Upon UV-irradiation, cis-configuration is formed to undergo nucleophilic attack of the ester bond leading to liberation of the fluorescent coumarin and gemcitabine (GMC) (Feng *et al*., 2016). The NIR photocaged prodrug composed of dialkylamine-based tricarbon cyanine as a light trigger tagged with camptothecin via a carbamate bond has been utilized to trace the location of the prodrug through NIR-fluorescence (820 nm) and controlled drug treatment through photo-decaging and self-elimination with NIR light-irradiation, and bond cleavage to produce active drug and new emission (820-535 nm) against cancer (Guo *et al*., 2018).

Forster resonance energy transfer (FRET) has been applied for ratiometric fluorescence imaging to detect enzyme activities, protein-protein interactions and drug deliveries, while two different emission channels are used to change the fluorescent intensity to monitor and provide the distribution and quantitative information of active drug in a non-invasive manner (Li *et al*., 2016; Hu and Zeng, 2017; Liu *et al*., 2017; Liu *et al*., 2016) (Figure 5). A few drugs such as doxorubicin, camptothecin and SN-38 having their own fluorescent characteristics as either FRET acceptor or donor, have been utilized for imaging and drug delivery therapy against cancer through separation of the pair (fluorophore and drug) after cleavage of linkers to cause the fluorescent changes of both parts i.e. different signals from two channels to get better resolution.

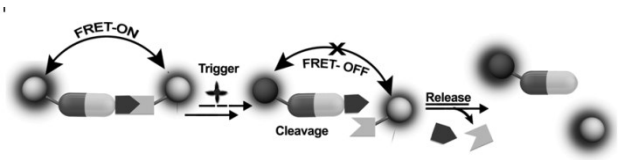

Figure 5. Design principle of FRET-based conjugates

CONCLUSION

Fluorescent image-based drug delivery, implicating diagnosis and therapy, provides great advances for a real-time evaluation of targeted drug-efficacy at the specific site/s of interest focused on its distribution and accumulation correlating the development of drug via rational design of conjugate. However, further challenges still remain: 1. As the conjugates need multiple strides of organic synthesis accompanying laborious purification and low yields related to limitation of large scaling-up processes, it is required to develop readily operable and highly efficient organic reactions to assemble rapidly the conjugates (Dong *et al*., 2014; Aubert *et al*., 2019). 2. As the bioavailability of fluorophores affects the drugs' activity adversely into the biological system in non-breakable or breakable-liberation manners, the metabolism of fluorophores requires comprehensive analysis during the development of fluorophore-drug conjugates for further biological application. 3. As few NIR fluorophores can act as drug monitoring as well as synergistic cancer inhibition therapy, the invention of potent multifunctional dyes with lower tissue auto-fluorescence, light scattering and photon attenuation is necessary for future photo-based therapy (Luo *et al*., 2019; Li *et al*., 2019; Tian *et al*., 2019). 4. The different Janus-faced molecules have been developed for their fluorogenic reactions in disease models (Han *et al*., 2018; Chen *et al*., 2019; Qin *et al*., 2020). It is needed to further enlarge triggers over-expressed in sub-cellular organelles with high sensitivity and selectivity for stimulating future drug invention (Fang *et al*., 2020; Huang *et al*., 2019). Moreover, owing to hydropohobic and positive charges of fluorophores, the site-specific characteristics of fluorophores and activations may change the original targets of drugs and diminish their efficiency (Wisnovsky *et al*. 2016; Dong *et al*., 2019). Therefore, the integrated modification of drugs-dyes may improve further the bioavailability of original drugs for their better therapeutic efficacy as well as accumulation-related diagnostic monitoring (Wang *et al*., 2018).

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