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Biorthogonal Click Chemistry: Pioneering Targeted Cancer Therapeutics

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Abstract: The characteristics of click chemistry include a high yield, a wide scope, less cytotoxic byproducts, high stereospecificity, and simple reactions under physiological conditions and the resulting irreversible chemical bonds. They modify biomolecules with various compounds, such as nucleic acids, lipids, and proteins. This review highlights the multiple reactions of click chemistry, and drug delivery systems by enabling the precise conjugation of therapeutic agents to target ligands or carriers. Click chemistry targets molecules and pathways involved in cancer progression; and several technologies are used in cancer with evidence *in vitro, in vivo,* and clinical studies. These cutting-edge strategies offer unparalleled precision in targeting cancer cells, which could lead to more effective treatment and greater patient outcomes.

Keywords: Click chemistry, Molecular targets, Drug delivery systems, Glycoengineering, Cancer

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Introduction

Carolyn Bertozzi gave the term Bioorthogonal chemistry in the late 1990s. It advertises chemical reactions that occur quickly in biological environments, around ambient temperature, in aqueous solutions, and in the presence of biological molecules at low concentrations. This type of chemistry enables selective reactions with a single functional group, which is useful in understanding biological systems. Bioorthogonal chemistry has become evident as an optimistic approach in anticancer applications, by enabling targeted tumor detection, imaging, and therapy. Metal-free click reactions are preferred over metal-catalyzed reactions due to their biocompatibility and reduced risk of cytotoxicity. Despite the development of various approaches, there is a lack of extensive reviews highlighting the advantages of the biorthogonal chemistry-based strategies for cancer theragnostic. This review focuses on the latest development in cancer theragnostic led by metal-free bioorthogonal click chemistry, providing

comprehensive insight into these advances.

Chemical reactions that occur under physiological conditions resulting in the formation of irreversible chemical bonds are called click chemistry reactions. Thus, to modify biomolecules like lipids, nucleic acids, and proteins with various compounds, click chemistry is predominantly used. This technology has emerged as a breakthrough in bioorthogonal chemistry, which depends upon chemical reactions that take place inside living beings without interfering with natural biological processes. Click reactions emerged as a crucial component of bioorthogonal chemistry, as click reactions take place exclusively between two synthetic molecules, resulting in quick and irrevocable joining of molecules collectively. The copper-catalyzed azide-alkyne cycloaddition is an example of a click reaction, while Bertozzi opened new frontiers with the creation of the bioorthogonal strain-promoted azide-alkyne cycloaddition. The above-mentioned reactions have transformed biological and chemical sciences by promoting exclusive, high-yielding, quick, and precise ligations and also by presenting exceptional methods to alter living systems.

This technology has been thoroughly examined for oncology labeling and targeting. On the other hand, since biochemical labeling can happen in both tumor cells as well as nontumor cells, to develop selective cancer therapy it is crucial to achieve targeted cancer marking. To facilitate selective marking of tumor cells or specifically delivered to cancerous tissues, rational designing of unnatural sugars can be done.

Reactions of click chemistry

- Copper (I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC)
- Strain-promoted [3 + 2] azide-alkyne cycloaddition (SPAAC) reaction
- Strain-promoted alkyne-nitrone cycloaddition (SPANC)
- Transition metal catalysis (TMC)
- Inverse electron demand Diels-Alder (iEDDA) reaction

In chemical biology, generally, two types of reactions are commonly used for various purposes. The first type of reaction is bond-formation reactions,which include CuAAC, SPAAC, photo-click cycloadditions, and SPANC. These reactions are often used for biomarker identification as well as protein-targeted modifications. Bond-release reactions are the second type of reaction which include Staudinger ligation, TMC, and iEDDA. These reactions especially aid in studying protein activation and drug discovery.

While the SPAAC reaction with cyclooctyne (OCT) requires a long reaction time, under systemic conditions, the reactions of CuAAC and SPAAC cyclooctyne with azide take place without the need for copper catalysis. To deal with this issue, scientists have advanced enhanced OCTs like biarylazacyclooctynone, bicyclononyne (BCN), azadibenzocyclooctyne (ADIBO/DIBAC/DBCO), dibenzocyclooctyne, and difluorinated cyclooctyne (DIFO). These modified OCTs result in faster copper-free click chemistry.

The iEDDA reaction is also faster copper-free click chemistry, which involves the cycloaddition of s-tetrazine and trans-cyclooctene (TCO) derivatives. Bioorthogonal chemical reporters of iEDDA reactions such as cyclopropane, norbornene, N-acylazetine, or vinylboronic acid have been developed by researchers. The reporters have shown potential in cell marking with fluorophores as well as functional molecules under physiological conditions upon reaction with tetrazines (Tz).

For molecular analysis, targeted imaging, and drug delivery, the “copper-free” click chemistry has been predominantly used to bind individual molecules and nanoparticles. For example, for cell monitoring and imaging, various probes might be used to implement color changes. Moreover, by installing specific target molecules, like antibodies or peptides, on the cell surface, the efficiency or

potency of cell-based therapy can be enhanced as they improve targeting moiety. Besides, a rapid quantitative evaluation of cell monitoring efficiency can be utilized by azide-tagged total proteins with click-reaction.

Molecular Targets

Click chemistry has been utilized to target various molecules and pathways involved in cancer progression.

Cell Surface Receptors

The current approach for treating cancer primarily depends upon targeting cancerous extracellular receptors. Thus, the side effects on normal tissue can be avoided as the specific identification of cell-surface receptors facilitates targeted delivery of therapeutic agents to the site of interest. Although, protein receptors are not always unique to tumor cells, and in some cases, the expression level between cancer and normal cells is not significant.

Moreover, targeted ligands including folate, peptides, sugars, and aptamers have comparatively low binding affinity. Furthermore, in cancer treatment targeting is difficult and vulnerable due to the therapeutic resistance mutation of protein receptors. To overcome these limitations, cell surface glycans subjected to chemical modifications by the metabolism of concomitant artificial sugars is a feasible tool. This approach allows for the incorporation of synthetic chemical receptors on the surface of cells for targeted therapeutic delivery and non-invasive cell imaging. With the help of biorthogonal chemistry, the installed cell surface receptors can be targeted with high selectivity. For non-invasive cell imaging and targeted therapy, the metabolic glycan has been investigated. Many functional groups including clickable moieties for click reactions like ketone, azide, diazoalkane, isonitrile, alkyne, and strained cyclopropane, BCN, DBCO, and trans-cyclooctyne have been engineered on the cell surface glycoproteins metabolically. However, these functional groups lack release capacity for drug delivery. The inverse electron demand Diels-Alder (iEDDA) reaction with the use of tetrazine and trans-cyclooctene, attains high efficiency of targeting and drug delivery at low concentration in complicated biological conditions due to their fast reaction kinetics and biorthogonality. Click chemistry has been employed to develop ligands that target overexpressed receptors on cancerous cell surfaces, such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), folic acid receptor, and prostate-specific membrane antigen (PSMA). These ligands are generally conjugated to therapeutic agents or imaging probes to selectively target cancer cells.

Therapeutic Agents Acting via Cell Surface Receptors

(i) *Angiogenesis Inhibitors*: Click chemistry has been utilized to create molecules that aim to target angiogenesis; the tumors generally promote the creation of angiogenic vessels through this process to sustain their progression. By targeting angiogenic molecules likevascular endothelial growth factor (VEGF) or its receptors, it is possible to inhibit tumor vascularization and restrict tumor growth. The process of angiogenesis plays a crucial part in tumor growth, which is directed by several intermediaries like VEGF. For tumor angiogenesis, Cyclo-peptide CBO-P11 can be used as a targeting ligand as it has proven to selectively bind to receptors of VEGF. The conjugation of CBO-P11 and novel nanoparticles together can selectively target the tumor location. Near-infrared cyanine dye bearing an alkyne function was used to modify CBO-P11, allowing both “click” coupling on azido-modified nanoparticles and fluorescent tagging (Deshayes *et al.*, 2011).

During tumor blood vessel formation, the CD13 receptor acts as an essential modulator of endothelial vessel formation, as per various research. CD13 receptor, a zinc-dependent membrane-bound ectopeptidase, amplified expression of which is associated with the advancements of many tumors, such as prostate, colon, and pancreatic cancer. Using click chemistry, the dimeric NGR peptide (NGR2) was

conjugated with an azide-terminated Cy5.5 fluorophore (Cy5.5-N3) to rapidly produce Cy5.5-NGR2 in a quantitative yield. The Cy5.5-NGR2 peptide provides highly sensitive and target-specific imaging of CD13 receptor expression in tumors. The excellent tumor-to-normal tissue ratio and fast tumor targeting ability of Cy5.5-NGR2 have proved it a promising molecular probe, not only allowing the NIRF imaging of CD13 overexpressed tumor angiogenesis but also having the potential to facilitate non-invasive monitoring of CD13-targeted anti-angiogenesis therapy (Huang *et al*., 2016).

It has also been reported that click-chemistry-derived triazole ligands of arginine-glycine-aspartate (RGD) mimetics by Cu(I)-catalyzed 1,3-dipolar alkyne-azide coupling reaction, that showed binding affinity properties toward α(v)β(3)/α(v)β(5) integrins, inhibited the adhesion of integrin-expressing human melanoma cells to RGD-containing proteins of the extracellular matrix, such as vitronectin, fibronectin, and osteopontin, and to impede adhesion of melanoma cells and both *in vitro* and *in vivo* angiogenesis (Trabocchi *et al*., 2010). Click chemistry facilitates the conjugation of anti-angiogenic agents to target ligands for selective delivery to tumor vasculature.

(ii) *Apoptosis Inducers*: Researchers have utilized click chemistry to develop molecules that can induce apoptosis (programmed cell death) in cancer cells. By targeting key regulators of apoptosis, such as Bcl-2 family proteins or inhibitors of apoptosis proteins (IAPs), click chemistry enables the creation of pro-apoptotic agents that can selectively trigger cell death in cancer cells while sparing normal cells (Qian *et al*., 2022). Tam *et al*. (2007) designed a novel "clickable" affinity-based probe called DZ-1, an adenosylhomocysteine hydrolase inhibitor known as 3-Deazaneplanocin A (DZNep). This probe induces efficient apoptotic cell death in cancer cells without affecting normal cells. DZNep effectively reduces PRC2 components EZH2, SUZ12, and EED, and inhibits associated histone H3 Lys 27 methylation. By integrating RNA interference (RNAi), genome-wide expression analysis, and chromatin immunoprecipitation (ChIP) studies, a group of genes selectively repressed by PRC2 in breast cancer that can be reactivated by DZNep was identified. Furthermore, it was demonstrated that a set of these genes, including a novel apoptosis effector called FBXO32, is preferentially reactivated by DZNep. The reactivation of these genes by DZNep contributes to the induced apoptosis in breast cancer cells (Tan *et al*., 2007).

(iii) *DNA Damage Response* (*DDR*) *Inhibitors*: Using click chemistry, scientists have been able to target molecules involved in the DNA damage response pathway, which is often disrupted in cancer cells. Defects in the DNA damage response pathway can lead to tumor formation by promoting DNA mutations. However, this also creates vulnerabilities that can be targeted by synthetic lethality-based therapies. PARP inhibitors like olaparib have been successful in treating cancers with DNA-repairing deficiency, such as BRCA1 or BRCA2 mutations. This success has led to a search for more targetable components in the DDR signaling pathways. Inhibitors of DNA repair enzymes, such as PARP or DNA-PK, can sensitize cancer cells to DNA-damaging agents or radiation therapy.

Nucleoside analogs like 5-vinyl-2′-deoxyuridine (VdU) can be metabolically incorporated into cancer cell DNA and "clicked" to form a toxic product. The combination of VdU and an acridine-tetrazine conjugate (PINK) synergistically induces cytotoxicity in cultured human cells by inducing DNA damage through DSB formation, which leads to S-phase accumulation and apoptosis. Click chemistry facilitates the development of DNA repair inhibitors for targeted cancer therapy.

The use of click chemistry has made it easier to analyze the cell cycle by flow cytometry. Scientists can now use click chemistry to analyze DNA replication, RNA synthesis, and other cellular events. For example, a lanthanide-chelated, azide-

containing probe allows click-chemistry-mediated labeling of target molecules. Following the incorporation of the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) during DNA synthesis in the S-phase of the cell cycle, scientists can measure DNA synthesis in single cells using mass cytometry. In fluorescence microscopy, scientists can confirm the click-chemistry reaction between an azide-containing fluorophore and EdU molecules that have been incorporated into the DNA during the replication phase of the cell cycle. This can be done through colocalization with the DNA counterstain 4′,6-Diamidino-2-Phenylindole (DAPI). Finally, click chemistry enables the conjugation of CDK inhibitors to target ligands for selective delivery to tumor cells.

(iv) *Immune Checkpoint Inhibitors*: A major problem with using immune checkpoint blockade (ICB) for solid tumor treatment is that the antibodies have poor penetration capability. To address this issue, researchers have used peptides that can penetrate deeper into the tumor to develop a new strategy called click reaction-assisted peptide immune checkpoint blockade (CRICB). This involves using peptides that can assemble *in situ*, leading to better accumulation and longer PD-L1 occupancy, resulting in improved tumor inhibition. The strategy works by using a free DBCO-modified targeting peptide (TP) to efficiently bind PD-L1 in a deep solid tumor. Then, when an azide-tethered assembled peptide (AP) is introduced, a reagent-free click reaction occurs, causing the peptides to self-aggregate *in situ*, resulting in enhanced accumulation and prolonged occupancy. This new approach was shown to be highly effective in inhibiting tumor growth in experiments conducted by Xiao *et al.* (2020).

Drug Delivery Systems

Targeted therapy of drugs at effective concentrations to specific carcinoma sites is considered highly beneficial, yet challenging, in cancer therapeutics development. To achieve specific site prodrug activation, drug delivery systems rely on altered pH, intracellular redox potential (e.g. H_2O_2 and glutathione), or exaggerated release of enzymes (e.g. esterases, and proteases) that abnormal conditions exist in the tumor's unique microenvironment. However, some of the cleavable linkers that have been employed for this plan of action have proven to be uneven in circulation and may cause premature drug release, causing various undesirable side effects before drug delivery to the desired site.

In a pre-targeting delivery system, an antibody is used to guide prodrugs which act as a biomarker and is connected to a prodrug through a chemically labile linker. Significantly, the former Antibody-Drug Conjugate (ADC) remains stable and inactive in the systemic circulation. Upon reaching the target tumor site, the linker is selectively cleaved through a biorthogonal reaction (e.g. IEDDA (or) trans-cyclooctene (TCO)-azide cycloaddition), releasing the active drug in response to a secondary administered trigger.

The localized delivery and activation utilize a trigger that is designed to accumulate within localized tumor tissue that can subsequently activated by a systemically administered prodrug and triggered by a bioorthogonal reaction. The trigger can be a nanoparticle-based system (NP) that is directly applied to the tumor tissues by a surgeon or preferentially delivered to the tumor through the Enhanced Permeability and Retention (EPR) effect, or this can be a biocompatible hydrogel modified with a chemically activated and directly injected into tumor tissue.

The enrichment-triggered prodrug plan of action uses two reaction partners with a moderate reaction rate that are both modified with the same targeting molecule. The bioorthogonal reaction kinetics are designed to simultaneously administer each reaction partner, never reaching effective concentrations for an energetically favorable reaction to proceed. After both the biorthogonal components are accumulated at the desired site, the local high concentration surpasses the energetic barrier threshold and initiates the click reaction to release the active drug. Overall, the remarkable advancements in the

design of biorthogonal chemistry for prodrug activation represent a significant enhancement to current drug-delivery strategies.

(a) *Nanoparticles*: Nanoparticles are widely used in drug delivery due to their ability to encapsulate and deliver therapeutic agents to target sites. Click chemistry allows for the surface functionalization of nanoparticles with targeting ligands (e.g., antibodies, peptides) for specific recognition and uptake by cancer cells. Additionally, click chemistry can be used to attach drugs or imaging agents to the nanoparticle surface, enabling controlled release or imaging of the payload.

(b) *Liposomes*: These are lipid-based vesicles that can encapsulate hydrophilic or hydrophobic drugs for delivery. Click chemistry can be employed to functionalize liposome surfaces with targeting ligands for specific interaction with cancer cells. Furthermore, click chemistry enables the conjugation of therapeutic agents to liposome surfaces, allowing for targeted drug delivery and controlled release at the tumor site.

(c) *Polymeric Micelles*: Polymeric micelles are self-assembled nanoparticles formed from amphiphilic block copolymers, which can encapsulate hydrophobic drugs in their core. Click chemistry enables the functionalization of polymeric micelles with targeting ligands for tumor-specific delivery. Moreover, click chemistry can be used to conjugate therapeutic agents to the surface of polymeric micelles, providing a means for targeted drug delivery and controlled release.

(d) *Dendrimers*: Dendrimers are highly branched macromolecules with well-defined structures, which can encapsulate drugs within their interior or conjugate drugs to their surface. Click chemistry allows for the precise functionalization of dendrimer surfaces with targeting ligands for specific recognition of cancer cells. Additionally, click chemistry can attach therapeutic agents to dendrimer surfaces, enabling targeted drug delivery and controlled release.

(e) *Hydrogels*: Hydrogels are three-dimensional networks of hydrophilic polymers capable of absorbing and retaining large amounts of water. Click chemistry can be employed to crosslink or functionalize hydrogel networks with targeting ligands for localized drug delivery to tumor sites. Furthermore, click chemistry allows for the conjugation of therapeutic agents or imaging probes to hydrogel matrices, enabling sustained release or imaging of the payload.

(f) *Exosomes*: Exosomes are small extracellular vesicles released by cells that can serve as natural drug delivery vehicles. Click chemistry enables the surface modification of exosomes by targeting ligands for specific interaction with cancer cells. Moreover, click chemistry can be used to load therapeutic agents into exosomes or conjugate drugs to exosome surfaces, facilitating targeted drug delivery and enhanced therapeutic efficacy.

Click chemistry plays a crucial role in the development of various types of drug delivery systems by enabling the precise conjugation of therapeutic agents to target ligands or carriers. These click chemistry-based drug delivery systems offer opportunities for targeted drug delivery, controlled release, and enhanced therapeutic efficacy in the treatment of cancer and other diseases.

Glycoengineering

In metabolic glycan engineering, N-azidoacetyl-mannosamine (Ac4ManNAz) is commonly employed for modifying the cell surface. Ac4ManNAz is processed into N-azidoacetyl neuraminic acid and it is incorporated into glycans, resulting in the expression of azide groups on the glycans present on the surface of viable cells

(i) *Compound*: *Ac4ManNAz 5μm for 3 days incubation*

In vitro: *Breast cancer cell* (*B16*): Cell surface glycosylation can be achieved using click chemistry and DNA rolling circle amplification (RCA). This technique involves a fluorescence resonance energy transfer (FRET)-based methodology that enables imaging of the glycan in specific proteins present on the cell surface. This

method is widely applicable for imaging the glycosylation of cellular proteins in living cells. GPC3 is a crucial cell surface proteoglycan that features two potential heparan sulfate attachment sites. It is a specific biomarker for several common cancers, particularly hepatocellular carcinoma (Wang *et al*., 2014).

(ii) *Compound*: *Ac4ManNAz 10μm for 3 days incubation*

In vitro: *Lung cancer*: *Human pulmonary adenocarcinoma cells* (*A549*): The study found that certain genes were upregulated by Ac4ManNAz, which is related to the stimulation of MAPK activity (TLR4, THBS1, FPR1), apoptotic program (NLRP3), fever elicitation (PTGER3, AFRB3), promotion of apoptosis (COL4A3), and responses of immune and inflammatory systems (IL15, KNG1, CD28, ITGAL). On the other hand, energy-generating genes (CYBB), transport (GABRA1, GABRQ, SLC6A2, AKAP7), neuroactive communication (CHRM4, PTGDR2, P2RX7), the JAK-STAT signal transmission pathway (CNTF), the PI3K-Akt signaling pathway (PIK3CG, ESR2, CCR2), phosphate incorporation (PTPRE, TTBK2), the cell cycle (NFATC1, SPDYA, CAMK2A, CAMK2B, OTX2, CDON), Cellular expansion and proliferation (ESR1), together with migration and angiogenic activity (FLT1, ANGPT1, FN1, LGI4) were suppressed in expression. The results showed that Ac4ManNAz has an impact on a wide range of cellular functions along with signaling pathways. The study also suggests that elevated concentrations of Ac4ManNAz induce the programmed cellular death process and immune response, and inhibit the cellular cycle, replication, and adhesion (Han *et al*., 2017).

(iii) *Compound: Ac4ManNAz 10μm for 3 days incubation and 10 μm* (*iv*) *in mice*

In vitro: *Mesenchymal stem cells* (*MSCs*): Cyclooctyne-based reagent (DBCO) and nanoparticles or fluorophores tagged with dibenzyl cyclooctyne are often used in mice azide functional groups) in the tumor tissue, subsequently facilitating the delivery of compounds conjugated with DBCO (DBCO-conjugates). Glycoconjugated-engineered MSCs exhibited actively targeted tumor localization in subcutaneous and orthotopic lung and ovarian tumor models. Following systemic administration of dibenzyl cyclooctyne (DBCO)-conjugated fluorophores or nanoparticles to mice pretreated with MSCs resulted in enhanced tumor deposit of these agents through bioorthogonal copper-free click chemistry. Further, administration of glycoconjugate-engineered MSCs along with paclitaxel-loaded nanoparticles functionalized with DBCO produced significant ($p < 0.05$) suppression of tumor growth along with improved survival rates ($p < 0.0001$) demonstrated in an orthotopic model of ovarian tumors with metastasis (Layek *et al*., 2016).

(iv) *Compound: Tz–DBCO 10μm about 30 min*

In vitro: *Jurkat T lymphocytes* (*human*), *and A549 lung carcinoma cells* (*human*): A click chemistry strategy has been developed for efficient and robust cell-to-cell adhesion. Before the conjugation of Tz and TCO molecules, cells were modified using glycoconjugate metabolic engineering to azide groups onto the cell floor along with minimal biological disruptions. Exposure to Tz-DBCO or TCO-DBCO showed minimal impact on cell viability. Among the four cell lines tested, Jurkat T and A549 cells had significantly higher incorporation than NIH3T3 cells, while EL4 cells showed the lowest incorporation (Koo *et al*., 2015).

(v) *Compound: Ac4ManNAz 50 μm for 3 days*

In vivo: *Incubation with chondrocyte* (*rabbit*) *for 3 days later implanted in mice*; *DBCO-650 50 μM for 1 h chondrocyte* (*rabbit*): Chondrocytes, which are cells found in cartilage, were treated with Ac4ManNAz to create non-natural azide groups (-N3) on their surface. Then, these unnatural azide groups were specifically conjugated with near-infrared fluorescent (NIRF) dye-conjugated cyclooctyne-based-reagent (DBCO-650) using bio-orthogonal copper-suspended click chemistry. This produced robust NIRF signals with relatively minimal cytotoxic effects. The levels of azide

groups and DBCO-650 could be readily adjusted by adding different amounts of Ac4ManNAz and DBCO-650 to the cell culture system. To track these cells *in vivo*, DBCO-650-labeled chondrocytes (1 × 10(6) cells) were implanted into mice on a 3D scaffold. The grafted DBCO-650-labeled chondrocytes could be productively tracked for up to 4 weeks using NIRF imaging technology (Yoon *et al*., 2016).

(vi) *Compound: Ac3ManNAz in <5 μM in 2 days*

In vitro: Primary hippocampal neurons (*rat*): The researchers incorporated a non-natural monosaccharide, peracetylated N-azidoacetyl-D-mannosamine, into the sialic acid biochemical pathway to display N-azidoacetyl sialic acid to PSA-NCAM. The conventional metabolic labeling method using dissociated neuron cells caused significant neurotoxicity. However, this modified strategy eliminated the neurotoxicity, allowing the investigation regarding the time and location of PSA distribution in primary hippocampal neurons. The study found that PSA-NCAM was continuously synthesized and recycled during neuronal development. The two-color labeling revealed that newly formed PSA-NCAMs were mainly delivered and integrated into growing neurites and not significantly into the cell body (Kang *et al*., 2015).

(vii) *Compound: DBCO-Cy5 100 μM for 48 h*

In vitro: *A549*; *NIH3T3*: DBCO-Cy5 5 nmol in mice i.v administration for 4 days. A chemical process called glycoengineering can be used to artificially induce non-natural sialic acids along with azide groups localized on the outer membrane of target cells. To visualize these labeled cells, a tetra-acetylated N-azidoacetyl-D-mannosamine is used, followed by the transplantation of these cells into a mouse liver. Intravenous injection of DBCO-conjugated Cy5 (DBCO-Cy5) administered to mice then employing chemical interaction it binds with the azide groups on the target cells' surface for real-time observation in living organisms. This labeling technique is highly useful for cell tracking with improved labeling efficacy and reduced false signals caused by macrophages' phagocytosis (Kang *et al*., 2014).

(viii) *Compound: DBCO-ZnPc-LP*

In vivo: *In mice 2.5 mg/kg (i.v) for 4 days*: DBCO-ZnPc-LP is a nanoagent composed of lipid-poly(ethylene glycol) that contains a solitary lipophilic near-infrared (NIR) dye zinc(II)-phthalocyanine (ZnPc). It is designed to target tumors through a two-step chemical process that involves glycan metabolism engineering along and click chemistry. Once injected intravenously, DBCO-ZnPc-LP can efficiently deliver to tumor cells and solid tumors. By combining the targeting approach, photoacoustic visualization, and the amplified outcome interaction between PTT and PAT, it is possible to accurately locate and eliminate solid tumors *in vivo*. This information comes from a study by Du *et al*. (2017).

(ix) *Compound: DBCO-Cy5*

In vivo: *5 nmol (i.v) in mice for 4 days*: The researchers altered a cell surface protein called CsgA to contain a specific codon. They then incorporated an amino acid called pAzF and displayed the engineered strains outside the cell. The modified strains could be labeled incorporating a Cy5 dye conjugated to the DBCO element via copper-free click reaction. To test the labeling strategy, the researchers used a mouse model and observed the labeling of the engineered strain by administering the dye orally. The labeling was still visible several days after the colonization of the gastrointestinal tract. This study provides a basis for developing strategies to track microbial activity *in vivo* that are compatible with non-invasive imaging techniques and can monitor specific microbial populations over time. (Praveschotinunt *et al*., 2018).

(x) *Compound: BCN-Lipo*

In vivo: *10 mg/kg* (*i.v.*) *mice for 4 days*: Attached are azide group-containing metabolic building blocks, and triacetylated N-azidoacetyl-d-mannosamine to generate 4 poly(amidoamine) dendrimer backbone. The nanoparticle-sized dendrimer of Nano metabolic precursors (Nano-MPs) could generate azide groups on the surface

of tumor cells homogeneously regardless of cell types via metabolic glycoengineering. Additionally, in mice models with tumors, Nano-MPs could be targeted to the tumor tissues owing to the enhanced permeation and retention (EPR) impact, and they successfully generated azide groups upon carcinoma cells *in vivo* post to an intravenous injection administration. Hence, the overall outcomes demonstrated indicate that our nanoscale metabolic building blocks could be extensively applied toward an innovative tumor-targeting strategy for molecular imaging and drug delivery, irrespective of the phenotype of diverse tumor cells (Lee *et al*., 2017).

(xi) *Compound: BCN-CNPs 3 days*

In vitro: *BT-474, MDA-MB231, MCF-7*: *In vivo:* mice: U87 tumor-bearing mice 5 mg/kg, i.v. (48 h). Azide-functionalized chemical reporters have been successfully introduced onto surface glycans of various carcinoma cells such as lung adenocarcinoma (A549), glioblastoma (U87), and breast carcinoma (BT-474, MDA-MB-231, MCF-7) via metabolic engineering *in vitro*. In addition, the tumor targeting capabilities of synthetic azide reporters in conjunction with bicyclononyne (BCN)-conjugated glycol chitosan nanoparticles (BCN-CNPs) and compared with integrin αvβ3-targeted cyclic RGD-conjugated CNPs (cRGD-CNPs), utilizing both *in vitro* and *in vivo* methodologies. Additionally, in the isolated heterogeneous U87 cells, BCN-CNPs could connect with artificial azide markers on carcinoma cells more uniformly (~92.9%) compared to cRGD-CNPs. Therefore, the artificial azide-reporter-targeting strategy can address mixed tumor cell populations via bio-orthogonal click reaction (Yoon *et al*., 2017).

(xii) *Compound: Ac4ManNAz*

In vivo: *40 mg/kg/day* (*i.v.*) *daily for 3 days in mice*: Ac4ManAz by converting the acetyl group at its anomeric position to a confined ether linkage that can be specifically broken down by cancer-upregulated enzymes and thus enables the overexpression of azido groups on the surface of carcinoma cells. HDAC (histone deacetylase) and acetylated azidomannosamine responsive to cathepsin L a similarly enzymatically activatable synthesized Ac4ManAz analog, governed cancer-targeted labeling *in vivo*, which enhanced carcinoma aggregation of a dibenzocyclooctyne-doxorubicin couple via click chemistry and enabled targeted therapy counter to LS174T colon carcinoma, MDA-MB-231 triple-negative breast carcinoma and 4T1 metastatic breast cancer in mice (Wang *et al*., 2017).

(xiii) *Compound: TCOK- Me2Tz,*

In vivo: *4 nmol* (*i.v*), *mice*: Synthesized the axial isomer of trans-cyclooctene conjugated lysine (TCOK-a, 2) and 3,6-dimethyl-1,2,4,5-tetrazine (Me2Tz, 3) serving like a bio-orthogonal cleavage duo for invDA-mediated lysine uncaging and subsequent protein activation inside cells. Thus, it is deduced that the site-specific integration of TCOK-a into place of the catalytic lysine residue may deactivate the kinase, in contrast, the Me2Tz-triggered invDA reaction and the following decaging of TCOK-a could reintroduce the essential lysine in the kinase activity chemically preserved. In this context, bio-orthogonal chemical activation plan of action for selective preservation of kinase activity within living cells in addition to the living animals (Zhang *et al*., 2016).

Conclusion

Click chemistry is an emerging trend in biomedical research and cancer therapeutics, which offersunparalleled precision, efficacy, and versatility. The key advantage of click chemistry ability to facilitate high-yield stereospecific reactions which often occur under mild physiological conditions i.e. neutral pH and ambient temperature, which makes it suitable for sensitive biological molecules without any damage and has revolutionized the modification of biomolecules and the design of drug delivery systems achieved by enabling precise conjugation of cancer therapeutic agents to target ligands or carriers. Their applications in cancer therapeutics

via targeting cancer-specific molecules and pathways are evidenced in *in vitro* and *in vivo* studies. As research advances, click chemistry will be pivotal in developing innovative strategies for combating cancer and other complex diseases.

Ethical Statement

This is a review article hence no Ethical Approval needed.

Author Contributions

Each author has contributed equally in designing the research, conducting thorough analysis, drafting the manuscript, and refining its content. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

References

Deshayes S, Maurizot V, Clochard MC, Baudin C, Berthelot T, Esnouf S, Lairez D, Moenner M and Déléris G. (2011) "Click" conjugation of peptide on the surface of polymeric nanoparticles for targeting tumor angiogenesis. Pharm Res. 28(7): 1631-1642.

Du L, Qin H, Ma T, Zhang T and Xing D. (2017) In vivo imaging-guided photothermal/photoacoustic synergistic therapy with bioorthogonal metabolic glycoengineering-activated tumor targeting nanoparticles. ACS Nano 11(9): 8930-8943.

Han SS, Lee DE, Shim HE, Lee S, Jung T, Oh JH, Lee HA, Moon SH, Jeon J, Yoon S, Kim K and Kang SW. (2017) Physiological effects of Ac4ManNAz and optimization of metabolic labeling for cell tracking. Theranostics 7(5): 1164-1176.

Huang R, Conti PS and Chen K. (2016) In vivo tumor angiogenesis imaging using peptide-based near-infrared fluorescent probes. Methods Mol Biol. 1444: 73-84.

Kang K, Joo S, Choi JY, Geum S, Hong SP, Lee SY, Kim YH, Kim SM, Yoon MH, Nam Y, Lee KB, Lee HY and Choi IS. (2015) Tissue-based metabolic labeling of polysialic acids in living primary hippocampal neurons. Proc National Acad Sci USA. 112(3): E241-E248.

Kang SW, Lee S, Na JH, Yoon HI, Lee DE, Koo H, Cho YW, Kim SH, Jeong SY, Kwon IC, Choi K and Kim K. (2014) Cell labeling and tracking method without distorted signals by phagocytosis of macrophages. Theranostics. 4(4): 420-431.

Koo H, Choi M, Kim E, Hahn SK, Weissleder R and Yun SH. (2015) Bioorthogonal click chemistry-based synthetic cell glue. Small 11: 6458-6466.

Layek B, Sadhukha T and Prabha S. (2016) Glycoengineered mesenchymal stem cells as an enabling platform for two-step targeting of solid tumors. Biomaterials 88: 97-109.

Lee S, Jung S, Koo H, Na JH, Yoon HY, Shim MK, Park J, Kim JH, Lee S, Pomper MG, Kwon IC, Ahn CH and Kim K. (2017) Nano-sized metabolic precursors for heterogeneous tumor-targeting strategy using bioorthogonal click chemistry in vivo. Biomaterials. 148: 1-15.

Praveschotinunt P, Dorval Courchesne NM, den Hartog I, Lu C, Kim JJ, Nguyen PQ and Joshi NS. (2018) Tracking of engineered bacteria *in vivo* using nonstandard amino acid incorporation. ACS Synth Biol. 7(6): 1640-1650.

Qian S, Wei Z, Yang W, Huang J, Yang Y and Wang J. (2022) The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. Front Oncol. 12: 1-16.

Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, Karuturi RK, Tan PB, Liu ET and Yu Q. (2007) Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev. 21(9): 1050-1063.

Trabocchi A, Menchi G, Cini N, Bianchini F, Raspanti S, Bottoncetti A, Pupi A, Calorini L and Guarna A. (2010) Click-chemistry-derived triazole ligands of arginine-glycine-aspartate (RGD) integrins with a broad capacity to inhibit adhesion of melanoma cells and both *in vitro* and *in vivo* angiogenesis. J Med Chem. 53(19): 7119-7128.

Wang H, Wang R, Cai K, He H, Liu Y, Yen J, Wang Z, Xu M, Sun Y, Zhou X, Yin Q, Tang L, Dobrucki IT, Dobrucki LW, Chaney EJ, Boppart SA, Fan TM, Lezmi S, Chen X, Yin L and Cheng J. (2017) Selective *in vivo* metabolic cell-labeling-mediated cancer targeting. Nat Chem Biol. 13(4): 415-424.

Wang Y, Tang L, Li Z, Lin Y and Li J. (2014) *In situ* simultaneous monitoring of ATP and GTP using a graphene oxide nanosheet-based sensing platform in living cells. Nat Protoc. 9(8): 1944-1955.

Xiao WY, Wang Y, An HW, Hou D, Mamuti M, Wang MD, Wang J, Xu W, Hu L and Wang H. (2020) Click reaction-assisted peptide immune checkpoint blockade for solid tumor treatment. ACS Appl Mater Interfaces 12(36): 40042-40051.

Yoon HI, Yhee JY, Na JH, Lee S, Lee H, Kang SW, Chang H, Ryu JH, Lee S, Kwon IC, Cho YW and Kim K. (2016)

Bioorthogonal copper free click chemistry for labeling and tracking of chondrocytes *in vivo*. Bioconjug Chem. 27(4): 927-936.

Yoon HY, Shin ML, Shim MK, Lee S, Na JH, Koo H, Lee H, Kim JH, Lee KY, Kim K and Kwon IC. (2017) Artificial chemical reporter targeting strategy using bioorthogonal click reaction for improving active-targeting efficiency of tumor. Mol Pharm. 14(5): 1558-1570.

Zhang G, Li J, Xie R, Fan X, Liu Y, Zheng S, Ge Y and Chen PR. (2016) Bioorthogonal chemical activation of kinases in living systems. ACS Cent Sci. 2(5): 325-331.