European Journal of Medicinal Chemistry 315 (2026) 118967

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: www.elsevier.com/locate/ejmech

Review article

Beyond conventional 5-Fluorouracil: Recent medicinal chemistry strategies to enhance its anticancer efficacy

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ARTICLE INFO

Keywords:
5-Fluorouracil
Anticancer agents
Mutual prodrugs
Hybrids
Prodrugs

ABSTRACT

Antimetabolites are anticancer drugs designed to mimic endogenous substrates, thereby inducing irreversible damage that ultimately leads to tumor cell death. 5-Fluorouracil (5-FU) belongs to this class, as it is an uracil analog capable of interfering with both DNA and RNA. Despite its widespread use in the treatment of several solid tumors, such as pancreatic, stomach, lung, breast, esophageal, and colorectal cancers, 5-FU suffers from limitations such as poor bioavailability, a short half-life, and low selectivity. This review aims to discuss the main medicinal chemistry strategies developed to overcome these drawbacks, and to highlight emerging trends over the past five years. Unlike recently published reviews, which predominantly focus on single strategies, this work aims to offer a comparative analysis of structural modifications performed on 5-FU to develop its analogs, hybrid compounds, and prodrugs. Collectively, the available evidence identifies mutual prodrugs as a leading strategy to address the long-standing clinical challenges associated with 5-FU therapy, with future efforts likely to focus on improved tumor targeting and translational potential.

1. Introduction

5-Fluorouracil (5-FU) was initially discovered in 1957 and then approved by the Food and Drug Administration (FDA) in 1962 [1]. It is used worldwide as a cancer treatment for solid tumors, including pancreatic, stomach, lung, breast and esophageal cancer, but it is especially used as a first-line treatment for colorectal cancer (CRC) [2]. However, some factors limit its use and clinical efficacy, such as its short half-life, low selectivity, various side effects, and poor bioavailability, reducing CRC cure rates to only 15% [3,4]. 5-FU belongs to the class of fluoropyrimidines, which were developed following studies on rat hepatocytes, showing that these cells utilized uracil more rapidly than normal cells [5]. 5-FU differs from uracil due to the presence of a fluorine atom, which acts as a bioisoster of the hydrogen atom at the 5-position of the pyrimidine ring [6,7]. 5-FU is an antimetabolite, a molecule capable of being incorporated into macromolecules, thereby inhibiting their normal function. Therefore, it can be incorporated into DNA and RNA, disrupting key biosynthetic processes, while also inhibiting thymidylate synthase (TS) [8]. It is known that 5-FU, being an uracil analog, enters the cell using the same facilitated transport system and then follows the anabolic and catabolic pathway (Fig. 1) [9,10].

In the anabolic pathway, 5-FU is converted into several active metabolites, such as 5-fluorodeoxyuridine monophosphate (FdUMP), which inhibits TS, an essential enzyme for the synthesis of thymidine, necessary for DNA replication (Fig. 1) [11]. TS catalyzes the conversion of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP) thanks to the transfer of a methyl group from 5,10-methylenetetrahydrofolate (MTHF), a folate cofactor (Fig. 1) [12]. FdUMP forms a stable ternary complex with TS and MTHF, thereby blocking the synthesis of deoxynucleotides, such as 2′-deoxythymidine-5′-triphosphate (dTTP), which is essential for the incorporation of thymine into DNA [13,14]. This causes an imbalance in the deoxynucleotide pool, with an excess of dUMP and a reduction of dTTP, potentially leading to further DNA damage [15]. Two other important metabolites, fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP), induce DNA and RNA dysregulation, causing damage and ultimately leading to cell death (Fig. 1) [9,16]. 5-FU also interferes with RNA processing by inhibiting pseudouridine synthase. When uracil is replaced by 5-FU in tRNA, the conversion of uridine into pseudouridine is prevented [17]. Similarly, methylation pathways are

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https://doi.org/10.1016/j.ejmech.2026.118967
Received 16 February 2026; Received in revised form 2 May 2026; Accepted 14 May 2026
Available online 19 May 2026

also blocked by fluorinated tRNA, resulting in reduced nucleoside modifications [18].

In contrast, the catabolic pathway is the main degradation route of 5-FU. Here, the enzyme dihydropyrimidine dehydrogenase (DPD) plays a key role, irreversibly converting 5-FU into dihydrofluorouracil (DHFU) through a rate-limiting step (Fig. 1) [8]. Overexpression of the DPD gene in CRC is one of the main factors associated with 5-FU resistance, leading to the need for higher doses and the development of drug resistance [19]. About 85% of administered 5-FU is transformed into its inactive metabolite, meaning that DPD significantly influences both the anti-cancer effect and the adverse effects of 5-FU [20]. Therefore, intra- and inter-individual variability in DPD activity is an important pharmacokinetic factor that must be considered in clinical treatment [21]. In patients with DPD deficiency, treatment with 5-FU can cause severe gastrointestinal and neurological toxicities, and even death [22]. Moreover, since 60-90% of intravenously administered 5-FU is excreted as α-fluoro-β-alanine (FBAL) within 24 h (Fig. 1) [23], this metabolite is considered the main driver of the observed toxicities, especially those involving the nervous and cardiovascular systems [24,25]. DPD activity is also linked to circadian rhythms, which have been observed in animals and are likely present in humans. In patients receiving continuous 5-FU infusion via automatic pumps, serum drug concentrations follow a circadian pattern, inversely correlated with DPD activity in peripheral blood mononuclear cells. This variation may explain the wide range of 5-FU half-life, which can vary from 4 to 25 min after intravenous infusion. For this reason, some oncologists, especially in Western Europe, suggest timed infusion regimens to optimize drug administration over 24 h [26,27].

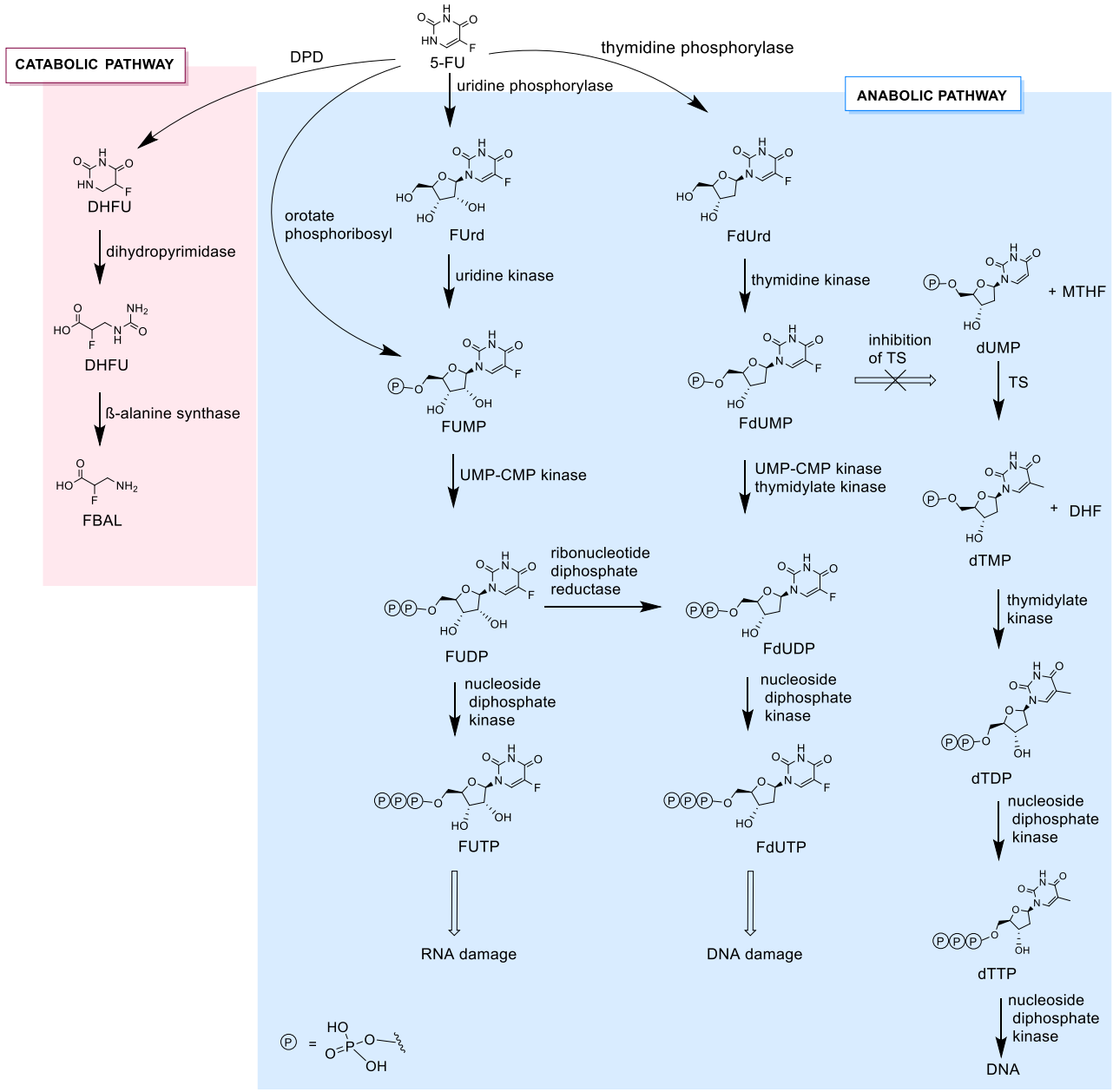


Fig. 1. Anabolic and catabolic pathways of 5-FU. Once inside the cancer cell, 5-FU converts into active metabolites (5-fluorouridine, FUrd; 5-fluorouridine monophosphate, FUMP; 5-fluorouridine diphosphate, FUDP; 5-fluorouridine triphosphate, FUTP), leading to RNA damage and (5-fluorodeoxyuridine, FdUrd; 5-fluorodeoxyuridine monophosphate, FdUMP; 5-fluorodeoxyuridine diphosphate, FdUDP; 5-fluorodeoxyuridine triphosphate, FdUTP) causing DNA damage. Additionally, FdUMP inhibits TS necessary for DNA replication. Concurrently, the catabolic pathway, mediated by dihydropyrimidine dehydrogenase (DPD), deactivates 5-FU into dihydrofluorouracil (DHFU) and then α-fluoro-β-alanine (FBAL) limiting its efficacy and reducing its half-life.

5-FU is primarily administered intravenously because this method is easier to manage compared to other routes, avoiding gastrointestinal absorption issues and rapid degradation. It can be given as an intravenous bolus or continuous infusion for up to 5 days, either alone or in combination with folic acid (FA), other chemotherapy drugs, and/or radiotherapy. Since 5-FU clearance is faster with continuous infusion, this method allows for higher total doses. However, a major limitation of 5-FU therapy is its high pharmacokinetic variability, which is observed in both infusion methods [28]. On the other hand, oral administration has low bioavailability due to the previously mentioned circadian and genetic variability in DPD activity [29]. This has led to the development of prodrugs in order to overcome these disadvantages [30]. In recent years, enzymatically activated prodrugs, polymer-drug conjugates, and controlled-release systems have been explored as alternative approaches [31,32]. An example of a 5-FU prodrug is capecitabine, which is converted into 5-FU through a three-step enzymatic process that includes carboxylesterase (located in the liver), cytidine deaminase (cyd deaminase, present in the liver and various solid tumors), and thymidine phosphorylase (dThd phosphorylase), which is predominantly expressed in tumor tissues (Fig. 2A) [33].

Another widely used strategy is to combine 5-FU with DPD inhibitors for oral administration [33]. This approach has multiple benefits, including inhibition of 5-FU catabolism during first-pass metabolism and gastrointestinal absorption. Moreover, DPD inhibition improves the pharmacokinetics of 5-FU, reducing inter-individual variability and prolonging the drug's half-life [28]. A first example is UFT, a combination of tegafur (another 5-FU prodrug) and uracil, which competes with the released 5-FU for the active site of DPD, leading to higher and sustained 5-FU levels (Fig. 2B) [34]. A second one is emitefur (Fig. 2B), a mutual prodrug composed of a 5-FU derivative (1-ethoxymethyl-5-FU, EM-FU) covalently linked to a potent DPD inhibitor (3-cyano-2,6-dihydroxypyridine, CNDP). This mutual prodrug is worth mentioning because it progressed through phase I and II clinical evaluations, even if it failed to advance further due to an unfavorable toxicity-to-benefit profile, which did not demonstrate sufficient improvement over existing fluoropyrimidine analogs [35]. A more recent pre-clinical polymeric conjugate is EMC-5-FU, a 5-FU prodrug that rapidly binds to albumin in the bloodstream, forming a conjugate that significantly prolongs the half-life and systemic exposure of 5-FU (Fig. 2C). This results in an enhanced 5-FU tumor accumulation and greater therapeutic efficacy compared to traditional intravenous administration [36]. Fig. 3 provides a schematic representation of some the prodrugs discussed and their

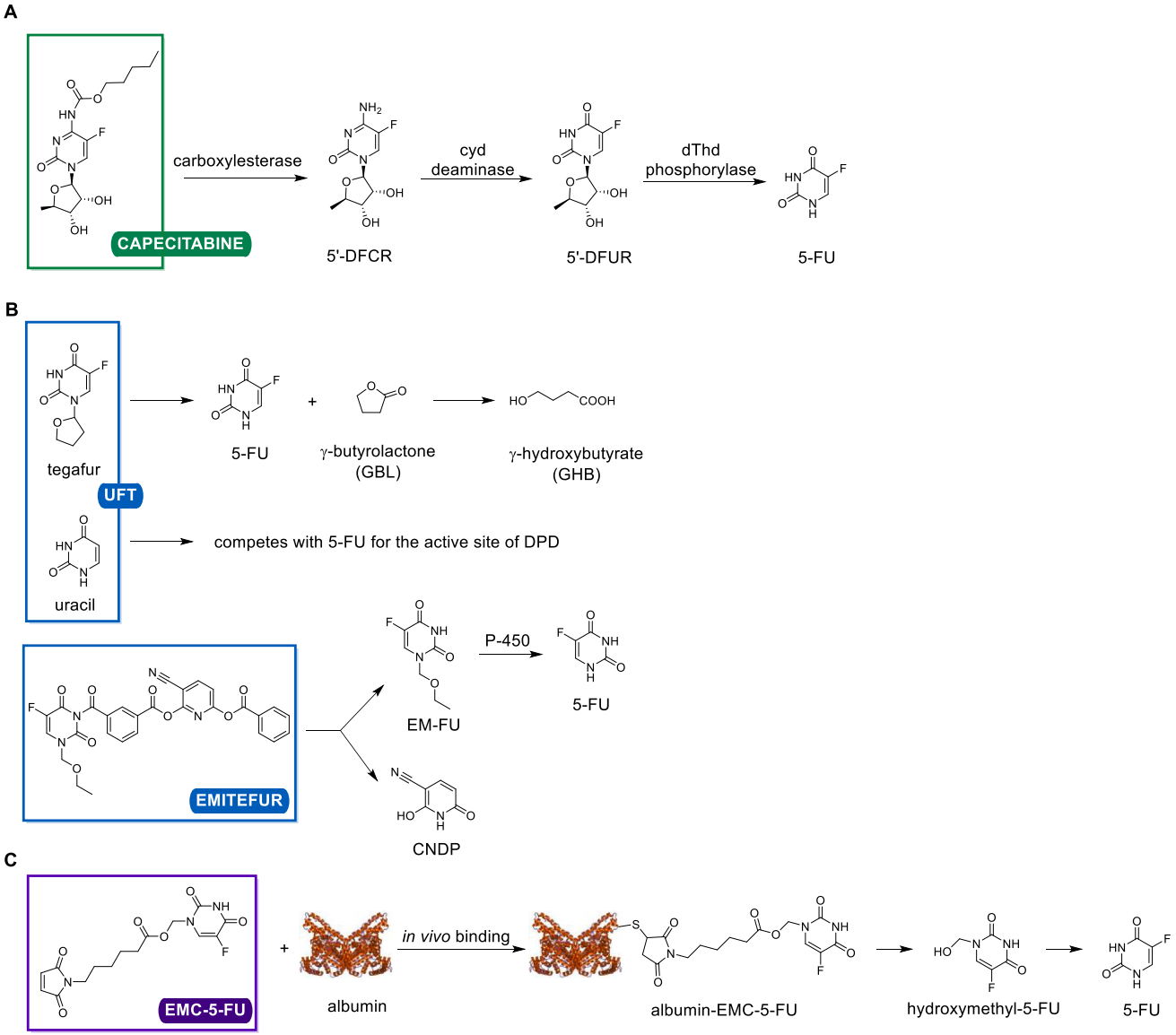


Fig. 2. Mechanism of activation of A) capecitabine, B) UFT and emitefur, C) EMC-5-FU.

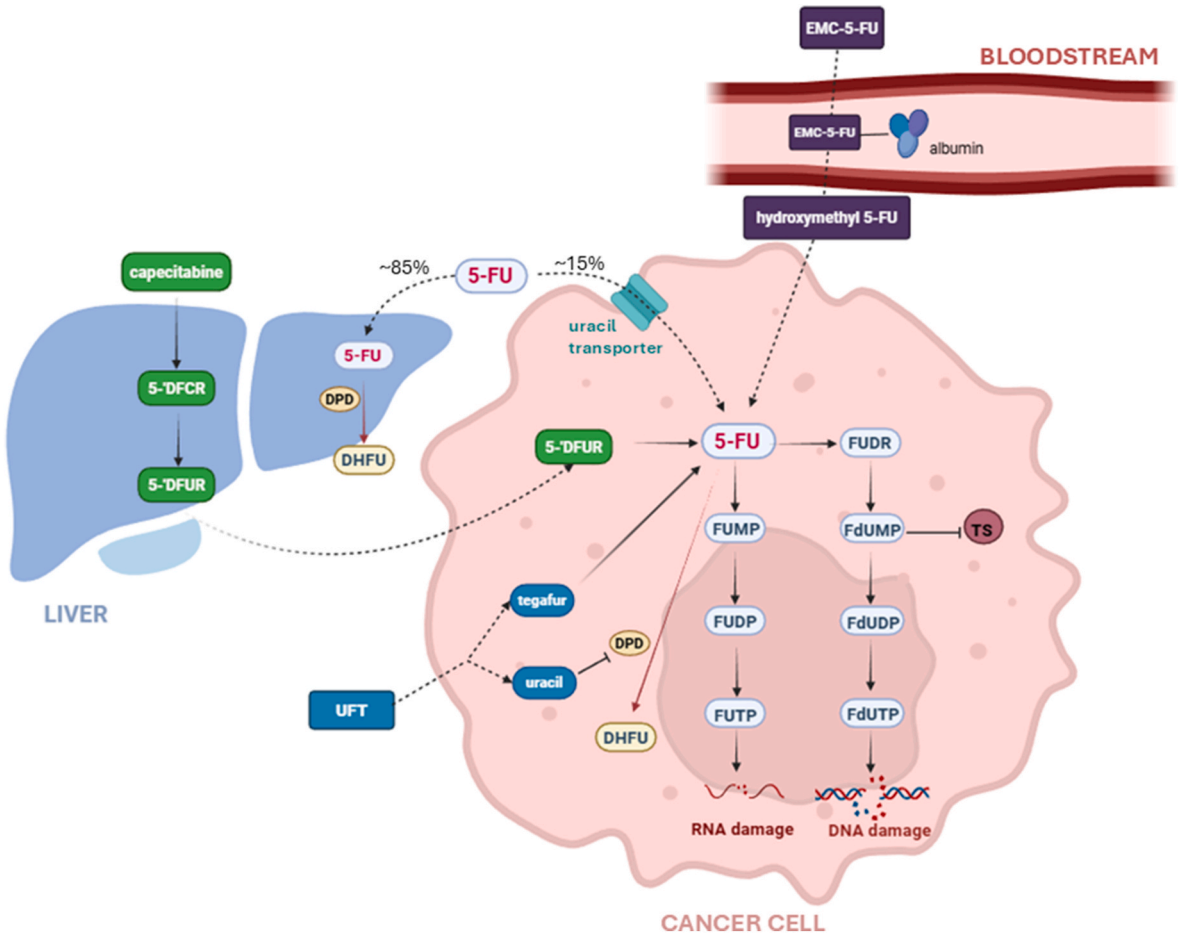


Fig. 3. Anabolic and catabolic pathways of 5-FU and its prodrugs. The diagram illustrates the metabolic activation and degradation of 5-FU within cancer cells. Capecitabine and UFT (tegafur + uracil) are prodrugs converted into 5-FU in the liver and tumor tissues. EMC-5-FU is a polymeric conjugate that enhances delivery since it rapidly binds to albumin, leading to better pharmacokinetic control. Created with BioRender.com.

respective activation mechanisms to generate 5-FU.

Recently, beyond its well-established anticancer activity, 5-FU has increasingly been recognized as an important immunomodulatory agent within the tumor microenvironment, a feature that is particularly relevant in the era of cancer immunotherapy. Several studies have demonstrated that 5-FU can selectively deplete immunosuppressive cell populations, such as myeloid-derived suppressor cells (MDSCs), thereby restoring antitumor immune surveillance and enhancing T cell-mediated responses [37]. In addition, 5-FU has been shown to promote immunogenic cell death, resulting in the release of tumor-associated antigens and danger-associated molecular patterns, including calreticulin, high-mobility-group box-1 protein (HMGB1), and adenosine triphosphate (ATP), which promote dendritic cell maturation and efficient antigen presentation [38]. These effects provide a rationale for combining 5-FU with immune checkpoint inhibitors or other immunotherapeutic strategies [39]. Importantly, emerging 5-FU-based derivatives may further amplify these immunomodulatory properties by improving tumor-selective delivery, reducing systemic toxicity, and enabling sustained drug exposure [3]. From this perspective, advanced formulations and prodrug strategies have the potential to increase not only the direct cytotoxic effects of 5-FU but also its ability to reshape the tumor immune microenvironment, thereby increasing the chance of durable therapeutic responses. A noteworthy example is provided by fosifloxuridine nafalbenamide (NUC-3373, Fig. 4), a phosphoramidate derivative of FdUrd, which shows how rational prodrug design can enhance both the pharmacological profile of fluoropyrimidines and the immune-modulating potential [40].

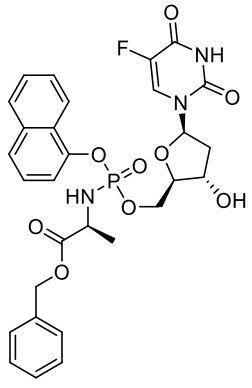


Fig. 4. Structure of NUC-3373, a pyrimidine nucleotide analog and TS inhibitor.

By overcoming resistance mechanisms associated with conventional 5-FU therapy, such as reliance on thymidine kinase activity and rapid degradation by DPD, NUC-3373 achieves sustained intracellular TS inhibition [40]. In addition, the enhanced pharmacokinetic characteristics of NUC-3373 allow its administration using a markedly shorter infusion duration than that required for 5-FU (2 *vs* 46 h) [41]. As a result, NUC-3373 is associated with a more consistent and predictable

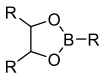
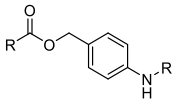
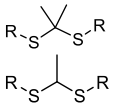
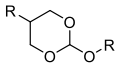
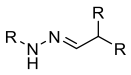
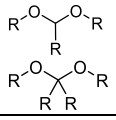
metabolic behavior while also mitigating the logistical and clinical burdens that restrict the use of 5-FU. Importantly, prolonged TS inhibition has been associated with enhanced cytotoxic efficacy and improved induction of immunogenic cell death as described above, supporting its clinical evaluation in combination with immune checkpoint blockade such as pembrolizumab [42].

Given these considerations, rational chemical modification of 5-FU has emerged as a key strategy to overcome its pharmacokinetic and toxicity limitations. These approaches include the development of analogs, hybrids, and classical prodrugs, the latter encompassing mutual prodrugs. Prodrugs are chemically modified derivatives of 5-FU designed to undergo enzymatic or chemical conversion *in vivo*, releasing 5-FU or its active metabolites in a controlled and, ideally, tumor-selective manner (Fig. 5) [43]. Mutual prodrugs, a specific subclass of prodrugs, consist of 5-FU covalently and reversibly linked to another bioactive agent, allowing reciprocal enhancement of pharmacological or pharmacokinetic properties (Fig. 5) [7]. Although 5-FU itself is a pyrimidine analog, further structural modifications can be explored, generating new analogs to achieve improvements in potency, bioavailability, and safety profiles (Fig. 5) [44]. Hybrid compounds, on the other hand, can be considered as intermediate entities between analogs and mutual prodrugs, integrating multiple pharmacological functions within a single molecular framework and potentially modulating biological activity without necessarily releasing the parent drug (Fig. 5) [45].

A key difference between hybrids and mutual prodrugs lies in the presence or absence of a cleavable linker. In hybrid compounds, the two components are connected by a structure that acts merely as a spacer and is not designed to release the two molecules *in vivo*. On the other hand, in mutual prodrugs, the pharmacologically active molecules are connected by a cleavable linker, which is a chemical structure capable of selectively breaking in response to a biochemical stimulus characteristic of a given tissue or microenvironment. These linkers are essential for the development of "smart" prodrugs capable of targeted drug delivery, as their selective activation enables discrimination between healthy and tumor cells, thereby increasing therapeutic efficiency while reducing systemic toxicity [46]. The main classes of cleavable linkers and the corresponding tumor-microenvironment stimuli that trigger their cleavage are summarized in the following table (Table 1).

Despite their potential, linkers may present certain limitations. These include: *i)* biological variability, as the concentration of enzymes responsible for their cleavage may differ between patients; *ii)* challenges in design, since each linker must remain stable in circulation yet cleave rapidly upon reaching the target site; and *iii)* the possibility of generating toxic fragments after cleavage [46].

Table 1
Cleavable linkers and tumor-associated stimuli enabling selective prodrug activation.

Linker	Structure	Stimulus
disulfide		glutathione (GSH)
peptide		metalloproteinases, cathepsin, hyaluronidase, legumain
ester		esterases
azo		hypoxia, enzymatic reduction of azo bond
boronic ester		reactive oxygen species (ROS)
p-aminobenzyl l		enzymes, ROS, pH
thioketal/ thioacetal		ROS (H_2O_2, radicals)
orthoester		pH
imine (Schiff base)		pH
hydrazone		pH
ketal/acetal		pH

In this review, we analyzed the most recent advancements in each of the approaches mentioned above by providing a detailed examination of innovations reported in the literature over the past five years. Specifically, the main focus was on medicinal chemistry strategies, while delivery-based approaches such as nanoparticles and liposomes have not

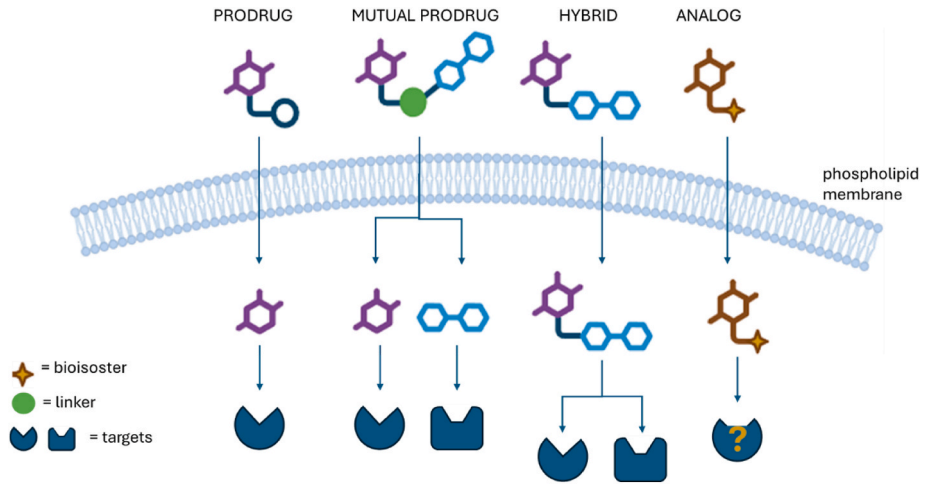


Fig. 5. Schematic overview of chemical strategies for 5-FU modification, including prodrugs, mutual prodrugs, hybrid compounds, and analogs. Some elements in this image are created with BioRender.com.

been addressed, since already been extensively covered in recent literature [47–49]. Whereas other review articles specifically focused on describing a single approach [7,50], the present article aims to explore different cutting-edge strategies by highlighting the most promising trends in the field. In contrast to clinically oriented reviews [51,52], which primarily address treatment optimization strategies such as therapeutic drug monitoring, dose individualization, and comparison of approved therapies, the present work focuses on how structural modifications of 5-FU can be exploited to overcome its intrinsic limitations at the molecular level. With this in mind, particular emphasis was placed on early-stage design approaches, especially in light of the very limited number of 5-FU derivatives that have advanced to clinical stages, which underscores the need for continued preclinical optimization.

2. 5-FU prodrugs and mutual prodrugs

2.1. Bioorthogonal strategies for 5-FU prodrug

A useful approach to bypass the degradation of 5-FU mediated by DPD and improve its bioavailability is the use of bioorthogonal prodrugs [53,54]. This strategy improves pharmacokinetic properties by designing prodrugs that can resist physiological metabolic mechanisms, while being activated instead by alternative triggers such as metal catalysis, photoinduced cleavage, or click-to-release reactions [53,54]. In a study conducted by Bray et al., one of these catalytic triggers is explored, specifically investigating the ability of palladium (Pd) to release *N*- and *O*-alkylated compounds in a biological environment. These Pd-loaded microdevices can be easily implanted into tumors under real-time ultrasound guidance, promoting *ex vivo* prodrug activation [55]. In order to resist physiological metabolic mechanisms, initial molecular docking has been carried out to determine which groups are essential for binding to DPD, and indicated that *O,O*-disubstituted 5-FU bound poorly to the DPD enzyme, as its structure does not fit within the enzyme's narrow binding pocket. Therefore, the two carbonyl groups are essential structural features for the interaction with the enzyme (Fig. 6A) [19,56].

In a previous study, a 5-FU prodrug (compound **1**, Fig. 6B) activated by Pd-mediated catalysis had been developed. In it, a selective masking of the *N*1-glycosylation site was introduced to prevent indiscriminate entry into the anabolic pathway [57]. Additionally, the lactam tautomer of the pyrimidine ring in 5-FU proved to be essential for recognition by DPD and other metabolic enzymes. Therefore, its masking in a lactim form was then adopted to further preclude interactions with DPD enzyme, preventing its recognition and inhibiting both the catabolic and anabolic pathways. In a subsequent study, a new prodrug (compound **2**, Fig. 6C) was generated, in which both critical recognition sites were masked, leading to very low affinity for DPD as confirmed by docking studies [56]. The compounds 5-FU, **1**, and **2** were tested toward hepatic enzymes comprising DPD, to determine the presence of DHFU, the reduced and inactive form of 5-FU. Substantial amounts of DHFU were detected for 5-FU and compound **1**, whereas compound **2** demonstrated remarkable stability, remaining completely unaltered under the reaction conditions. Subsequently, compound **2** was tested to assess the ability of Pd to convert it into the active form under physiological conditions, monitoring its conversion into 5-FU by UPLC. A rapid onset of activation was observed, with detectable conversion after only 10 min and complete transformation within 1 h. Cytotoxicity assays showed that the novel prodrug alone does not affect cell viability, nor do Pd resins; however, when combined, they displayed significant cytotoxicity in both pancreatic adenocarcinoma BxPC3 and human colon HCT116 cells [56]. Pharmacokinetic studies conducted in rats via oral and intravenous administration further revealed that this prodrug can be administered orally, exhibiting excellent bioavailability. Moreover, considering an efflux ratio (PappB-A/PappA-B) of 1.38, it can be inferred that intestinal absorption is favorable and that efflux is limited [56].

This work demonstrated the feasibility of creating an orally available 5-FU prodrug capable of bypassing hepatic catabolic mechanisms. Nevertheless, further investigations are required to optimize the intratumoral implantation and long-term stability of the Pd-based activating device [55].

2.2. 5-FU-dichloroacetate mutual prodrugs

Tumor cells employ various mechanisms to survive and continue growing. Normally, glucose is oxidized to produce ATP in the mitochondria; however, a common feature of rapidly proliferating cells is their tendency to consume large amounts of glucose and convert it into lactate via lactic fermentation, even in the presence of oxygen, thereby acidifying the surrounding environment. This mechanism, known as the Warburg effect, creates an acidic microenvironment that promotes tumor invasiveness and influences the immune system [58]. Dichloroacetic acid (DCA) is a structural analog of pyruvate that has been used for the treatment of lactic acidosis because it can inhibit mitochondrial pyruvate dehydrogenase kinase (PDK), consequently shifting glucose metabolism from glycolysis and lactate production toward oxidative phosphorylation [59–61]. Due to this property, DCA has been used as a chemotherapeutic agent for the treatment of various types of tumors [62], both alone and in combination with other anticancer agents. In particular, in the work of Mironiuk-Puchalska et al. DCA was linked to 5-FU (**3a-d**, Fig. 7) [63].

The use of different linkers enabled an investigation into how spacer length affects 5-FU release. It was observed that increasing the linker length also increased the time required to reach 50% of 5-FU concentration, consistent with slower ester hydrolysis for longer spacers. Among the series, the shortest spacer derivative (compound **3a**) exhibited the fastest 5-FU liberation and therefore the lowest chemical stability under these conditions. Consequently, stability studies were conducted on compound **3a** at pH 2, typical of the gastric environment, to evaluate its suitability for oral administration. Drugs sensitive to acidic pH are generally administered as film-coated pellets, so being the most sensitive, compound **3a** was subjected to stability tests in bovine blood plasma and in an enteric-coated (gastric-resistant) capsule in a pH

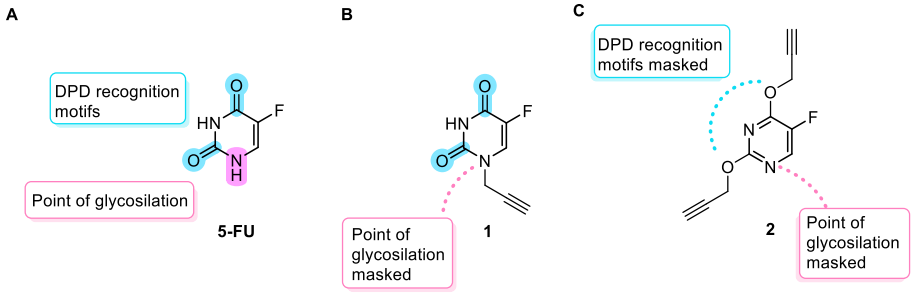


Fig. 6. A) Highlights of DPD recognition motifs and the point of glycosylation of 5-FU. B) Structure of compound **1**, highlighting masking of the *N*1-glycosylation site. C) Newly designed 5-FU prodrug (compound **2**) with both critical recognition motifs and point of glycosylation masked.

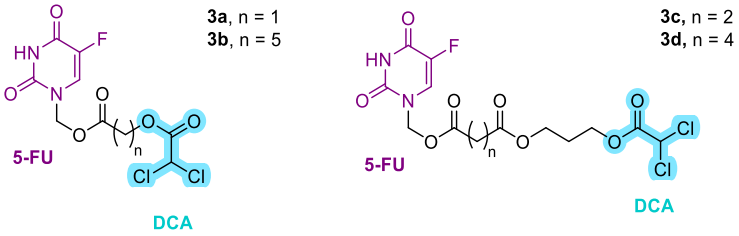


Fig. 7. General structures of the 5-FU-DCA mutual prodrugs **3a-d**.

2 citric buffer, showing that no hydrolysis products were detected in the buffer after 2 h [64]. Compounds were then tested using MTS assays on both 2D and 3D cell lines using spheroids to closely mimic the tumor environment. Specifically, their antiproliferative activity was assessed on two cell lines with different aggressiveness: human melanoma SK-MEL-28, which is p53-negative and more aggressive, and melanoma WM793, which is p53-positive and less aggressive. The cytotoxic efficacy of the new prodrugs showed that all compounds were capable of reducing cell viability, generally in a concentration-dependent manner. The maximum enhancement for all compounds was observed in WM793 cells at 24 h, with compound **3a** showing the highest overall potency ($IC_{50} = 108.7$ *vs* 707.1 μM for 5-FU, corresponding to a 6.5-fold improvement), followed by **3c** ($IC_{50} = 323.9$ μM, 2.2-fold improvement), **3b** ($IC_{50} = 435.0$ μM, 1.6-fold improvement), and **3d** ($IC_{50} = 559.7$ μM, 1.3-fold improvement) [63]. In addition to their ability to induce cancer cell death, their effects on reducing tumor cell size and disrupting cytoskeletal organization were evaluated. This latter effect is significant because cytoskeletal disruption can promote cancer cell migration and invasiveness [65]. Changes were observed in both cell lines in terms of morphology and cell number, with increased apoptosis signals, particularly in the more aggressive tumor line (i.e., WM793), likely due to the regulatory role of p53 in the cellular damage response [63]. Regarding 3D cell assays, human lung adenocarcinoma A549 and breast adenocarcinoma MDA-MB-231 cell lines were used, with increased efficacy observed in lung cancer cells. Importantly, studies on normal cells, LL24 and HMF spheroids, showed that the compounds were significantly less toxic, with **3a** displaying the lowest toxicity, making it the selected candidate for a further experiment using Organ-on-Chip technology. Accordingly, microsystems were created in which cells grew in 3D and were treated under dynamic flow. Spheroid viability on the chip was monitored for seven days, showing an overall decrease in viability for all compounds. Notably, on day seven, a dose increase was required due to a resurgence in spheroid proliferation, an effect not typically observed in traditional assays. In contrast, normal cell spheroids maintained high viability with compound **3a** (approximately 95%) compared to 5-FU (approximately 80%) [63].

These findings highlight DCA-5-FU mutual prodrugs, particularly compound **3a**, as promising therapeutic candidates that effectively target tumor metabolism through complementary mechanisms provided by both conjugated moieties, while preserving selectivity toward cancer cells, supporting their further development in advanced anticancer strategies.

2.3. 5-FU-folate-polyethylene glycol mutual prodrug

Among modern targeting strategies, folate receptor-mediated delivery has gained particular attention. Folate receptors, especially the FR-α isoform, are highly overexpressed on the surface of many cancer cells but show limited expression in normal tissues [66]. FA binds these receptors with very high affinity, making it an effective targeting ligand. When anticancer drugs are conjugated to FA, they can preferentially enter cancer cells via receptor-mediated endocytosis [67]. Small molecular size and strong receptor affinity make FA suitable for conjugation with various therapeutic agents, such as 5-FU. To improve the selectivity of 5-FU and its bioavailability, biodegradable polymers, especially polyethylene glycol, are commonly employed [68].

Therefore, in the study conducted by Sarwar et al., polyethylene glycol (PEG) was employed to improve solubility, biocompatibility, and circulation time of 5-FU, while FA can be efficiently combined with such carriers to enhance tumor-specific delivery and therapeutic performance (Fig. 8) [69].

The resulting 5-FU-PEG-FA mutual prodrug (Fig. 8) was systematically evaluated through *in vitro* release studies, cytotoxicity assays, cellular uptake experiments, and *in vivo* antitumor testing. The conjugate exhibited a controlled and sustained drug release profile driven by both diffusion and hydrolysis, in contrast to the rapid diffusion of free 5-FU. Indeed, over an extended period, the PEG linker gradually degraded, ensuring continuous release of the active drug [69]. Cytotoxicity assays confirmed that the conjugate retained strong anticancer activity while achieving enhanced uptake in folate receptor-positive HeLa cells (human cancer cells derived from cervical carcinoma) compared with normal Vero cells (non-cancerous cells derived from the kidney of the African green monkey). These findings indicate that PEGylation and amide linkage did not compromise folate receptor targeting or the cytotoxic efficacy of 5-FU. Cells treated with free 5-FU showed high initial toxicity that declined over time, whereas the prodrug induced a slower but progressively stronger inhibitory effect [69]. *In vivo* experiments using a murine hepatoma model further validated the therapeutic potential of the conjugate. Compared with saline and free 5-FU controls, the 5-FU-PEG-FA-treated group showed a sustained reduction in tumor volume over time. While both free 5-FU and the mutual prodrug initially suppressed tumor growth, only the folate-targeted prodrug maintained long-term antitumor effects, leading to significantly smaller tumors after prolonged treatment and confirming *in vitro* results [69]. Molecular docking and dynamic studies, conducted on the crystallized structure of folate receptor, have shown that the 5-FU-PEG-FA maintains the binding with the FRα receptor while forming a more stable and less flexible complex. Key residues enhanced stabilization through hydrogen bonding; indeed, free energy and Molecular Mechanics/Generalized Born Solvent Area (MM/GBSA) analyses confirmed stronger binding than FA alone, with a total free binding energy of −65.2 kcal/mol for 5-FU-PEG-FA mutual prodrug and −52.73 kcal/mol for FA, supporting improved receptor affinity and targeting efficiency of the prodrug [69].

5-FU-PEG-FA mutual prodrug has shown prolonged tumor retention, superior *in vitro* and *in vivo* antitumor efficacy than 5-FU alone, and stable FR binding. The observed improvement is primarily driven by enhanced tumor targeting and drug exposure, while the intrinsic mechanism of action of 5-FU remains unchanged. However, competition experiments with an excess of free FA could offer further insight, as well as simulations of the tumor microenvironment (including pH and enzymatic conditions) to validate both receptor-mediated uptake and the controlled release mechanism of the prodrug.

2.4. 5-FU-polysaccharide-carboxymethyl-folate mutual prodrugs

Polymer-drug conjugates provide multiple benefits; these include better drug solubility, longer residence time in the bloodstream, lower immune response, more controlled drug release, and an overall

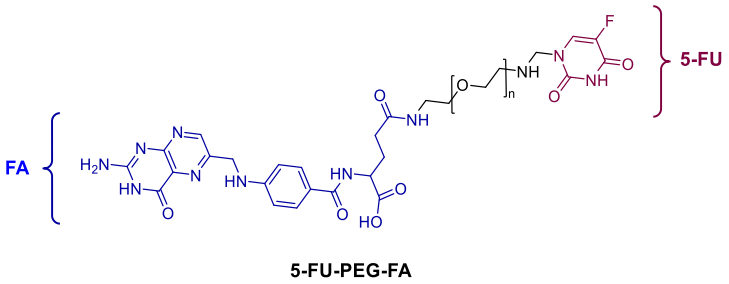


Fig. 8. General structure of the 5-FU-PEG-FA mutual prodrug.

improvement in treatment safety [70]. In particular, polysaccharides can serve as effective drug delivery systems by combining natural biocompatibility and biodegradability with protective, targeting, transport, and bioavailability-enhancing functions, as well as versatile fabrication strategies that preserve their properties [71]. Indeed, they can be linked to small-molecule drugs through covalent or non-covalent bonds, leading to an improved antitumor response [72–74]. In a previous study conducted by Ma et al. [75], the polysaccharide GFP1, derived from *G. filicina*, has shown immunostimulatory activity that may help counteract the side effects of 5-FU, such as leukopenia and thrombocytopenia [76,77]. Since folate receptors are membrane proteins with strong affinity for FA and are highly expressed in many tumor types, rather than in normal tissues [78–80], to take advantage of all of these features, a more recent study by Ma et al. [81] aimed at conjugating FA to 5-fluorouracil-1-acetic acid (5-FA) through the mutual prodrug strategy (Fig. 9).

GFP1 is a sulfated polysaccharide characterized by the presence of both sulfates, mainly responsible for its immunomodulatory activity, and hydroxyl functional groups, chosen as the reaction sites for esterification, which lead to the release of 5-FA under alkaline conditions. Since hydroxide ions act as strong nucleophiles that promote ester bond cleavage, higher pH values lead to accelerated hydrolysis, explaining the reduced stability of the conjugate under alkaline conditions [81]. To further assess the stability of compound **4**, its behavior was examined not only under different pH conditions but also in the presence of biologically relevant enzymes, specifically α-amylase and proteinase K, showing that the conjugate does not undergo significant structural breakdown under enzymatic conditions [81]. The cytotoxic effects of GFP1, GFP1-FA, GFP1-5-FA, and compound **4** were assessed in folate receptor-positive human cervical carcinoma HeLa and gastric adenocarcinoma AGS cell lines using the WST-1 assay. The results demonstrated that compound **4** exhibited higher dose-dependent cytotoxicity

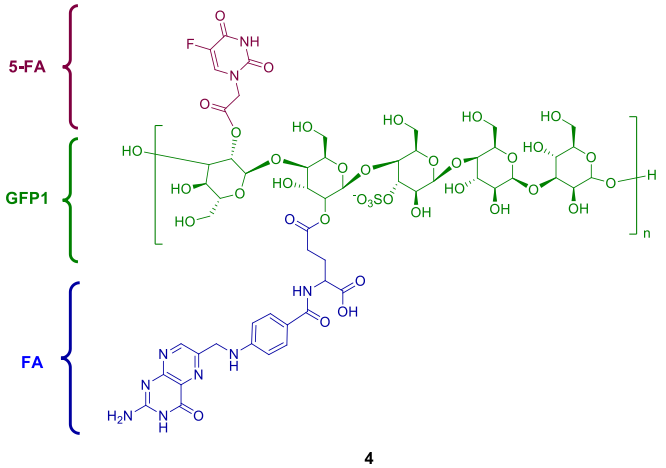


Fig. 9. General structure of the 5-FA-GFP1-FA mutual prodrug **4**.

than free 5-FU, which can be attributed to improved cellular internalization of the conjugate. Both GFP1 and GFP1-FA showed negligible toxicity, with cell viability remaining above 80% even at higher concentrations, confirming their good biocompatibility [81]. However, since both the efficient cellular uptake and intracellular accumulation of drug conjugates are critical determinants of their cytotoxic activity [82], in this study, the internalization of the compound **4** was suggested to occur mainly through folate receptor-mediated endocytosis, a mechanism known to be inhibited by the presence of excess free FA [81]. Morphological evaluation and cell imaging analyses revealed that treatment with compound **4** induced pronounced apoptotic features in both HeLa and AGS cells compared to untreated controls and free 5-FU. Moreover, the mutual prodrug remained biologically active after cellular uptake, as compound **4** not only reached tumor cells but also triggered intracellular signaling mechanisms, such as the NF-κB and MAPK pathways, that are essential for targeted and efficient cancer cell killing [81].

Compound **4** has shown promising targeted anticancer activity in HeLa and AGS cells; this enhanced efficacy should be interpreted as arising from the synergistic interplay between folate-mediated cellular uptake, the polysaccharide-based carrier, and the controlled intracellular release of 5-FU, rather than from intrinsic optimization of 5-FU alone. Future studies should address long-term immunogenicity and evaluate drug release behavior under dynamic physiological conditions to better assess its clinical potential.

2.5. 5-FU-methotrexate mutual prodrugs

Methotrexate (MTX) is an FA analog that differs primarily by the substitution of a methyl group at the N-10 position and an amino group in place of the keto group at the C-4 position of the ring. It can bind folate receptors expressed on cancer cell membranes, making it a valuable targeting ligand in anticancer drug design [83,84]. In addition to its targeting capability, MTX is a well-established chemotherapeutic agent and has been shown to potentiate the antitumor efficacy of 5-FU [85]. This enhancement occurs through MTX-mediated inhibition of folate-dependent enzymes, which increases intracellular phosphoribosyl pyrophosphate (PRPP) levels. PRPP is essential for the conversion of 5-FU into FUMP, its active metabolite, via orotate phosphoribosyltransferase, thereby amplifying 5-FU's cytotoxic activity [86].

Intending to overcome 5-FU's drawbacks, Zhao et al. described a novel mutual prodrug formed by chemically linking 5-FU with MTX, generating 5-FU-methotrexate mutual prodrug (MF, Fig. 10) [87].

The conjugation strategy is designed to prolong 5-FU circulation time and improve tumor targeting while minimizing systemic toxicity. However, because of the limited solubility in water and stability of MF, sulfobutyl ether-β-cyclodextrin (SBE-β-CD) was employed to create an inclusion complex suitable for injection (MF-SEBCD) [87]. Cyclodextrins (CDs) are cyclic oligosaccharides composed of glucose units connected through α-1,4-glycosidic linkages. Their distinctive toroidal structure features a hydrophobic inner cavity and a hydrophilic outer surface, enabling them to encapsulate a wide range of guest molecules

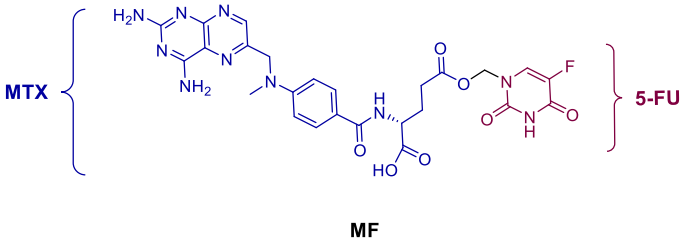


Fig. 10. Structure of the mutual prodrug MF.

through inclusion complex formation. The chemically modified cyclodextrin SBE-β-CD is one of the most widely used because of its sulfobutyl ether group, which significantly enhances water solubility, improves drug-binding capacity, and reduces toxicity [88]. Phase-solubility analysis indicated the formation of a stable MF-SEBCD complex (molar ratio 1:1 of MF and SBE-β-CD) and confirmed the strong solubilizing capacity of the cyclodextrin [87]. *In vitro* plasma studies showed that MF-SEBCD did not alter the metabolic conversion of MF, which gradually released 5-FU and MTX through ester bond hydrolysis. This controlled release behavior suggests prolonged systemic exposure and improved pharmacological performance. Tissue distribution and pharmacokinetic studies further showed that MF-SEBCD enhanced 5-FU accumulation and retention in tumor tissue, different from the results given by 5-FU alone, and markedly prolonged half-life with sustained plasma concentration while avoiding high peak levels that may contribute to systemic toxicity. Both *in vitro* cytotoxicity and *in vivo* antitumor studies, conducted on human CRC HT29, mouse CRC MC38, and mouse breast 4T1 cells, confirmed that MF-SEBCD retained a good anticancer activity, with IC_{50} values of 1.29, 0.51, and 1.26 μM, respectively, producing a greater tumor inhibition than the physical mixture of 5-FU and MTX. Additionally, safety evaluations indicated no significant changes in body weight, organ indices, or hematological and biochemical parameters in MF-SEBCD-treated animals. In contrast, elevated liver enzyme levels were observed in mice receiving the physical drug mixture, highlighting the improved safety profile of the conjugate formulation [87].

Overall, the MF conjugate, particularly when formulated as the MF-SEBCD inclusion complex, effectively addresses the limitations of conventional 5-FU therapy. By improving solubility, stability, tumor targeting, and pharmacokinetics while reducing systemic toxicity, MF-SEBCD represents a promising strategy for CRC treatment and provides a strong basis for further preclinical and clinical development.

2.6. 5-FU-coumarin self-immolative mutual prodrugs

Given that enzyme-triggered prodrugs exploit the high substrate specificity of enzymes and given their selective overexpression in various organ-specific cancers, the strategy of mutual prodrugs offers significant advantages over non-enzymatic stimuli-responsive systems [89–91]. In particular, esterases have been reported to be overexpressed in cancer cells [92] due to their important roles in tumor growth, such as migration, invasion, and cellular survival [93]. Indeed, their activity in malignant CRC is higher compared to that in normal tissues [94]. In this study, Badirujjaman et al. exploited the enzymatic activity of esterases to trigger the release of 5-FU from a mutual prodrug. Specifically, the compound BJ-92 was developed, in which 5-FU is conjugated to 7-hydroxycoumarin (7HC) through a hydrolysable linker consisting of a benzyl carbonate group (Fig. 11) [95].

7HC is a well-known fluorophore widely employed in stimuli-responsive systems, since it primarily acts as a reporting and triggering moiety, threfore the observed antiproliferative effects are directly attributable to the controlled intracellular release of 5-FU rather than to

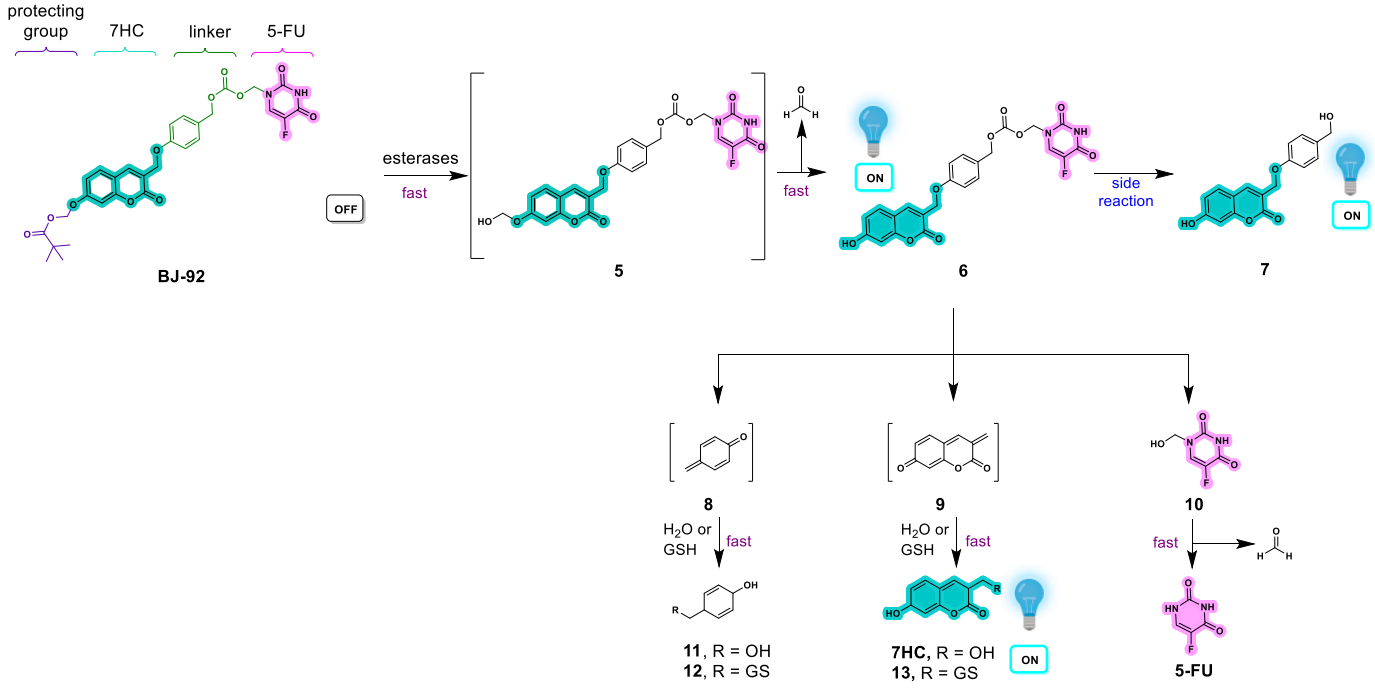


Fig. 11. Structure of the mutual prodrug BJ-92 and proposed pathway for its esterase-mediated activation leading to 5-FU and 7HC release.

an independent anticancer activity of the coumarin fragment. Its hydroxyl group was masked with a pivaloyloxymethyl moiety, ensuring that fluorescence is activated only upon esterase-mediated hydrolysis (Fig. 11). BJ-92 exhibits a characteristic absorption band (i.e., $\lambda = 310$ nm), which undergoes spectral changes upon incubation with the porcine liver esterase (PLE), indicative of coumarin activation through hydroxyl group release. This behavior can be explained by a self-immolative cleavage mechanism, whereby, as shown in Fig. 11, BJ-92 is firstly converted into intermediate **6** and subsequently into the reactive species 4-benzoquinone and 4-naphthoquinone methides (intermediates **8** and **9**, respectively). Only after nucleophilic attack by water molecules or GSH, the fluorescent 7HC is released. This process, further confirmed by HPLC analysis, results in a slow and controlled release of 5-FU, a feature highly favorable for therapeutic applications [95]. An important feature of BJ-92 is its high specificity toward esterases. Fluorescence was observed exclusively in the presence of PLE, while no activation occurred with other biomolecules (ions, amino acids, antioxidants, GSH, H_2O_2, NADPH, etc.). Moreover, the compound demonstrated remarkable stability, showing no spontaneous degradation in blood or culture media [95]. The antiproliferative activity of BJ-92 was assessed via MTT assay on A549 lung carcinoma cells and healthy HEK-293 cells. BJ-92 displayed potent and selective cytotoxicity toward cancer cells ($IC_{50} = 0.64$ and 27.3 μM, in A549 and HEK-293 cells, respectively), with a selectivity index of 42.6 (HEK/A549), which is significantly higher than that of free 5-FU (0.65). Importantly, the released byproducts were found to be non-toxic, confirming that the cytotoxic effect derives exclusively from 5-FU release. These results highlight the improved efficacy and selectivity of BJ-92, which is able to overcome the resistance exhibited by A549 cells to 5-FU alone [95]. Western Blot analysis further revealed modulation of key proteins involved in apoptosis and cell survival. The anti-apoptotic protein Bcl-2 was dose-dependently downregulated, while the tumor suppressor p53 was upregulated. Additionally, PARP, a protein involved in DNA repair, was also downregulated [95].

In conclusion, this study demonstrates that the proposed strategy allows for selective activation of the prodrug, ensuring targeted release of the active agent within tumor tissue. Furthermore, the concomitant fluorescence signal provides a valuable tool for real-time monitoring of the activation process.

2.7. Triple 5-FU-coumarin mutual prodrugs

An interesting approach was developed by Mustafa et al., who designed two different generations of 5-FU mutual prodrug bearing a coumarin-based linker. In particular, in the first one, the design addressed the objectives of selecting a prodrug type that improves 5-FU's lipophilicity and reduces its degradation by DPD. So, to improve bioavailability, 5-FU was linked through the coumarin-based linker to the 5-ethynyluracil (5-EU), a known DPD inhibitor, to give compound **14** (Fig. 12A) [96].

5-EU functions as a mechanism-based inactivator, meaning that it does not simply block the enzyme's active site, but instead takes advantage of the natural catalytic process of DPD to achieve inhibition. By binding to the pyrimidine site and inducing a reductive activation with NADPH, 5-EU positions its ethynyl group near the critical cysteine residue, allowing a covalent cross-link to form. This covalent modification disables the enzyme's active site, effectively preventing DPD from processing its normal substrates [97,98]. Building on this strategy, subsequent studies have further investigated the chemical stability of compound **14**, which remained stable in HCl buffer and

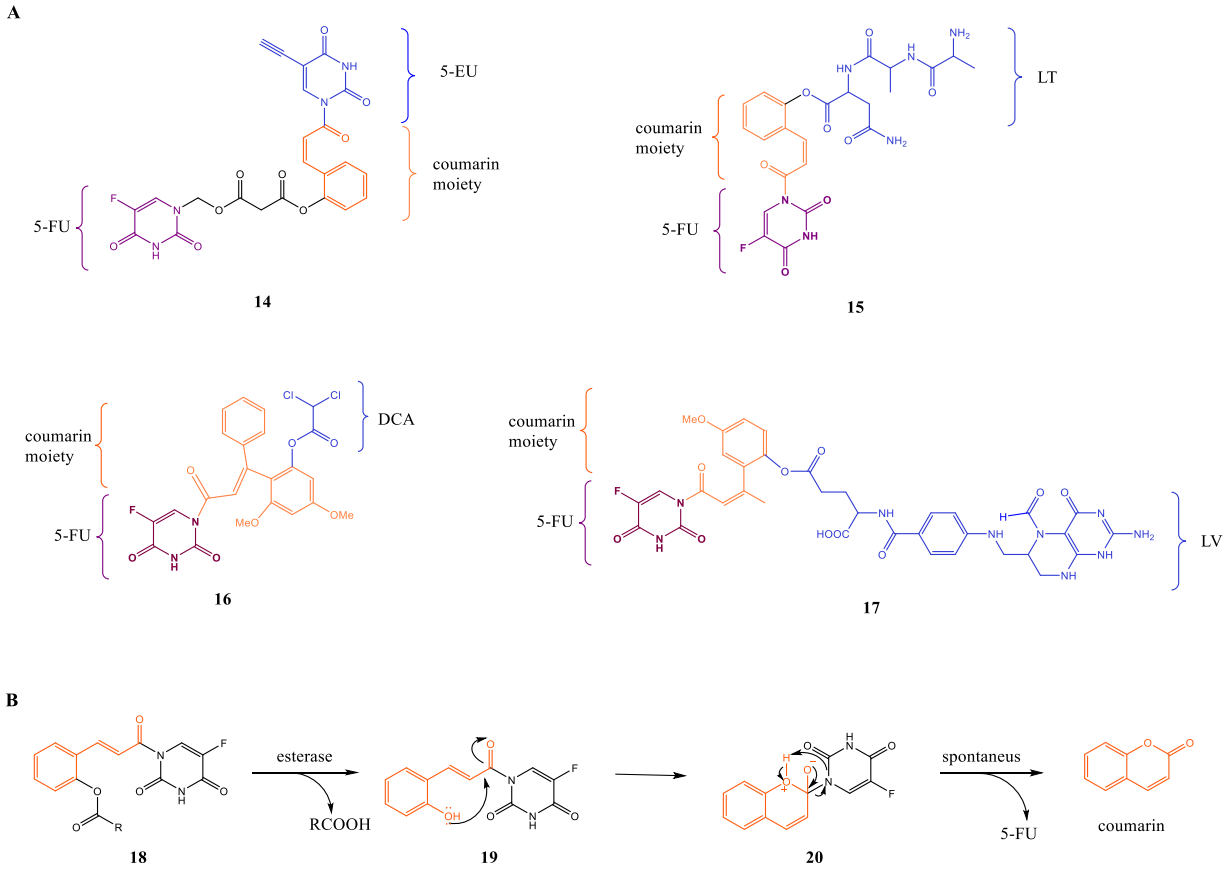


Fig. 12. A) Design and structures of the triple coumarin-based 5-FU mutual prodrugs **14**–**17**. B) Proposed mechanism of bioreversible intramolecular cyclization leading to 5-FU, 5-EU, coumarin, and biochemical modifiers release.

phosphate-buffered saline (following pseudo-first-order kinetics with half-lives of 33.19 and 18.13 h, respectively), likely due to steric hindrance around the ester linkages. This suggests the prodrug can pass intact through gastrointestinal-like environments; on the other hand, release studies demonstrated that compound **14** undergoes controlled enzymatic cleavage in human serum, enabling sustained release of 5-FU and 5-EU. Given that the logP values of 5-FU, 5-EU, and compound **14** are −0.90, −0.51, and 1.76, respectively, this indicates that this mutual prodrug has superior lipophilicity, which could enhance oral bioavailability. In a second generation, three further mutual prodrugs were developed (compounds **15**, **16**, **17**, Fig. 12A), where 5-FU was linked to three biochemical modifiers through a different coumarin counterpart for each of them [99]. In particular, biochemical modifiers are a class of compounds that alter specific biochemical or cellular processes in order to modulate the effect of another drug or treatment. Accordingly, over the past 40 years, several biochemical modifiers were widely employed, including the legumain-specific tripeptide (LT) [100]. Legumain is a cysteine endopeptidase capable of cleaving proteins at asparagine residues [101], reckoned as a molecular target in cancer since it is overexpressed in tumor cells while absent or only weakly expressed in normal cells [102]. The second biochemical modifier employed is DCA, previously mentioned for its antitumor activity as a PDK inhibitor [103]. The third one is leucovorin (LV), a reduced folate derivative that enhances the antitumor activity of 5-FU by stabilizing the ternary complex with TS, thereby preventing its rebound activation, and also facilitates cellular uptake by exploiting reduced folate carriers (Fig. 12A) [104]. These biochemical modifiers were conjugated to the coumarin counterpart, which in turn was linked to 5-FU, as illustrated in Fig. 12A [99]. It is noteworthy that these two generations of mutual prodrugs contain a latent form of the coumarin-based linker, subsequently activated through a bioreversible intramolecular cyclization system [105]. Hydrolysis studies mediated by esterases and phosphatases demonstrated that the ester group undergoes the first hydrolytic cleavage, releasing the biochemical modifier. The consequent exposure of the hydroxyl group triggers intramolecular coumarin cyclization, ultimately enabling the release of 5-FU (Fig. 12B) [106]. This design was aimed at improving the therapeutic profile of 5-FU by enhancing its oral bioavailability and tumor-targeting properties. Stability studies confirmed the ability of these prodrugs to withstand passage through the gastrointestinal tract; experiments were conducted in simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and in human serum to assess the activity of amidases against these prodrugs. The synthesized prodrugs exhibited good stability, with relatively high half-lives, ranging from 6.52 to 8.63 h [99].

However, in both studies, further investigations are required to evaluate the oral bioavailability, tumor selectivity, safety, and overall anticancer effectiveness of newly synthesized compounds since only preliminary stability and release studies have been conducted so far.

2.8. 5-FU-platinum (IV) mutual prodrugs

FDA-approved platinum (II) complexes include cisplatin and its analogs carboplatin and oxaliplatin, which are effective against a variety of cancers either alone or in combination with other agents [107,108]. However, their clinical utility is limited by severe side effects from off-target interactions, as well as intrinsic and acquired drug resistance [109]. Third-generation platinum (II) compounds, such as nedaplatin, lobaplatin, and heptaplatin, retain cisplatin's square planar geometry but face similar therapeutic limitations [110]. Oxidation of platinum (II) to platinum (IV) introduces two additional axial ligands, generating a low-spin, d^6 octahedral geometry that enhances kinetic stability and reduces undesired reactions with extracellular biomolecules [111,112]. The axial positions in platinum (IV) prodrugs also allow modulation of pharmacological properties, including selectivity, lipophilicity, bioactivity, and reduction potential [113–115]. Therefore, to overcome the constraints of conventional platinum drugs, structurally distinct platinum complexes are being developed. Among these, $[Pt(PL)(AL)]^{2+}$ complexes, where PL is a polyaromatic ligand such as 1,10-phenanthroline (Phen) and AL is a chiral ancillary ligand like 1*S*,2*S*-diaminocyclohexane (SSDACH), have demonstrated enhanced *in vitro* cytotoxicity with nanomolar GI_{50} values across multiple cell lines. These compounds exert multimodal mechanisms, affecting the cytoskeleton, nuclear DNA, mitochondrial membrane potential, and epigenetic processes [116–118]. Due to their limited *in vivo* efficacy, subsequent oxidation to platinum (IV) $[Pt(PL)(AL)(OH)_2]^{2+}$ has improved their activity [119]. 5-FU has been widely used, either alone or in combination with platinum-based drugs [120]. This established efficacy makes 5-FU an attractive candidate for incorporation into platinum (IV) prodrugs. Accordingly, in a study conducted by Khoury et al., two carboxylic acid derivatives of 5-FU were synthesized, 5-FA and 5-fluorouracil-1-methoxy-4-oxobutanoic acid (5-FU-succ) and coordinated to different platinum complexes at one or both axial positions, as shown in Fig. 13 [121].

Determination of lipophilicity of complexes **21**–**26** by RP-HPLC ($\log k_w$) revealed that compounds bearing a 5-FU-succ axial ligand (i.e., compounds **25** and **26**) are the most lipophilic (and more lipophilic than the corresponding 5-FA analogs **21**–**24**). Complexes **22** and **26**, containing the dimethyl-substituted Phen (5,6Me_2Phen), are consistently more lipophilic than corresponding Phen-based derivatives **21** and **25**. Additionally, lipophilicity results of compounds with axial modifications compared with the ones bearing equatorial modifications show that the latter have a greater impact on lipophilicity [121]. The *in vitro* cytotoxicity of selected prodrugs and ligands was assessed, using the MTT assay, across a broad panel of cells, including colon HT29, glioblastoma U87, breast MCF-7, ovarian A2780, lung H460, skin A431, prostate DU145, neuroblastoma BE2-C, glioblastoma SJ-G2 cisplatin-resistant, ovarian ADDP, and breast MCF10A cancer cell line. Derivative **26** was the most potent, displaying GI_{50} values ranging from 0.001 (DU145 cells) to 0.086 μM (BE2-C cells), significantly higher than the 5-FU treatment in the same cell lines (i.e., 5.3 and 4.0 μM, respectively). Derivatives containing the 5-FU-succ axial ligand were generally more active than their 5-FA counterparts, while mono-substituted 5,6Me_2Phen (i.e., compounds **22** and **26**) derivatives exhibited higher cytotoxicity than Phen-based analogs. Although lipophilicity did not correlate linearly with activity, compounds **22** and **26**, mentioned as the most lipophilic complexes, tended to be more cytotoxic, suggesting enhanced cellular uptake [121]. Based on their superior cytotoxicity, derivatives **25** and **26** were further investigated for reactive oxygen species (ROS) generation and mitochondrial effects in HT29 CRC cells. Both platinum (IV) complexes induced higher ROS levels than platinum (II) analogs and cisplatin, with derivatives **25** and **26** further amplifying ROS production over time. This sustained oxidative stress suggests activation of intrinsic apoptotic pathways. Mitochondrial membrane potential studies revealed pronounced depolarisation following treatment with derivatives **25** and **26**, exceeding the effects observed for platinum precursors, free ligands, and cisplatin. Derivative **26** caused the greatest mitochondrial dysfunction, consistent with its enhanced cytotoxicity. The data indicate that incorporation of the 5-FU-succ axial ligand into platinum (IV) scaffolds synergistically increases oxidative stress and mitochondrial damage, ultimately promoting cancer cell death [121].

Overall, these findings highlight the potential of axial 5-FU-functionalised platinum (IV) prodrugs as a versatile platform for enhancing anticancer activity through the combined contribution of platinum-driven redox and mitochondrial effects and fluoropyrimidine-based cytotoxicity rather than through sole optimization of 5-FU. Although a general trend between increased lipophilicity and cytotoxicity was observed, it remains unclear whether this effect arises from enhanced cellular uptake; therefore, cellular accumulation studies would be required [121].

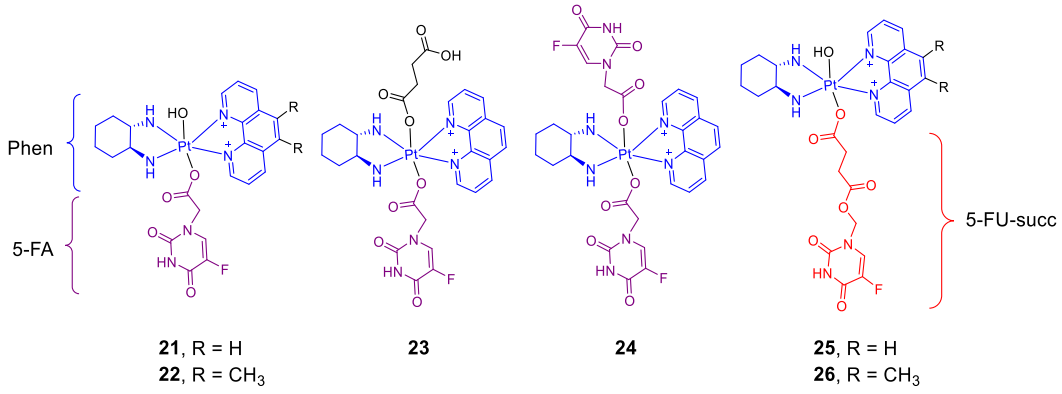


Fig. 13. General structures of the 5-FU-platinum (IV) mutual prodrugs **21**–**26**.

2.9. 5-FU-heme oxygenase 1 inhibitor mutual prodrugs

Heme oxygenase-1 (HO-1), an inducible stress-response enzyme involved in heme degradation, plays a key role in cancer progression by regulating oxidative stress, apoptosis, and cell proliferation. Its overexpression has been associated with tumor growth and resistance to chemotherapy, making HO-1 inhibition a promising anticancer strategy [122]. Indeed, HO-1 inhibitors have shown antiproliferative effects and the ability to enhance the efficacy of conventional chemotherapeutics [123]. Among the known HO-1 inhibitors, three arylethanolimidazole-based analogs (SI 1/09, LS 4/28, LS 6/42, Fig. 14) were selected as suitable candidates for conjugation, due to their potent inhibitory activity and favorable structural features [124].

Based on this rationale, novel 5-FU/HO-1 inhibitor mutual prodrugs were designed by Salerno et al. The two active moieties were linked via a biodegradable succinic acid spacer to generate a polypharmacological agent capable of releasing both parent drugs under physiological conditions (Fig. 14) [125]. The enhanced anticancer efficacy reflects a genuine dual-targeting strategy, in which inhibition of the HO-1 cytoprotective pathway cooperates with the cytotoxic action of 5-FU.

In silico analyses of SI 1/17, the first compound of the series, indicated that, despite minor violations of Lipinski's and Veber's rules, it can

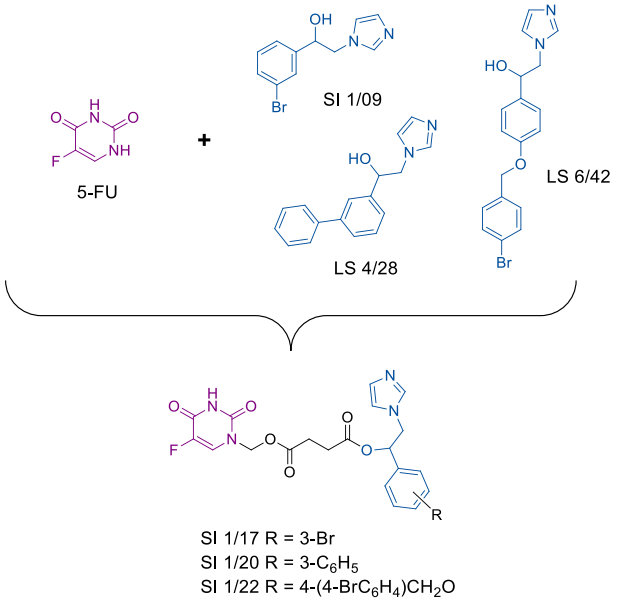


Fig. 14. Structures of the 5-FU, heme oxygenase 1 inhibitors (SI 1/09, LS 4/28, LS 6/42), and general structures of the corresponding mutual prodrugs (SI 1/17, SI 1/20, SI 1/22).

retain a favorable drug-like profile with improved predicted ADME and toxicity properties compared to 5-FU [125]. Additionally, chemical stability studies demonstrated that it is stable under acidic conditions, supporting oral administration, while undergoing faster hydrolysis at neutral and alkaline pH. Enzymatic assays were also conducted and confirmed effective cleavage by esterases, ensuring the timely release of the active components. Although SI 1/17 displayed reduced direct HO-1 inhibitory potency relative to the parent inhibitor, this was consistent with its role as a prodrug [125]. Antiproliferative evaluation revealed that SI 1/17 exerted dose-dependent cytotoxic effects on human prostate DU145 ($IC_{50} = 46.93\ \mu M$) and lung A549 cancer cell lines ($IC_{50} = 1.45\ \mu M$), comparable to 5-FU ($IC_{50} = 31.65$ and $0.98\ \mu M$, in DU145 and A549 cells, respectively) and to the physical combination of the parent compounds (5-FU and SI 1/09). Importantly, derivative SI 1/17 showed reduced toxicity toward non-tumorigenic lung epithelial BEAS-2B cells, suggesting improved selectivity and mitigated 5-FU's aspecific toxicity [125]. Encouraged by the results obtained with SI 1/17, additional derivatives, named SI 1/20 and SI 1/22, were designed and further investigated by Salerno et al. (Fig. 13). Stability studies carried out in porcine esterase solution demonstrated that both compounds, SI 1/20 and SI 1/22, undergo enzymatic hydrolysis following pseudo-first-order kinetics, with approximately half of each compound cleaved within a few hours. While SI 1/20 showed high stability in phosphate-buffered saline, SI 1/22 was more susceptible to hydrolysis in aqueous conditions, likely due to differences in aromatic substituents affecting electron density [126]. *In silico* evaluation showed that SI 1/20 and SI 1/22 possess favorable drug-likeness properties and predicted oral bioavailability, with high intestinal absorption, moderate blood-brain barrier penetration, and strong plasma protein binding. While a potential interaction with CYP3A4 was predicted, no CYP2D6 liability emerged, and toxicity profiles indicated improved safety compared to 5-FU [126]. Importantly, treatment of DU145 cells resulted in a marked reduction of intracellular HO-1 activity, confirming effective release of the active inhibitor within cells. Furthermore, both SI 1/20 and SI 1/22 significantly downregulated HO-1 protein expression without affecting HO-2 levels, suggesting dual modulation of the HO system through enzymatic inhibition and suppression of protein expression [126]. Notably, HO-2 represents the constitutively expressed isoform, and its preservation supports the selectivity of these compounds toward the inducible, cancer-associated HO-1 [127]. Consistent with HO-1 inhibition, treatment with the conjugates led to a time- and concentration-dependent increase in ROS levels in DU145 cells, providing a mechanistic explanation for the observed cytotoxicity. Elevated ROS generation is known to impair cancer cell survival, supporting the proposed mode of action of these compounds [126]. The anticancer activity of the mutual prodrugs was evaluated in lung A549 and prostate DU145 cancer cell lines characterized by HO-1 overexpression. Both SI 1/20 and SI 1/22 reduced cell viability more

effectively than the corresponding HO-1 inhibitors LS 4/28 and LS 6/42, with DU145 cells showing enhanced sensitivity to mutual prodrugs compared to 5- FU. Notably, SI 1/22 produced a dose-dependent and statistically significant reduction in DU145 cell viability, outperforming both 5-FU alone and the physical combination of 5-FU with HO-1 inhibitors. In contrast, the mutual prodrugs exhibited reduced cytotoxicity toward non-tumorigenic BPH-1 cells, indicating a favorable selectivity profile [126]. More recently, the antitumor potential of all these compounds (SI 1/17, SI 1/20, and SI 1/22) was also extended to CRC [128]. In HCT116 cells, SI 1/22 emerged as the most effective derivative compared to SI 1/17 and SI 1/20 (cell viability at 5 μM after 72 h = 77%, 85%, and 93%, respectively), displaying antiproliferative activity superior to 5-FU and comparable to the combination of 5-FU with the parent HO-1 inhibitor. This effect was associated with efficient intracellular HO-1 inhibition, increased oxidative stress, and the activation of apoptotic and autophagic pathways, supporting the relevance of dual targeting also in CRC models.

In conclusion, the 5-FU/HO-1 mutual prodrugs, particularly SI 1/22, showed enhanced anticancer activity and improved selectivity compared to 5-FU. Their efficacy in prostate, lung, and CRC models highlights the advantage of this targeting within a single molecular entity, supporting further development of this multitarget strategy.

2.10. 5-FU and glucose transporter inhibitor mutual prodrugs

We previously discussed the Warburg effect, highlighting how cancer cells rely more heavily on anaerobic glycolysis than on oxidative phosphorylation [58]. Cancer cells also exhibit increased expression of glycolytic enzymes and glucose transporters, which results in enhanced glucose uptake [129,130]. Two main classes of glucose transporters are involved: *i)* GLUT, which mediate facilitated diffusion; and *ii)* SGLT, which are secondary active sodium-glucose cotransporters [131,132]. Targeting these transporters has been shown to overcome the resistance to 5-FU associated with their overexpression, as demonstrated by compounds such as phloretin, a GLUT1 inhibitor, and phlorizin, an SGLT-1 inhibitor (Fig. 14) [133,134]. However, because these transporters are widely expressed across many tissues, Chang et al. proposed conjugating phlorizin and phloretin to 5-FU using redox-sensitive linkers, specifically designed to respond to GSH, which is present in high levels in cancer cells (Fig. 15) [135].

The stability and release behavior of the synthesized conjugates were first investigated in human plasma and under different GSH conditions. Among the compounds analyzed, compound **28b** demonstrated markedly superior plasma stability compared to compounds **27b** and **29b**, remaining intact for a longer period under physiological conditions. The lower stability observed for the latter compounds is likely related to the chemical nature of their linkers, which appear more prone to degradation at neutral pH. Compounds **27a**, **28a**, and **29a** showed comparable results to their phloretin counterparts [135]. The ability of the conjugates to release 5-FU in response to a reductive environment was carefully evaluated, with compound **28b** demonstrating significant sensitivity to reductive conditions. The latter showed a clear GSH-dependent release profile, highlighting the effectiveness of the disulfide linker in enabling selective drug release within cancer cells, while minimizing premature release in circulation. The biological activity of the mutual prodrugs was further assessed through GLUT1 inhibition, and cytotoxicity studies suggest that conjugation does not significantly impair GLUT1 binding. Cytotoxicity assays conducted on CRC HCT-116 and HT-29 cell lines revealed that compound **28b** displayed antiproliferative activity with an IC_{50} of 10.5 μM in HCT-116 and 3.8 μM in HT-29, comparable to free 5-FU in cancer cells (IC_{50} = 13.9 and 5.2 μM, respectively). In NHDF normal cells, compound **28b** showed reduced cytotoxicity compared to 5-FU, as reflected by its higher IC_{50} value (90.0 μM) relative to that of 5-FU (70.4 μM) [135]. Based on these promising *in vitro* findings, compound **28b** was selected for *in vivo* evaluation using an orthotopic mouse model of CRC. Treatment with compound **28b** led to stronger tumor suppression than free 5-FU and, notably, animals treated with this mutual prodrug did not exhibit the weight loss commonly associated with 5-FU administration, suggesting an improved safety profile [135]. Pharmacokinetic and biodistribution

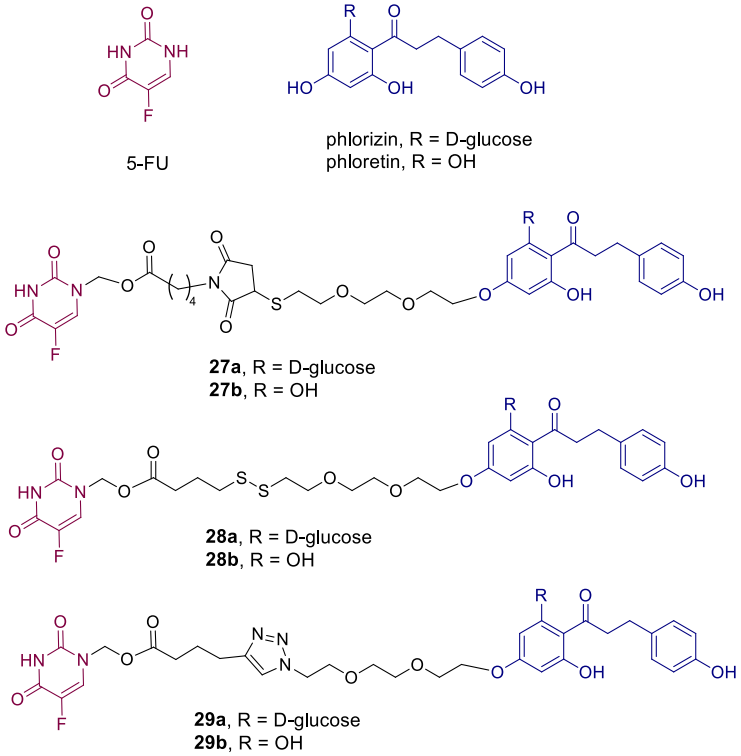


Fig. 15. Structures of the 5-FU, phlorizin, phloretin, and their corresponding mutual prodrugs **27a**,**b**, **28a**,**b**, **29a**,**b**.

studies supported the penetration of the compound in tissues, with the release of 5-FU accumulated predominantly in the colon, whereas free 5-FU showed a broader and less selective tissue distribution [135].

These findings indicate that conjugation to a glucose transporter-binding moiety enables preferential delivery of 5-FU to CRC, likely mediated by GLUT1, with enhanced efficacy and reduced systemic toxicity driven by targeted delivery rather than intrinsic optimization of 5-FU.

3. 5-FU hybrids and analogs

3.1. 5-FU-pterostilbene and 5-FU-biphenyl hybrids

Several FDA-approved anticancer drugs used to treat advanced solid tumors, including epithelioid and basal cell carcinomas, contain a biphenyl structural motif; among these, there are tazemetostat, sonidegib, and daclatasvir (Fig. 16) [136–138]. These drugs have emerged as promising anticancer agents with mechanisms distinct from existing therapies [139].

Stilbenes represent another important class of bioactive compounds, consisting of two aromatic rings connected by an ethylene bridge. They have recently shown noteworthy anticancer activity by modulating multiple intracellular pathways [140]. Among these, resveratrol is a promising natural stilbene with antioxidant, anti-inflammatory, and anticancer activities that can overcome multidrug resistance and enhance chemotherapy (Fig. 16) [141]. However, its clinical use is limited by poor pharmacokinetics, low potency, and potential nephrotoxicity, so research efforts are focused on the design and development of more potent analogs [142]. Pterostilbene, a dimethylated analog of resveratrol, exhibits comparable or superior antitumor activity, including enhanced colon cancer prevention and a longer *in vivo* half-life, due to the increased lipophilicity (caused by its methoxy groups), which improves bioavailability and biological potency [143, 144]. Since molecular hybridization is a potentially effective medicinal chemistry strategy that combines distinct pharmacophores to achieve dual biological activity (Fig. 5) [145], biphenyl and pterostilbene scaffolds were connected respectively to 5-FU to obtain compounds with comparable or improved cytotoxicity and reduced off-target toxicity (Fig. 16) [136]. It has been reported that resveratrol synergizes with 5-FU in CRC cells by inducing S-phase arrest, apoptosis, inhibition of STAT3/Akt signaling, suppression of epithelial-mesenchymal transition, and anti-inflammatory effects, findings that motivated the study conducted by Becerra-Quintana et al. [146]. The synthesized 5-FU hybrids were initially screened on SW480 CRC cells at a single concentration of 100 μM, revealing that stilbene-based derivatives exhibit markedly higher cytotoxic activity than their biphenyl analogs, with a 2- to 4-fold increase in potency. Structure-activity relationship (SAR) analysis highlighted the critical role of methoxy substitution patterns in

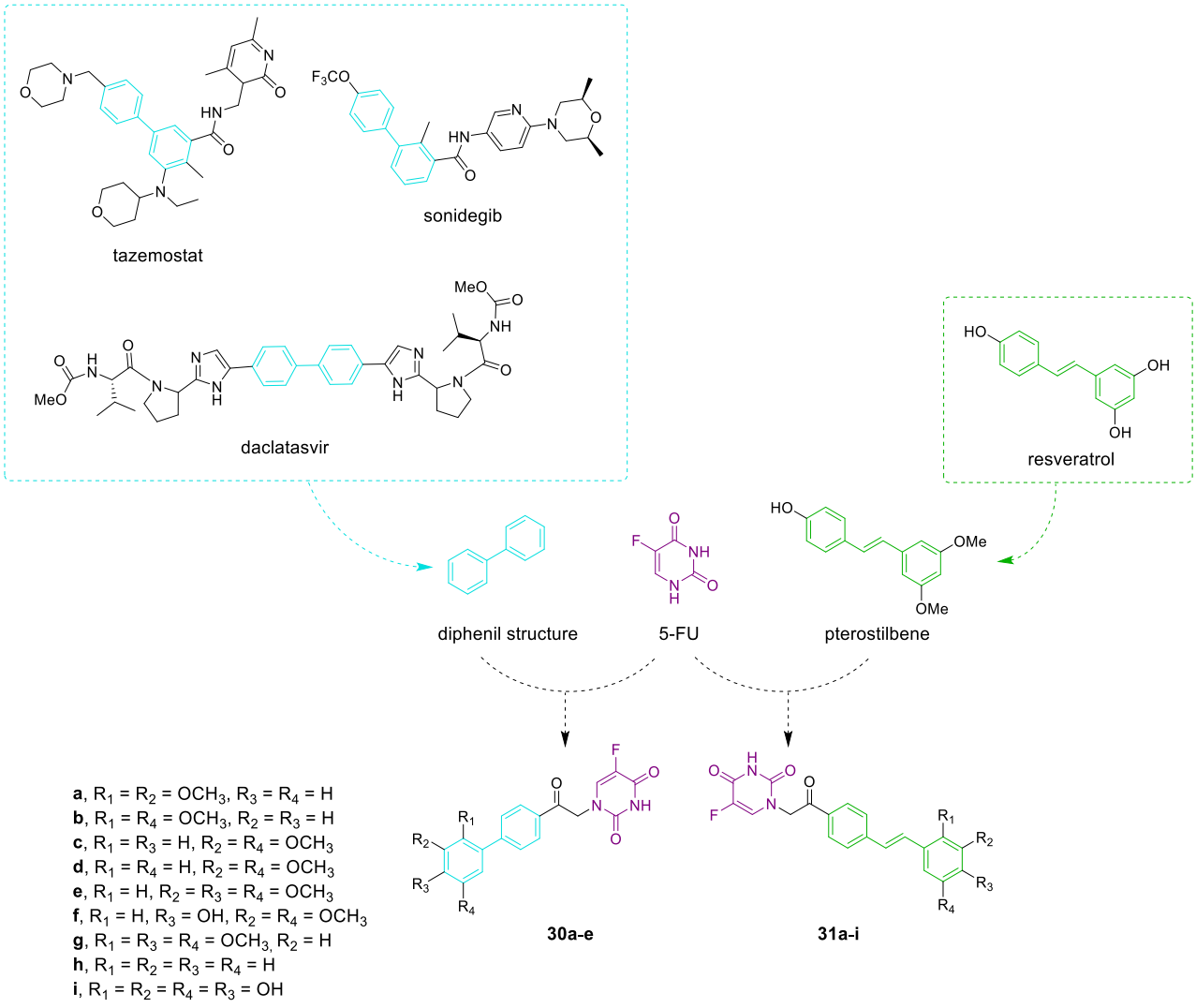


Fig. 16. Structures of tazemetostat, sonidegib, daclatasvir, and resveratrol. Design of 5-FU-diphenil and 5-FU-pterostilbene hybrids, **30a-e** and **31a-i**, respectively.

modulating biological activity. In particular, stilbene hybrids bearing trimethoxy substitution displayed superior cytotoxic effects compared to dimethoxy or unsubstituted analogs, with maximal inhibition observed for 2,4,5- and 3,4,5-trimethoxy derivatives (% inhibition at 100 μM of 100 and 78.27, respectively). Conversely, removal of methoxy groups or the presence of a hydroxy substituent at the C-4 position markedly reduced activity, while a methoxy group at C-4 emerged as a key structural feature for optimal efficacy in stilbene-based compounds [136]. In contrast, biphenyl hybrids showed a distinct SAR profile since trimethoxy substitution did not enhance cytotoxicity, and the presence of a methoxy group at the C-4 position significantly diminished inhibitory activity. Instead, the concomitant substitution at C-2 and C-5 was associated with improved cytotoxic effects, whereas relocation of these methoxy groups to the C-3 and C-4 positions led to approximately a 2-fold reduction in activity. Furthermore, comparative analysis between stilbene and biphenyl series demonstrated that replacement of the *trans*-double bond with a single bond resulted in a substantial loss of potency [136]. Based on these results, compounds **31e** and **31g** were further evaluated using a seven-dose scheme in SW480 CRC cells and non-malignant NCM460 cells, revealing notable antiproliferative effects, with compound **31g** achieving total growth inhibition and partial lethality, while compound **31e** exhibited a superior selectivity index, causing minimal toxicity in normal cells compared to 5-FU [136]. The lead compound **31e** was evaluated for key parameters related to ADMET and associated with oral bioavailability and membrane permeability. The results indicate that hybrid **31e** exhibits a favorable pharmacokinetic profile, with lipophilicity, intestinal absorption, permeability, and total polar surface area all within ranges typical of oral drugs approved by the FDA. Moreover, comprehensive *in silico* toxicological modeling using multiple predictive platforms suggested that **31e** lacks major toxicity liabilities, including mutagenic, hepatotoxic, cardiotoxic, nephrotoxic, or neurotoxic effects, and does not trigger PAINS (Pan--assay interference chemicals) or DNA-reactive alerts [136].

In conclusion, among this new series of 5-FU-pterostilbene and 5-FU-biphenyl hybrids, compound **31e** emerges as a particularly promising lead due to its improved potency, selectivity, and favorable predicted pharmacokinetic and safety profile, which derive from the hybrid molecular framework as a whole. However, direct experimental comparisons between the physical combination of 5-FU and pterostilbene and the covalently linked hybrid compounds, in the same cellular models, would be necessary to clarify whether hybridization offers advantages over simple co-administration.

3.2. 5-FU-genistein hybrids

Genistein, the main metabolite of soy, has been extensively studied for its broad spectrum of biological activities, as a chemotherapeutic and chemopreventive agent across multiple cancer types [147]. Despite studies elucidating its ability to regulate key cellular processes such as cell cycle progression, apoptosis, angiogenesis, inflammation, and metastasis, the clinical use of genistein is limited by chemoresistance, poor aqueous solubility, and low bioavailability [148]. Chemical modification of the genistein scaffold, such as hydroxyl group masking through methylation, can enhance metabolic stability and membrane permeability [149].

Due to the urgent need for novel therapeutic strategies against CRC, and since 5-FU represents the first-line chemotherapeutic agent, a series of 5-FU-genistein hybrids (compounds **32a-h**) was designed and synthesized by Moreno-Quintero et al. (Fig. 17) [150,151].

These molecules were obtained through click chemistry by coupling propargyl-5-FU with various genistein-derived alkyl azides, exploiting the relevance of the 1,2,3-triazole ring in medicinal chemistry. This moiety is known not only for its pharmacological relevance but also for its ability to enhance molecular stability by reducing vulnerability to enzymatic degradation, oxidation, reduction, and hydrolysis [152,153]. The cytotoxicity of the synthesized 5-FU-genistein hybrids, along with controls (genistein, 5-FU, and their equimolar mixture), was evaluated in human CRC SW480 and SW620 cells and non-malignant HaCaT and CHO–K1 cell lines, using the sulforhodamine B assay. IC_{50} values shown in Table 2 indicated that hybrids **32a** and **32g** displayed selective cytotoxicity toward cancer cells over non-malignant cells, outperforming parental compounds and their simple mixture [150].

Hybrids **32a** and **32g** also demonstrated dose- and time-dependent antiproliferative effects, reducing cell viability even at low concentrations after 4 days, and induced morphological changes consistent with cell damage [150]. Flow cytometry revealed that these hybrids altered cell cycle distribution in SW480 and SW620 cells, decreasing G0/G1 or S-phase populations and suggesting cytostatic activity, also confirmed by mitochondrial membrane potential studies. However, annexin V/PI assays revealed loss of plasma membrane integrity, indicating induction of cell death rather than cytostatic activity. In addition, hybrid **32a** significantly increased active caspase 3 and caspase 8 levels in SW620 cells, supporting the involvement of an extrinsic apoptotic pathway. Moreover, hybrid **32a** modulated the tumor suppressor protein (Tp53) levels in metastatic cells, suggesting partial reactivation of p53-dependent signaling. These findings indicate that 5-FU-genistein hybrids, particularly **32a**, may induce cell death through a mitochondria-independent, extrinsic apoptotic mechanism, supporting their potential use as chemopreventive agents or adjuvants to conventional 5-FU-based chemotherapy [150]. Computational analyses supported experimental findings by indicating that hybrid **32a** interacts with multiple apoptosis-related targets, including caspase-3, caspase-8,

Table 2
Cytotoxic activity (IC_{50}, μM) of the tested compounds on cancer and non-cancer cell lines.

Compound	SW480	SW620	HaCaT	CHO–K1
32a	62.73	50.58	64.64	71.10
32g	>100	36.84	>100	>100
combination	32.71	37.76	5.89	15.45
genistein	48.13	75.84	45.91	48.44
5-FU	174.30	180.90	118.67	173.20

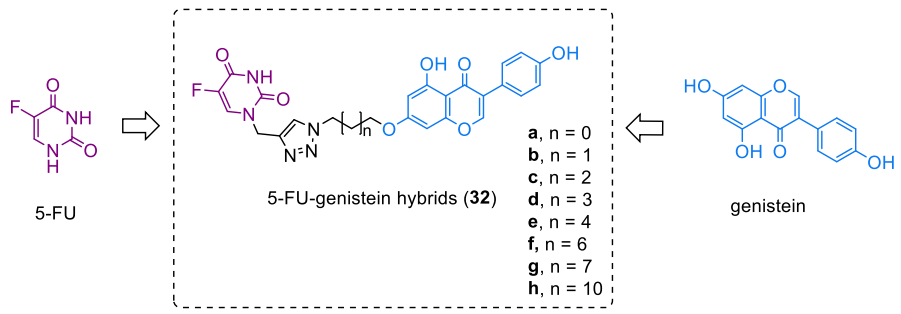


Fig. 17. Design and general structures of 5-FU-genistein hybrid **32a-h**.

and Tp53, with extensive hydrogen bonding and π-interactions. Molecular dynamics and molecular mechanics Poisson-Boltzmann surface area analyses highlighted the **32a**-caspase-3 complex as the most stable and energetically favorable, suggesting that caspase-3 modulation represents the primary molecular mechanism underlying the antiproliferative activity of hybrid **32a** in CRC cells [150].

5-FU-genistein hybrids showed to exert selective antiproliferative activity against SW480 and SW620 CRC cells, with hybrids **32a** and **32g** inducing cell cycle arrest and likely extrinsic apoptosis involving Tp53, while experimental and computational analyses identified caspase-3 as a key molecular target. This activity arises from the integrated pharmacological properties of the hybrid scaffold, where the genistein moiety contributes to apoptosis-related signaling, reflecting the generation of a new chemical entity with additional mechanisms beyond the canonical 5-FU pathway.

Accordingly, the lack of a clear correlation between linker length and cytotoxic potency suggests that structural factors other than linker-driven lipophilicity play a dominant role in defining the activity profile in this set.

3.3. 5-FU-curcumin hybrids

By adopting a similar approach to that used for genistein, Moreno-Quintero et al. also obtained curcumin-based hybrids. Curcumin has strong antioxidant properties and can reduce inflammation, both of which are linked to cancer development and progression [154]. It can trigger apoptosis in cancer cells by affecting critical cell signaling pathways such as NF-κB, which controls inflammatory responses and cell proliferation, and STAT3, involved in survival signaling in cancer cells [155]. It can inhibit several growth factors such as vascular epithelial growth factor (VEGF), urokinase plasminogen activator receptor (uPAR), and epithelial growth factor receptor (EGFR); thus, curcumin can be potentially useful to prevent cell differentiation, angiogenesis, and metastasis [156–158]. However, its clinical effectiveness is hindered by its moderate potency, limited solubility, low bioavailability, and rapid metabolism in humans [159].

Several hybrid molecules have been developed in which curcumin was linked to various small molecules, including 5-FU [160]. In particular, in the work of Moreno-Quintero et al., half of the curcumin molecular framework was specifically conjugated with 5-FU using the 1,2,3-triazole ring through click chemistry (compounds **33a-g**, Fig. 18) [161].

Overall, the hybrid compounds displayed a generally higher cytotoxic potency compared with curcumin and 5-FU in the colon adenocarcinoma SW480 cell line and in its metastatic derivatives SW620 cells. Microscopic examination revealed pronounced, dose- and time-dependent morphological alterations in tumor cells, including changes in cell size and shape as well as a marked reduction in cell density. However, the enhanced cytotoxicity over prolonged exposure was also observed in non-tumor HaCaT and CHO–K1 cell lines, leading to an overall decrease in selectivity indices for all compounds except for **33a**, **33d**, and **33e**. SAR analysis demonstrated that the length of the aliphatic linker plays a critical role in biological activity, with a three-carbon spacer yielding maximal cytotoxic effects. In contrast, further elongation of the linker resulted in a progressive decline in anticancer activity [161]. Based on cytotoxicity and selectivity data, compounds **33a**, **33d**, and **33e** were chosen for further studies (selectivity index at 48 h = 4.24, 11.56, and 1.90, respectively). These hybrids showed a clear concentration- and time-dependent antiproliferative effect in SW480 and SW620 cells, with significant reductions in cell viability observed from day 2 onward [161]. Cell cycle analysis revealed that compound **33a** induced S-phase accumulation in SW480 cells, indicating a cytostatic effect, whereas compounds **33d** and **33e** caused a marked increase in the sub-G0/G1 population in both cell lines, consistent with the induction of cell death. In agreement with these findings, mitochondrial membrane potential was not significantly altered, suggesting a mitochondria-independent mechanism of action. Annexin V/PI staining and caspase analysis further supported distinct biological profiles, with compound **33a** exerting mainly cytostatic effects, compound **33d** inducing caspase-independent cell death, and compound **33e** triggering caspase-mediated apoptosis in SW620 cells. Overall, these results indicate that the selected hybrids interfere with cell cycle progression and activate different cell death pathways, contributing to their anticancer activity in CRC cells [161].

These results highlight the anticancer potential of curcumin-based molecular hybrids, mainly due to their ability to affect cell-cycle progression and trigger different cell-death pathways, reflecting additional biological properties introduced by the hybrid structure; however, further studies of pharmacokinetic and bioavailability, as well as molecular docking or computational analyses to predict interactions with potential cellular targets, would be interesting for fully evaluating their therapeutic potential.

3.4. 5-FU-coumarin hybrids

Naturally occurring coumarins have attracted considerable attention because of their broad range of biological activities, prompting extensive efforts in medicinal chemistry to modify their core structure and explore their potential as novel therapeutic agents [162]. Among the strategies employed, the conjugation of coumarins with other small-molecule drugs to generate hybrid compounds has emerged as a promising approach to enhance or diversify their biological activity [163–165]. The 1,2,3-triazole ring represents, as said before, an effective and robust linker. It is highly resistant to metabolic degradation and oxidative or reductive conditions, while also playing an active role in interactions with biological targets, thereby enhancing overall bioactivity [152,153]. Owing to these features, 1,2,3-triazoles are well-established moieties that have been extensively utilized in drug discovery, with reported anticancer, antifungal, antibacterial, antitubercular, and antiviral properties [166,167]. With this in mind, López et al. employed a molecular hybridization strategy through click chemistry, by conjugating 5-FU with coumarin-based scaffolds through the triazole linkage (Fig. 19) [168].

Subsequently, newly synthesized CP1 and CP2 conjugates were

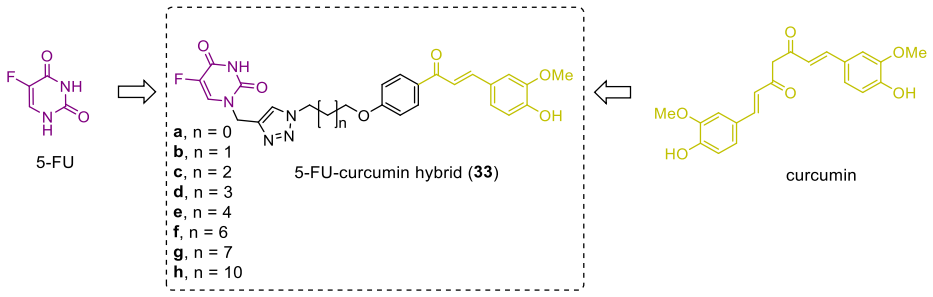


Fig. 18. Design and general structures of 5-FU-curcumin hybrid **33a-h.**

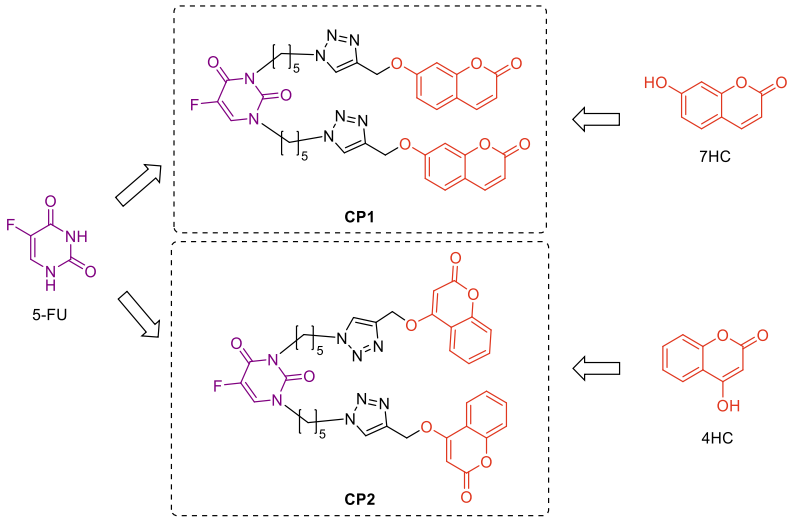


Fig. 19. Design and general structures of 5-FU-coumarin hybrid CP1 and CP2.

evaluated for their antioxidant activity through the DPPH radical scavenging assay, comparing them with the activity of ascorbic acid. Both compounds showed strong *in vitro* antioxidant activity, displaying a clear dose-dependent response with CP1 exhibiting a superior antioxidant efficiency [168]. Beyond their pronounced antioxidant properties, the amphiphilic nature of CP1 and CP2 prompted the investigation of their self-assembly behavior in aqueous environments. Indeed, the amphiphilic conjugates were able to self-assemble into nanoparticles, likely characterized by a hydrophobic coumarin core and a hydrophilic outer shell composed of 5-FU and triazole moieties. These nanoparticles were prepared via solvent evaporation and exhibited low critical aggregation concentrations, indicating a strong tendency toward aggregation even at minimal concentrations. In addition, stability studies indicated colloidal integrity for approximately one week, after which aggregation occurred as the systems approached their isoelectric point [168]. Cytotoxicity assays on the human pancreatic cancer cell line PANC-1 revealed an interesting hormetic effect at low doses. Hormesis refers to a biphasic response in which low levels of a potentially harmful agent elicit adaptive or protective cellular effects, whereas higher levels induce toxicity [169]. In this case, CP1 and CP2 showed a hormetic response at low concentrations and a dose-dependent antiproliferative activity at higher doses. CP1 demonstrated the strongest anticancer activity after 24 h of incubation compared to CP2, with an IC_{50} of 0.77 and 2.28 μM, respectively. However, since the IC_{50} of 5-FU could not be determined (cell viability >50%), a literature value (i.e., 12.66 μM, at 48 h in PANC-1) was used for comparison, indicating that CP1 was more effective than free 5-FU. Morphological changes observed in treated cells were consistent with these findings [168]. In addition, the cytotoxic effects of the coumarin derivatives were specifically evaluated because these natural compounds are known to possess biological and chemotherapeutic properties [170]. However, both 7HC and 4-hydroxycoumarin (4HC) exhibited negligible intrinsic toxicity over the investigated concentration range [168].

Although the coumarin moiety does not exhibit significant antiproliferative activity per se, its conjugation with 5-FU enables nanoparticle self-assembly and markedly enhances the overall anticancer efficacy through improved cellular uptake and synergistic antioxidant effects. Nevertheless, further investigations addressing cellular mechanisms, selectivity, uptake pathways, and *in vivo* performance would be appropriate to fully assess their therapeutic potential.

3.5. 5-FU analogs

Although biologics and immunotherapies have gained prominence due to their selectivity and immune-modulating properties, small-molecule drugs continue to play a central role because they can target diverse cellular pathways, are often orally bioavailable, easier to synthesize, and significantly less expensive [171]. For this reason, modifying established molecular scaffolds with known mechanisms of action represents a rational and efficient strategy to improve pharmacological performance and reduce toxicity [172]. For instance, 5-FU's limitations have driven the development of fluoropyrimidine analogs; however, resistance remains a critical obstacle, particularly in lung cancer [33, 173].

In this study, Harutyunyan et al. developed a series of novel 5-FU derivatives with designed structural modifications that may enhance plasma-protein binding, improve intestinal absorption and membrane permeability, and reduce predicted off-target toxicities, while maintaining favorable physicochemical properties. Thus, electron-withdrawing and electron-donating substituents were introduced to modulate pKa, lipophilicity, metabolic stability, and cellular uptake (Fig. 20) [174].

Computational ADMET profiling predicted increased plasma-protein binding for all derivatives relative to 5-FU, suggesting prolonged systemic exposure. SAR studies showed that *N1*- arylmethyl substitution is essential for enhanced anticancer activity, while balanced *N1*,*N*3- disubstitution is tolerated only within optimal physicochemical space. Introduction of a bromine atom at C5 (compounds **34c**, **35c**, and **36c**) and a 2-methoxy-5-formylbenzyl moiety (compounds **35a**, **36a**, **37a**, **35c**, and **36c**) markedly increased cytotoxicity and selectivity toward A549 cells, whereas nitro substituents and excessive polarity reduced activity and promoted oxidative stress. While aqueous solubility was reduced, values remained within typical drug-like ranges. Among the compounds, derivative **35c** showed the most promising balance of molecular weight, polarity, lipophilicity, and rigidity, alongside improved predicted human intestinal absorption. Toxicity predictions indicated reduced hepatotoxicity and neurotoxicity across most derivatives, with **35c** demonstrating no predicted risk for these endpoints [174]. *In silico* evaluation of long-term safety showed good agreement between predicted and experimental carcinogenicity data for 5-FU, supporting the reliability of the model. Although 5-FU is associated with a relatively low secondary cancer risk compared with DNA-reactive agents, this aspect remains relevant for prolonged treatments [175]. Among the derivatives, compounds **35e** and **37b** exhibited higher predicted carcinogenic risk, whereas the others showed reduced risk, with **35c** and **35d** emerging as the safest candidates [174]. In normal lung fibroblasts MRC-5 cells, 5-FU showed no cytotoxic effects at any tested concentration, with cell viability remaining close to 90% relative to vehicle

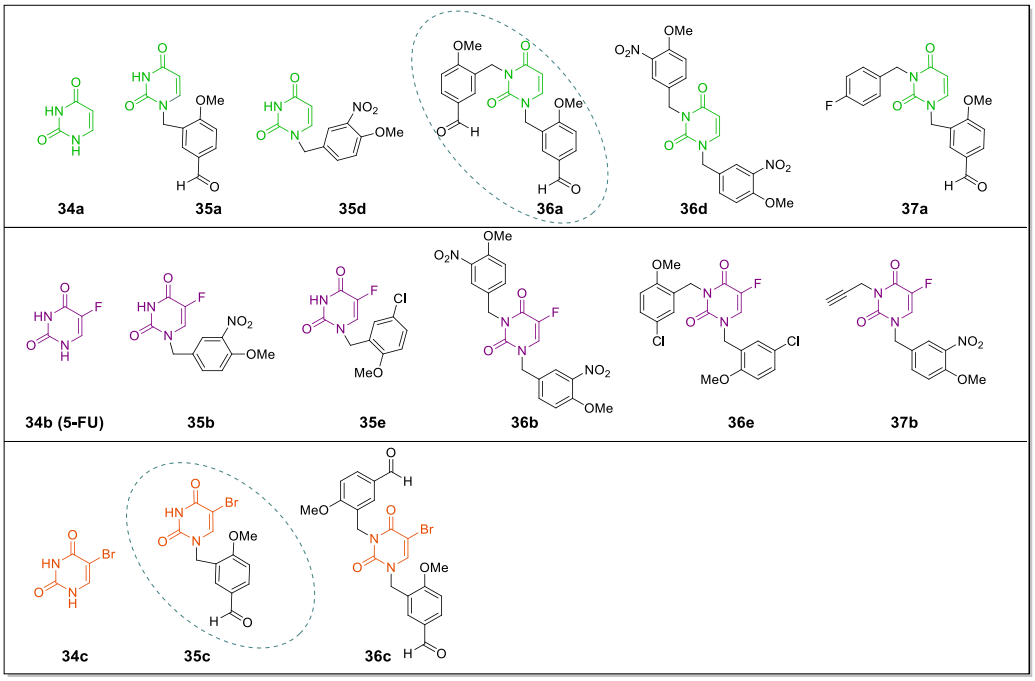


Fig. 20. Structures of a series of novel 5-FU derivatives (**34a-c**, **35a-e**, **36a-e**, **37a**,**b**) with electron-withdrawing and electron-donating substituents.

controls, confirming its known selectivity toward cancer cells. Biological evaluation revealed that derivatives **35c** and **36a** exhibited significantly greater cytotoxicity toward lung adenocarcinoma A549 cells than 5-FU, while maintaining minimal toxicity in normal MRC-5 lung fibroblasts. This selective anticancer activity suggests an improved therapeutic window. Unlike 5-FU, whose cytotoxicity is associated with oxidative stress induction, compounds **35c** and **36a** reduced cancer cell viability without increasing intracellular ROS levels. Instead, their activity correlated with alterations in antioxidant enzyme function, particularly with the inhibition of superoxide dismutase and catalase, actions that suggest disruption of metabolic pathways rather than oxidative damage [174]. Analysis of cellular ploidy further distinguished these derivatives from 5-FU. While 5-FU and some derivatives induced polyploidization, an effect linked to treatment resistance and tumor progression [176], compounds **35c** and **36a** shifted the cell population toward hypodiploidy, a hallmark of apoptosis [177]. This finding supports a more favorable mechanism of action that avoids adaptive resistance associated with polyploidy [174].

In conclusion, the study highlights rational structural modification of the 5-FU scaffold as an effective strategy to overcome key clinical limitations. Compounds **35c** and **36a** emerge as promising alternatives to 5-FU, warranting further investigation in preclinical models to assess pharmacokinetics, toxicity, formulation strategies, and therapeutic efficacy *in vivo*.

Table 3 summarizes information concerning all the compounds described in this review.

4. Conclusions and future perspectives

In recent years, the development of novel 5-FU derivatives has remained a central strategy to overcome the intrinsic limitations of this drug, including poor tumor selectivity, systemic toxicity, and unfavorable pharmacokinetic properties. As outlined in the introduction and detailed throughout this review, different medicinal chemistry strategies such as prodrugs, mutual prodrugs, hybrid compounds, and analogs have been explored to enhance the therapeutic profile of 5-FU. A critical analysis of the derivatives reported over the last five years reveals a clear shift in research focus toward the design of mutual prodrugs, which significantly outnumber the other categories. This emerging trend reflects the increasing interest in multifunctional systems capable of integrating 5-FU with a second bioactive moiety within a single molecular framework. The growing interest in this approach can be attributed to several factors, such as the advantage that mutual prodrugs offer by combining synergistic anticancer activity with improved tumor selectivity, or their ability to release the active compounds through tumor-specific cleavage mechanisms. Compared with conventional prodrugs and structural analogs, the mutual prodrugs strategy has the potential to maximize therapeutic efficacy while minimizing off-target toxicity, addressing some of the long-standing clinical challenges associated with 5-FU therapy. However, despite this potential, mutual prodrugs still face several inherent limitations, including the difficulty of achieving an optimal balance between chemical stability and efficient release of both counterparts, as well as the complexity of demonstrating the true therapeutic advantage and synergistic efficacy of the combined moieties.

To address these challenges, an emerging paradigm is the development of self-delivery systems, in which hybrids (i.e., 5-FU-coumarin hybrids) self-assemble into nanostructures and simultaneously integrate multiple therapeutic functions within a single molecular entity. In this context, the combinatory index of different bioactive species covalently linked to 5-FU is no longer determined solely by their pharmacological synergy, but also by their overall contribution to physicochemical properties (i.e., amphiphilicity, critical aggregation concentration, and nanoparticle stability in biological fluids). Moreover, the rational selection of complementary moieties, including targeting ligands and stimuli-responsive linkers, can allow the fine-tuning of self-delivery behavior that might combine enhanced biodistribution with preferential tumor accumulation [178]. Although still largely at a preclinical stage, these approaches highlight how the interplay between medicinal chemistry and pharmaceutics strategies can significantly boost the therapeutic potential of 5-FU hybrids as versatile platforms for selective tumor-targeting.

Regardless of the large number of 5-FU derivatives reported recently, only a very limited number of them have progressed to clinical studies in humans to date. This may be due to several factors related to existing treatment regimens, such as the previously mentioned capecitabine,

Table 3
Summary of 5-FU derivatives covered in this review.

Co-somministration	Strategy	Linker	Cell line	5-FU limitation addressed	Outcomes	Ref.
none	prodrug	none	BxPC3, HCT116	↓ pharmacokinetic ↓ selectivity	no DHFU formation activation only in the presence of Pd	[56]
5-FU and DCA	mutual prodrugs	ester group and diester group	SK-MEL-28, WM793	↓ efficacy ↓ selectivity	↑ cytotoxicity than 5-FU ↓ toxicity in normal cells	[63]
5-FU and FA	mutual prodrug	PEG	HeLa, Vero	↓ selectivity ↓ bioavailability	↑ uptake in cancer cells than normal cells controlled release	[69]
5-FU, GFP1 and FA	mutual prodrugs	ester group	HeLa, AGS	↓ efficacy ↓ bioavailability	↑ cytotoxicity than 5-FU conrolled release	[81]
5-FU and methotrexate	mutual prodrugs	ester group	HT29, MC38, 4T1	↓ solubility ↓ half-life systemic toxicity	↑ solubility prolonged circulation enhanced tumor accumulation ↓ toxicity	[87]
5-FU and coumarin	mutual prodrugs	carbonate group	A549, HEK-293	↓ selectivity ↓ bioavailability	↓ toxicity in normal cells and activation only with PLE controlled release	[95]
5-FU and 5-EU **5-FU and coumarin**	mutual prodrugs mutual prodrugs	ester group coumarin-based linker	None none	↓ bioavailability	↑ half-life	[96,99]
5-FU and platinum(IV)	mutual prodrugs	ester group and diester group	HT29, U87, MCF-7, A2780, H460, A431, Du145, BE2-C, SJ-G2, MIA, ADDP, MCF10A	↓ efficacy ↓ cellular uptake	↑ cytotoxicity than 5-FU partially demonstrated	[121]
5-FU and HO-1 inhibitors	mutual prodrugs	diester group	DU145, A549, BEAS-2B, HCT116	↓ efficacy ↓ selectivity ↓ bioavailability	↑ cytotoxicity than 5-FU ↓ toxicity in normal cells *in silico* favorable ADME stability under acidic pH, enzymatic hydrolysis at neutral pH	[125, 126, 128]
5-FU and glucose transporter inhibitor	mutual prodrugs	succinimidyl thioether, disulfide linker, 1,2,3-triazole ring	HCT-116, HT-29	↓ efficacy systemic toxicity	↑ tumor reduction no weight loss in mice	[135]
5-FU and pterostilbene, and 5-FU and biphenyl	hybrids	carbonyl group	SW480, NCM460	↓ selectivity ↓ bioavailability side effects	↓ toxicity in normal cells *in silico* favorable ADME predicted non-mutagenic, non-tumorigenic, non-hepatotoxic, non-neurotoxic	[136]
5-FU and genistein	hybrids	1,2,3-triazole ring	SW480, SW620, HaCaT, CHO–K1	↓ efficacy ↓ selectivity	↑ cytotoxicity than 5-FU ↓ toxicity in normal cells	[150]
5-FU and curcumin	hybrids	1,2,3-triazole ring	SW480, SW620, HaCaT, CHO–K1	↓ efficacy ↓ selectivity	↑ cytotoxicity than 5-FU ↓ toxicity in normal cells	[161]
5-FU and coumarin	hybrids	1,2,3-triazole ring	PANC-1	↓ efficacy	↑ cytotoxicity than 5-FU	[168]
None	analogs	none	A549, MRC-5	↓ efficacy ↓ selectivity ↓pharmacokinetic	↑ citotoxicity than 5-FU ↓ toxicity in normal cells ↑ predicted plasma-protein binding; ↑ predicted human intestinal absorption	[174]

which is also used in combination with other chemotherapeutic agents such as oxaliplatin [179], irinotecan [180]. This raises the bar even further for approval of new 5-FU derivatives, as they must not only be non-inferior to existing regimens but also provide significant advantages in terms of safety, convenience, and efficacy. Reduced toxicity, in particular, represents one of the key factors limiting clinical translation. The unexpected toxicity arising from released metabolites or generated intermediates from new 5-FU derivatives further complicates their progression into advanced development phases. For these reasons, as good practice, preclinical studies should assess cytotoxicity in both cancer and healthy cell models as well as evaluate metabolic profiling since the early discovery phase.

In addition, given the narrow therapeutic index of 5-FU, mutual prodrugs that combine 5-FU with inhibitors that affect its metabolism may be particularly risky, as small changes in concentration can lead to severe adverse effects that are not necessarily compensated by increased efficacy. The clinical failure of examples such as emitefur highlights that improvements in pharmacokinetics alone are not sufficient to replace established regimens. Therefore, simply aiming to improve half-life or oral bioavailability is not enough. Future candidates should therefore prioritize tumor selectivity, for example, through tumor-responsive linkers (e.g., GSH-responsive linkers) or conjugation with intrinsically tumor-selective agents (i.e., folate conjugates). While combining 5-FU with other bioactive compounds can enhance its properties, preliminary evaluation of the assessment of synergistic versus additive effects (e.g., by using the Chou-Talalay method) of the combined drugs is still mandatory, while early *in silico* and *in vitro* validation should be a priority.

Finally, regulatory constraints represent an additional challenge for prodrugs [181]. Comparison with existing regimens, not only in terms of efficacy and safety but also in terms of cost-benefit ratio, places significant pressure on the ability of new derivatives to successfully progress through clinical trials. Moreover, their biological effects may not be easily interpreted, as improved efficacy can arise from multiple overlapping mechanisms. This uncertainty can hinder mechanistic understanding and complicate further optimization of molecular design.

In conclusion, the primary objective of many of the studies discussed herein is the identification of novel drug-targeting modalities and mechanisms of action for 5-FU derivatives. In this framework, emphasis is placed on the development of broadly applicable medicinal chemistry strategies that can be extended to existing and future drugs, to establish generalizable therapeutic paradigms rather than achieving immediate clinical translation.

CRediT authorship contribution statement

Sara Mocka: Conceptualization, Investigation, Visualization, Writing – original draft. **Maria N. Modica:** Data curation, Supervision, Validation, Writing – review & editing. **Valeria Pittalà:** Writing – review & editing. **Loredana Salerno:** Writing – review & editing. **Giuseppe Romeo:** Writing – review & editing. **Agostino Marrazzo:** Writing – review & editing. **Laura Siracusa:** Writing – review & editing. **Sebastiano Intagliata:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was funding from theEuropean Union - Next-GenerationEU -National Recovery and Resilience Plan (NRRP) – Mission 4, Component 2, Investiment N. 1.1, call PRIN 2022 PNRR D.D. 1409 14-09-2022 – Project code: P2022F4PTE, Project title: HO-Hybrid2 – CUP: E53D23015960001.

Data availability

No data was used for the research described in the article.

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