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SuFEx chemistry for nucleosides, nucleotides, and nucleic acids

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Sulfur(VI) fluoride exchange (SuFEx) reactions were introduced as next-generation click transformations that form robust sulfur(VI)-based linkages under mild conditions. Their defining feature is the unusual behaviour of the S–F bond: it is thermodynamically stable, yet can be selectively substituted when a suitable nucleophile is properly positioned. This balance has made SuFEx a valuable platform in chemical biology, enabling novel covalent probes, inhibitors and conjugation strategies in complex aqueous environments. In contrast, SuFEx applications to nucleosides, nucleotides and nucleic acids remain comparatively scarce and are only now beginning to mature. Progress has been limited by scaffold-specific synthetic and workflow constraints, including the scarcity of broadly enabling methodological studies and limited compatibility with standard oligonucleotide workflows. Even so, recent reports show that these barriers can be overcome in selected settings and that SuFEx can be translated into functional nucleic-acid constructs. This review summarises current advances with a focus on concepts and practical design rules. The first part is chemistry-centered: it compares the most successful strategies for installing sulfur(VI)–fluoride electrophiles on nucleoside, nucleotide, and oligonucleotide frameworks, and discusses reagent choices, linker designs and warhead positioning. The second part focuses on applications, outlining how these synthetic advances are turned into chemical biology tools where proximity effects convert reversible recognition into durable capture. We conclude by highlighting key bottlenecks and the most promising opportunities for progress.

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Introduction

The sulfur(VI) fluoride exchange (SuFEx) reaction (Fig. 1A), introduced in 2014 by Sharpless and co-workers as a new-generation "click" transformation,[1] has rapidly evolved into a robust and widely applicable tool for covalent bond formation.[2] SuFEx exploits the unique balance between the high thermodynamic stability and controlled kinetic reactivity of S–F bonds, allowing efficient substitution of fluorine by diverse nucleophiles under mild conditions. Typical electrophilic partners (Fig. 1B) include sulfonyl fluorides (RSO_2F, **SF**), fluorosulfates ($ROSO_2F$, **FS**), sulfamoyl fluorides ($RNHSO_2F$) and iminosulfur oxydifluorides ($RN{=}SOF_2$), which undergo selective reactions with amines and phenols, to form stable linkages. The resulting S(VI)-containing motifs are chemically and biologically resilient, giving SuFEx chemistry its reputation as a reliable, modular, and "click-like" synthetic platform.[3]

Since its introduction, SuFEx chemistry has rapidly emerged as a versatile platform for constructing and modifying biologically relevant molecules.[4] In biochemistry and chemical biology, it has enabled the development of selective covalent probes, enzyme inhibitors, and bioconjugation strategies that exploit the controlled reactivity of sulfur(VI)–fluoride bonds toward nucleophilic residues such as lysine, tyrosine, serine, and histidine (Fig. 1C).[5,6] In medicinal chemistry, SuFEx-derived sulfonyl fluorides and sulfamoyl fluorides have been incorporated into drug candidates and fragment libraries, providing covalent yet tunable electrophiles with favourable pharmacokinetic profiles.[7] The robustness, selectivity, and modularity of SuFEx linkages make them particularly attractive for designing chemical tools, therapeutic agents, and biomolecular assemblies under physiologically relevant conditions.

SuFEx based strategies have become firmly established in peptide and protein chemistry, where their bioorthogonal nature and tunable reactivity have been exploited for a wide range of applications. SF and FS electrophiles react selectively with nucleophilic amino acid side chains to generate covalent adducts. This reactivity has been harnessed for activity-based protein profiling, ligand-directed covalent inhibition, and covalent probe design. Moreover, the controlled reactivity of specially designed reagents enables site-selective installation of electrophilic SuFEx warheads into amino acids, peptides and proteins under mild conditions.[4,8]

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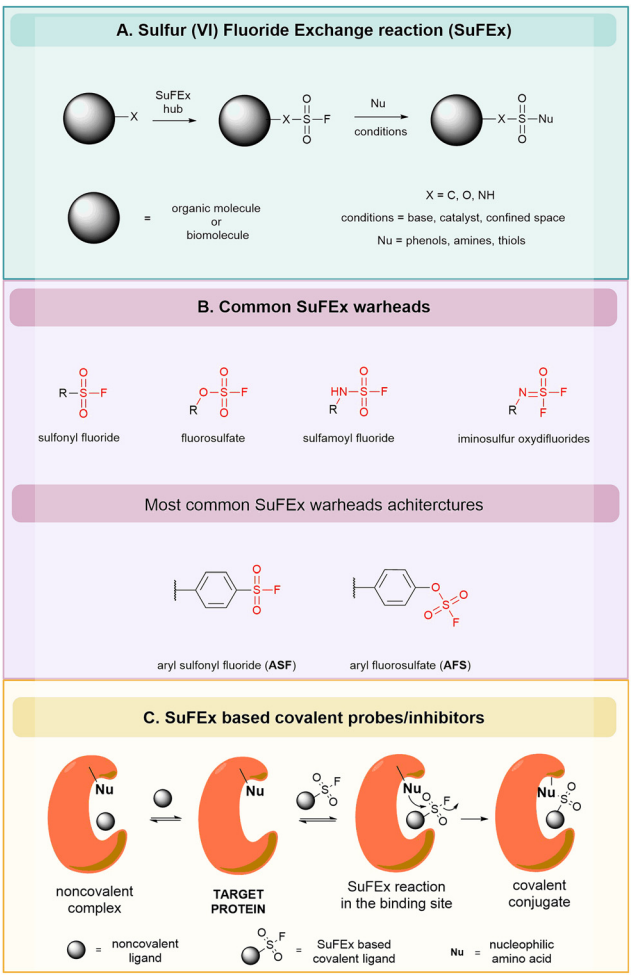

Fig. 1 SuFEx reaction – an overview (A) general scheme of SuFEx reaction; (B) representative classes of SuFEx electrophiles; (C) proximity-enabled covalent conjugation in a protein binding site.

Importantly, SuFEx has also been integrated into genetic code expansion technologies, which enable the site-specific incorporation of noncanonical amino acids bearing SuFEx warheads directly into recombinant proteins.[9–11] These engineered amino acids—carrying SF or FS groups—serve as latent electrophilic handles for subsequent conjugation or cross-linking reactions. This strategy provides precise control over SuFEx reactivity in biological systems and has opened new avenues for protein engineering and probe development. As a result, SuFEx chemistry is now regarded as a mature and versatile approach for studying and manipulating protein structure, function, and interactions.

In striking contrast to this well-developed area, applications of SuFEx chemistry to nucleosides, nucleotides, and nucleic acids remain comparatively scarce.

Despite the central biological roles and the wealth of reactive functional groups present in nucleic acid components---including hydroxyl groups on the sugar moiety, exocyclic amines on nucleobases, and phosphate oxygens—their intrinsic polyanionic character, structural rigidity, and aqueous environment have posed significant challenges to the direct application of SuFEx strategies. Consequently, only a handful of studies have demonstrated successful SuFEx-based ligations or modifications involving nucleic acid components. Yet, these emerging examples illustrate a promising and largely untapped potential: SuFEx could provide a general and orthogonal platform for nucleic acid conjugation, probe construction, and functional oligonucleotides design, mirroring its success in protein science.

Recent general reviews of applications of SuFEx chemistry in bioconjugation and chemical biology have typically treated nucleic-acid-related bioconjugation only briefly,[4,7] while more specialized reviews have tended to address SuFEx primarily in the context of protein and peptide systems[12,13] or discussing SuFExable nucleic acids alongside other covalent bioconjugation strategies and from multiple perspectives.[14–16] To the best of our knowledge, however, the application of SuFEx chemistry specifically to this field has not previously been the subject of a dedicated, detailed review. In this article, we therefore summarize and critically evaluate the current progress in applying SuFEx chemistry to nucleosides, nucleotides, and nucleic acids in a comprehensive manner, with particular attention to both the underlying synthetic logic and the constraints unique to (oligo)nucleotide scaffolds. Notably, several of the most advanced demonstrations in this area have been reported only very recently, largely within the past three years, underscoring both the timeliness of the topic and the need to consolidate the rapidly expanding literature.

Because this emerging field spans technically demanding synthetic methodology and application-driven chemical biology, we deliberately organise the review into two complementary parts to serve readers with different backgrounds and needs. The first, chemistry-focused part presents reported cases in which nucleic acids themselves act as nucleophilic partners in SuFEx-type ligations and summarises the main strategies for introducing SuFEx-reactive motifs on nucleoside, nucleotide and oligonucleotide scaffolds, with emphasis on reagent classes, handle installation, and compatibility with nucleic acid synthesis and enzymatic workflows. The second, application-focused part discusses how these SuFExable constructs are used in chemical biology, highlighting general design principles such as proximity-driven reactivity, warhead positioning, and multivalency rather than detailed synthetic procedures. Where a study is discussed in both parts, we provide brief in-text cross-references to guide readers between the chemistry and applications sections. In the Chemistry section, we also use textboxes, which allow for a better understanding of the described synthesis methodology in the context of specific applications, the details of which the reader will find in the Applications section. Finally, we outline future directions and challenges for expanding the reach of SuFEx chemistry within nucleic acid science.

Chemistry

Nucleic acids as nucleophiles in SuFEx reactions

Although SuFEx chemistry has been explored in the context of capturing biomolecules,[4] there are only a few examples in

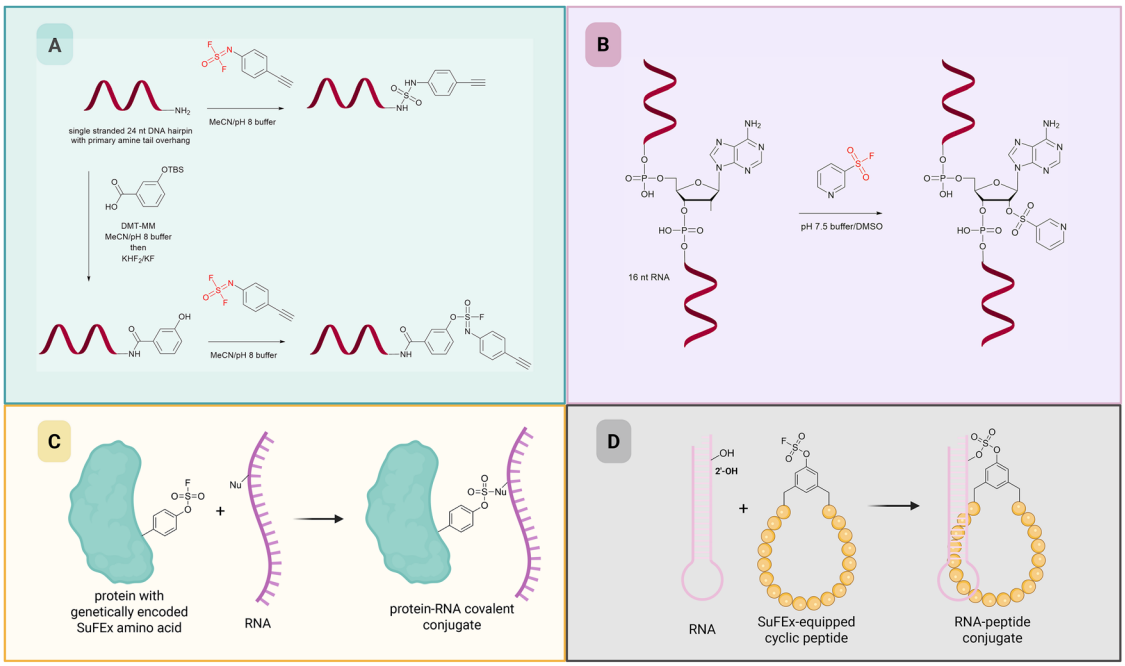

Fig. 2 Nucleic acids as nucleophiles in SuFEx reaction. (A) SuFEx bioconjugation of single-stranded DNA bearing terminal amine or phenol groups; (B) direct sulfonylation of RNA ribose 2′-hydroxyl groups; (C) proximity-driven protein–RNA crosslinking enabled by genetically encoded sulfur(VI)–fluoride amino acids installed in RNA-binding proteins; (D) covalent capture of RNA by macrocyclic peptides bearing aryl fluorosulfate warheads.

which nucleic acids themselves serve as nucleophilic partners in SuFEx-type reactions. The earliest such demonstration was reported by Sharpless and co-workers, who showed that amine and phenol functionalized single-stranded DNA could directly undergo SuFEx bioconjugation with SOF_4-derived iminosulfur oxydifluorides under mild aqueous conditions (Fig. 2A).[17] In this transformation, the terminal amine or phenol of the DNA acted as a nucleophile, forming a respective sulfamide or sulfurofluoridoimidate linkage upon reaction with the electrophilic sulfur centre. This early study provided the first clear proof that nucleic acid derivatives are compatible with SuFEx chemistry.

A later study by Kool and co-workers offered another perspective on nucleic acids acting as SuFEx nucleophiles, focusing on the direct sulfonylation of RNA ribose 2′-hydroxyl groups (Fig. 2B).[18] In developing this reaction, the authors systematically examined a range of electrophilic sulfonyl reagents that differed in the nature of the leaving group at the sulfur(VI) centre, including fluoride, chloride, and various heterocycles (mainly imidazole and triazole derivatives). In this broad screening sulfur–fluoride reagent appeared to be hydrolytically unstable and exhibited limited reactivity toward the weakly nucleophilic 2′-OH group of RNA, providing low conversion under tested conditions compatible with RNA. Consequently, for further investigations the authors adopted alternative leaving groups that offered higher intrinsic reactivity and better stability in aqueous media. This study clearly illustrated that, while the conceptual framework of sulfur(VI) exchange applies, the S–F bond can be too inert for effective reaction with inactivated nucleophilic sites in RNA under mild conditions.

The most advanced examples of nucleic acids acting as SuFEx nucleophiles were recently presented by Wang[11] and Dickinson.[19] In the first work (Fig. 2C), the authors established a general strategy for covalent protein–RNA crosslinking based on the genetically incorporating of two sulfur(VI)-based electrophilic amino acids [fluorosulfate-L-tyrosine (FSY) and *o*-sulfonyl fluoride-*O*-methyltyrosine (SFY)] into RNA-binding proteins. FSY was used in the previous studies of protein–protein interactions and enabled controlled and selective proximity-driven SuFEx coupling under physiological conditions.

SFY, introduced here for the first time, displayed much higher intrinsic reactivity enabling targeting weaker nucleophiles. In the initial experiments authors investigated if SFY could crosslink with nucleoside monophosphates and the traces of respective adducts were detected by MS after 16 h incubation under high concentrations. Next, both amino acids were incorporated site-specifically into RNA-binding proteins. In these systems, nucleophilic functionalities of RNA---principally the 2′-hydroxyls of ribose and, in some cases, exocyclic amino groups of nucleobases—attacked the electrophilic sulfur(VI) centres of the engineered amino acid side chains, yielding stable covalent protein–RNA conjugates.

A recent study by Dickinson and co-workers[19] provides another clear example of RNA functioning as a nucleophile through proximity-driven SuFEx reaction. In this work, SuFEx warheads containing linkers were embedded within a library of macrocyclic peptide scaffolds (Fig. 2D), enabling covalent reaction with RNA once binding brought the electrophile into close proximity to reactive sites. Chemically, the key SuFEx warhead employed was an aryl fluorosulfate (AFS), selected for its moderate reactivity

compared with aryl sulfonyl fluorides (ASF) and improved stability under aqueous and near-physiological conditions. Upon binding, the FS group underwent substitution by RNA nucleophiles, with detailed mass spectrometric analysis revealing selective reaction of ribose 2′-hydroxyl groups rather than nucleobase nitrogens. The reaction proceeded under mild aqueous conditions consistent with a proximity-enabled SuFEx mechanism rather than nonspecific bulk reactivity. Mapping of the covalent adducts showed formation of stable sulfate esters at defined dinucleotide motifs, demonstrating that RNA 2′-hydroxyl groups can act as competent nucleophiles in SuFEx chemistry when appropriately positioned relative to the sulfur(VI) electrophile underscoring their suitability for controlled SuFEx ligations on structured RNA substrates. From a chemical standpoint, these two works represent the most complex and mature examples to date of nucleic acids acting as intrinsic nucleophiles in SuFEx reactions.

Introduction of SuFExable groups into nucleosides and nucleotides

Installing S(VI)–F electrophiles on nucleosides and nucleotides creates covalent, nucleotide-mimetic reagents that retain the native recognition elements of the scaffold while adding a programmable electrophilic handle. In practice, these modifications are most often introduced by late-stage functionalisation using SuFEx hubs, because mononucleotides and nucleosides are sensitive to harsh conditions and suitable pre-functionalised building blocks remain limited. Here, "SuFEx hubs" refers to conveniently handled reagents that either (i) already contain a preinstalled sulfonyl fluoride or fluorosulfate group together with an orthogonal attachment handle, or (ii) act as fluorosulfurylating agents that transfer the sulfur(VI)–fluoride functionality directly onto nucleophilic sites such as amines or phenols. The key benefit is modularity: hubs allow precise placement of SF or FS groups (often as ASF/AFS motifs) through defined exit vectors and tunable linkers, enabling systematic optimisation of geometry and reactivity for nucleotide-binding sites. Representative functional uses of these modified nucleotides/nucleosides (*e.g.*, covalent probes and inhibitors) include ref. 20–23 and are further discussed in the Applications section.

The installation of sulfur(VI)–fluoride electrophiles on nucleotide scaffolds predates the coining of the SuFEx acronym and its later inclusion within the click chemistry framework by several decades. In the mid-1970s, Colman and co-workers reported the synthesis of 5′-fluorosulfonylbenzoyl adenosine,[23] the early example of nucleoside analogue bearing a reactive SF moiety. This compound was obtained through acylation of the 5′-hydroxyl of adenosine with *p*-fluorosulfonylbenzoic acid chloride (Fig. 3), generating a stable, isolable electrophile capable of engaging in controlled covalent labelling of enzymes (see section Applications/FSBA).

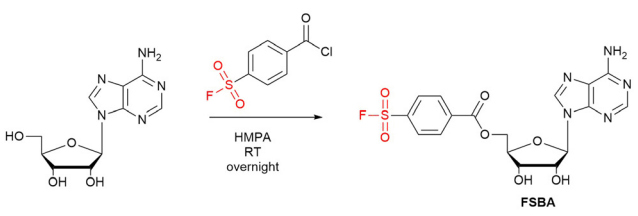

Fig. 3 The synthesis of FSBA.

The same approach was later extended to other nucleosides (including modified ones) demonstrating the generality of the benzoyl-linked SF strategy for purine nucleosides. Although these early examples predate the modern resurgence of sulfur(VI)–fluoride exchange chemistry in chemical biology, they represent foundational demonstrations of stable, isolable nucleosides functionalised with sulfonyl fluoride groups. Their synthesis and application, including more elaborated structures (see section Applications/FSBA derivatives and analogues), laid important groundwork for later developments in SuFEx-based bioconjugation of nucleosides and nucleotides.

After Sharpless and co-workers coined SuFEx in 2014 as an umbrella term that systematized a class of sulfur(VI)–fluoride substitution reactions within the click-chemistry framework, synthetic methods for accessing sulfonyl fluorides and fluorosulfates expanded rapidly, yielding numerous new and more practical strategies. Many of these protocols rely on rather harsh organic transformations, often unsuitable for the modification of fragile biomolecules such as nucleotides or oligonucleotides.[24,25] However, progress in the field has led to the development of specialized reagents known as SuFEx hubs (Fig. 4) that creates the possibility of selective introduction of reactive sulfur(VI) fluoride functionalities into complex molecules under mild conditions. These reagents are, in most cases, commercially available or readily accessible, and they offer considerable flexibility during the design of compounds for biochemical studies.

SuFEx hubs can generally be divided into two major classes. The first group comprises bifunctional building blocks that already contain a preinstalled SF or FS moiety, along with a second, typically electrophilic group that enables covalent attachment to the target molecule (Fig. 4). Common examples include aryl-based derivatives such as benzyl bromides, benzoic acid derivatives, and ethenesulfonyl fluoride (ESF), as well as more specialized reagents.[26–30]

The second group of SuFEx hubs (Fig. 4) includes reagents that act as SO_2F^+ synthons, capable of directly transferring the sulfur(VI) fluoride group to nucleophilic partners such as amines or phenols. The simplest representative of this class is sulfuryl fluoride (SO_2F_2);[1] however, due to its gaseous nature and high toxicity, its laboratory use requires specialized equipment and strict safety precautions. To overcome these limitations, several modern, bench-stable SuFEx reagents have been developed, including 1-(fluorosulfonyl)-2,3-dimethyl-1*H*-imidazol-3-ium trifluoromethanesulfonate (SuFEx-IT)[31] and (4-acetamidophenyl)(fluorosulfonyl)sulfamoyl fluoride (AISF).[8] These crystalline, easily handled reagents serve the same functional purpose as SO_2F_2 but allow precise control over reagent stoichiometry and reaction conditions.

Together, this diverse toolbox of convenient SuFEx reagents enables the late-stage introduction of SuFEx-reactive functional groups and facilitates the selective functionalization of complex molecules. Such strategies open the door to modular and efficient modification of nucleotide-based structures,

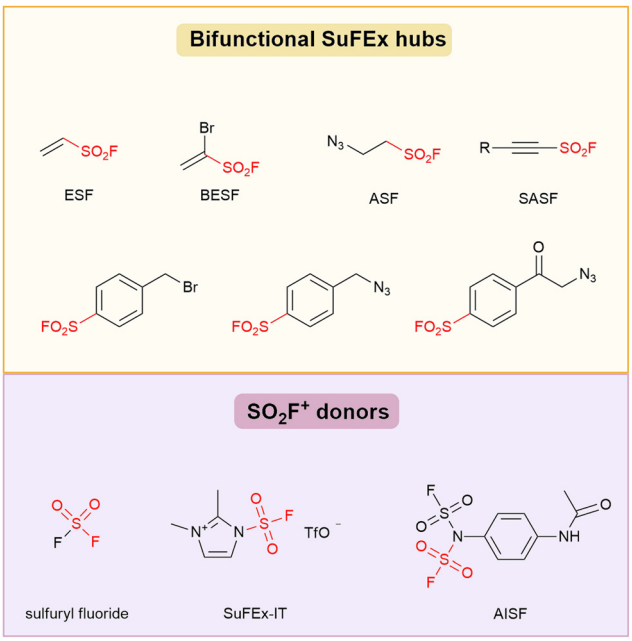

Fig. 4 Representative SuFEx hubs used to introduce S(VI)–F electrophiles into complex molecules.

expanding the scope of SuFEx chemistry beyond its applications in small-molecule research.

Despite this remarkable recent progress in SuFEx chemistry, examples involving the modification of nucleosides and nucleotides remain relatively scarce. The introduction of sulfur(VI) fluoride electrophiles into nucleosides and nucleotides has been explored far less extensively than analogous chemistry in peptides or small molecules, yet several studies demonstrate that these privileged biological scaffolds are compatible with SuFEx modifications. Nucleotides often appear only as single, advanced demonstrations within methodological studies aimed at introducing new SuFEx hubs. Such examples were presented, for instance, by Sharpless and co-workers, who applied the transformation of primary amines into iminosulfur oxydifluorides using SOF_4 to modify a reduced derivative of 3′-azidothymidine as well as the N^6-position of a protected adenosine analogue.[32] Azidothymidine also featured as a more advanced example in the studies by Moses[26] and Fokin,[27] where azides were converted into sulfonyl fluoride–containing triazoles using the BESF reagent.

The study by Scammells and Christopoulos[22] represents demonstrations that nucleoside analogues can accommodate ASF groups, although the work was not directed toward developing SuFEx methodology. Instead, the authors explored a broad panel of electrophilic warheads for generating irreversible A_1 adenosine receptor agonists (see section Applications/Other SuFEx nucleotide and nucleoside compounds), including isothiocyanates, Michael acceptors, and SFs. Within this larger set, the SuFEx-containing derivatives were prepared by first substituting 6-chloropurine riboside with diamine or aminobenzyl linkers to generate a family of N^6-modified intermediates. These were then acylated with 4-(fluorosulfonyl)benzoic acid under standard peptide coupling conditions. This strategy positioned the SF at the distal end of a linker, spatially removed from the nucleoside core so as not to interfere with receptor-binding determinants (Fig. 5A). While the chemistry effectively installs an ASF onto an adenosine scaffold, its role in the study was comparative rather than methodological without attempting to optimise or generalise the SuFEx installation itself.

Early systematic development appears in the study by Jemielity and Chromiński, (Fig. 5B)[21] which provides the first broadly applicable chemical method for preparing sulfamoyl fluoride nucleosides. In this work, the exocyclic amino groups of adenosine, guanosine and cytidine were directly converted into *N*-sulfamoyl fluorides. The authors evaluated multiple sulfur(VI) fluoride donors and ultimately adapted a protocol based on *ex situ* generation of sulfuryl fluoride gas from sulfonyl diimidazole and KF activated by trifluoroacetic acid.[33] The SO_2F_2 generated in this way was delivered into a reaction chamber containing protected nucleosides under basic conditions, enabling clean and high-yielding formation of sulfamoyl fluoride derivatives. The same study also utilized a following calcium-mediated activation mode of SuFExable derivatives: sulfamoyl fluorides were converted into sulfamides by reaction with various amines in the presence of $Ca(NTf_2)_2$/DABCO system.[34,35] The authors also demonstrated transformations of these nucleosides into nucleotides and short oligonucleotides, illustrating that SuFEx-derived groups can be carried through phosphorylation and solid phase chemistry. Taken together, this paper remains the only methodological study dedicated to the systematic preparation and reactivity of SuFExable nucleosides. Most other examples use SuFEx motifs in more application-driven contexts.

A structural integration of SuFEx chemistry into nucleotides was achieved in by Pflum and co-workers,[36] who designed a γ-modified ATP analogue bearing an AFS, but the SuFEx electrophile (Fig. 5C) was again only one of several warheads evaluated. The authors compared AFSs with other electrophilic groups to assess their suitability for proximity-driven kinase–substrate trapping (see section Applications/Other SuFEx nucleotide and nucleoside compounds). The SuFEx-reactive analogue was prepared by constructing a PEG-based linker, coupling it to 4-hydroxybenzoic acid, and converting the phenolic function into an AFS using AISF/DBU system. The FS-modified linker was attached to the γ-phosphate of ATP using EDCI/N-methylimidazole, generating a nucleotide in which the SuFEx warhead is displayed at the terminus of a flexible PEG–aryl chain. While this demonstrates that nucleotide triphosphates tolerate installation of an AFS at the terminal phosphate, the study treated the SuFEx warhead as one option among multiple electrophilic strategies and did not pursue broader optimisation or generalisation of SuFEx chemistry at the nucleotide level.

A conceptually SuFEx-oriented contribution appears in the study by Kowalska and Chromiński.[20] In this work, the authors designed covalent inhibitors of cNIIIB by installing an ASF at the N7-position of GMP (Fig. 5D). The strategy builds on earlier studies demonstrating that benzylated N7-guanosine

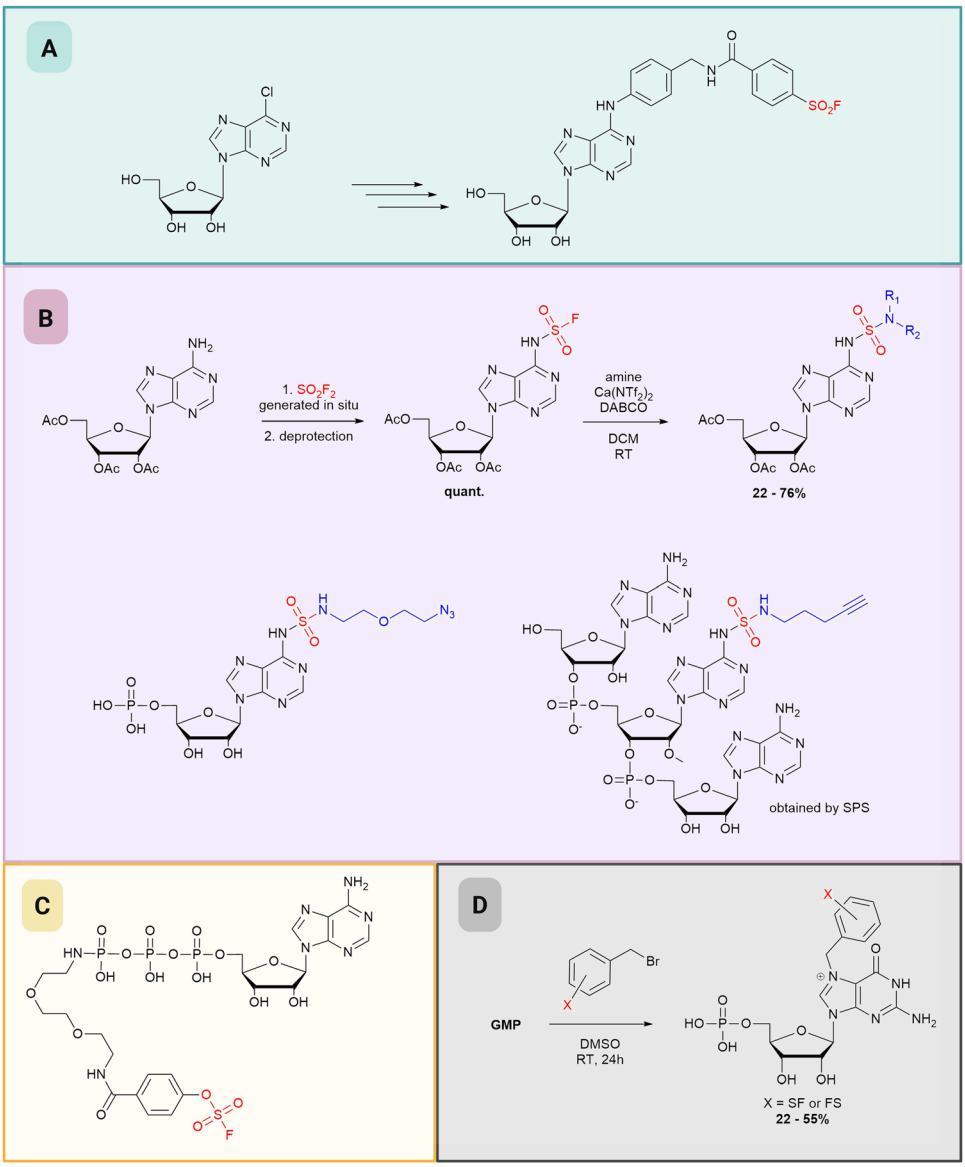

Fig. 5 Selected strategies for introducing sulfur(VI)–fluoride motifs into nucleoside and nucleotide scaffolds. (A) N^6-substituted adenosine derivatives bearing an aryl sulfonyl fluoride warhead; (B) "SuFNucs": preparation and diversification of sulfamoyl fluoride nucleosides and representative derivatives; (C) γ-Modified ATP analogue bearing an aryl fluorosulfate warhead (ATP–aryl fluorosulfate); (D) N7-functionalised GMP derivatives bearing aryl sulfonyl fluoride or aryl fluorosulfate groups.

monophosphate (Bn^7GMP) derivatives act as potent noncovalent inhibitors of this enzyme, and on the structural features identified as critical for high inhibitory activity.[37] The key synthetic step involves alkylating GMP with benzyl bromides bearing ASFs or AFSs under conditions that promote selective N7 substitution. This method enables direct introduction of a SuFEx electrophile without the need for protecting-group manipulation or multi-step linker construction, providing a concise and synthetically accessible route to SuFEx-modified guanosine nucleotides. The electrophile is positioned on an aromatic ring appended to N7, a site chosen to preserve the essential base-recognition elements of GMP while directing the sulfur(VI) warhead toward nucleophilic residues within the enzyme's binding pocket. The study is also mechanistically noteworthy, as it provides a rare example—predicted *in silico* and confirmed experimentally—of histidine tagging by an ASF electrophile (see section Applications/Other SuFEx nucleotide and nucleoside compounds).[38]

SuFExable oligonucleotides

The application of sulfur(VI) fluoride exchange (SuFEx) chemistry to oligonucleotides has opened a powerful route to constructing covalent aptamers—nucleic acid ligands equipped with sulfur(VI) electrophiles capable of forming irreversible bonds upon target engagement. Across multiple recent studies, ASFs and AFSs have been installed at defined positions within oligonucleotide sequences through synthetically robust and highly chemoselective reactions. Although these constructs

differ in sequence, target, and biological purpose, the underlying chemical strategies used to introduce SuFEx warheads fall into two categories: (i) click chemistry-based attachment of SuFEx-modified benzyl groups onto alkyne-bearing oligonucleotides (Fig. 6A), and (ii) direct backbone derivatisation through alkylation of phosphorothioate internucleotide linkages with benzyl bromides carrying sulfur(VI) fluorides (Fig. 6B). Together, these methods constitute a modular toolkit for engineering covalent aptamers with controllable warhead placement and tunable reactivity.

CuAAC and SPAAC reactions for introducing SuFEx warheads

CuAAC/SPAAC installation is most useful when a single SF/FS warhead must be positioned at a precisely defined site while keeping the underlying oligonucleotide sequence and fold largely unchanged. In this strategy, an alkyne handle is introduced either internally *via* incorporation of an alkyne-modified nucleotide (during solid-phase synthesis or enzymatic extension) or directly at a terminus (most commonly the 5′ end, *e.g.*, as a strained alkyne such as DBCO). The SuFEx electrophile is then delivered as an azide-bearing aromatic ASF/AFS reagent, affording a triazole-linked warhead. A key advantage is that the modification site can be strictly defined at the design stage (a single nucleobase position, multiple predefined positions, or a specific terminus), enabling controlled orientation and minimal structural perturbation. This precision is particularly useful in covalent aptamer design, where maintaining the native binding fold while directing the electrophile toward a productive region of the target interface is critical for proximity-driven reactivity. In functional settings, click-installed warheads support covalent capture and can also be adapted to library-compatible workflows discussed in the Applications section. Representative examples of this approach can be found in the ref. 39–41.

A straightforward strategy for SuFEx functionalisation involves the installation of an alkyne-type handle on the oligonucleotide, followed by its conjugation with an azidomethyl aryl bearing SuFEx type electrophile. Two variants of this approach have been employed: copper(I)-catalysed azide–alkyne cycloaddition (CuAAC, Fig. 6A) and strain-promoted azide–alkyne cycloaddition (SPAAC, Fig. 6A).

In the earliest example by Yang and Taki[40] an internal alkyne is introduced into predesigned aptamer structure during solid-phase synthesis by replacing a natural deoxythymidine with an 5-octadiynyl-dU (Fig. 6A). This provides a synthetically precise sites for CuAAC, in which an azide-containing SuFEx electrophile is linked *via* a triazole moiety. This method is particularly well suited for site-specific internal modifications because the alkyne can be positioned at any desired point within the sequence during automated synthesis. Building on this site-specific CuAAC strategy, the same group subsequently translated the alkyne-to-SuFEx click installation from a single, predesigned aptamer into a more advanced library-compatible format for screening workflows.[42] In this follow-up, an alkyne handle was embedded at a predefined position within an otherwise randomized DNA library by incorporating again terminal-alkyne-modified deoxyuridine as the sole chemically addressable nucleotide, and the resulting library remained compatible with polymerase handling. The SuFEx electrophiles were then introduced post-synthetically by CuAAC with azide-bearing aromatic ASF and AFS yielding triazole-linked warheads installed at a single, precisely defined site across the entire pool. The functional consequences of these constructs are discussed in the Applications section (Aptamers/Enzyme and PPI inhibitors and Aptamers/SuFEx *in vitro* selection).

A recent study by Wang and Zhang[39] further extends the use of CuAAC for installing SuFEx-reactive groups onto oligonucleotides in the context of covalent aptamer evolution (see section Applications/Aptamers/SuFEx *in vitro* selection). The central chemical concept relies on positioning an aryl sulfonyl fluoride

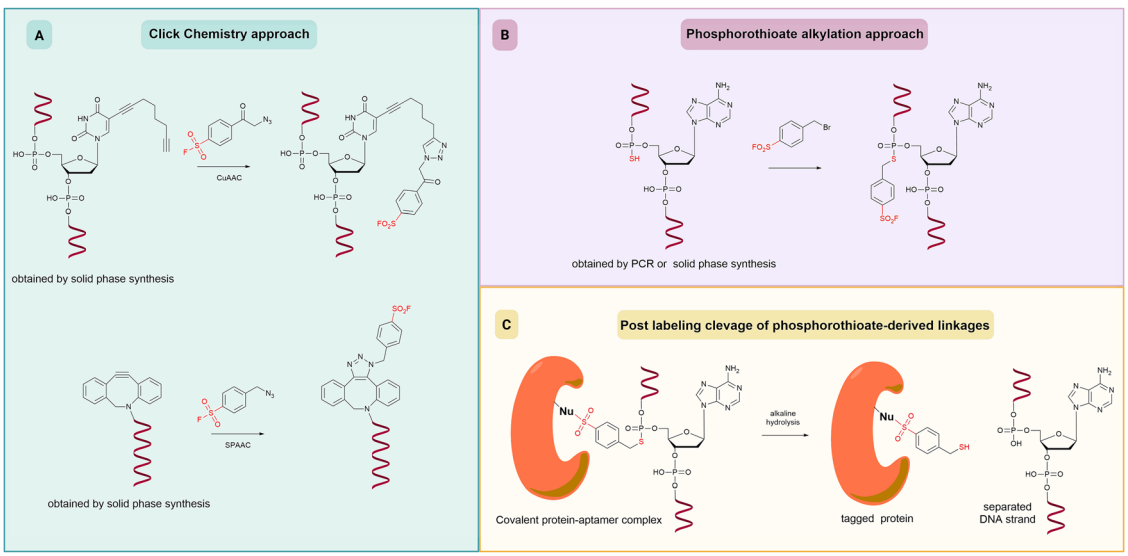

Fig. 6 Chemical strategies for installing SuFEx-reactive motifs on oligonucleotides. (A) CuAAC and SPAAC based installation of S(VI)–F warheads onto alkyne-modified DNA; (B) site-specific introduction of S(VI)–F warheads *via* phosphorothioate alkylation; (C) post-labeling processing feature of phosphorothioate-derived cross-links.

warhead at the 3′ terminus of DNA aptamers through a modular CuAAC based strategy that is fully compatible with amplification and selection workflows. To achieve this, the authors introduced a terminal alkyne handle by enzymatically extending the 3′ end of PCR-amplified DNA strands with 5-ethynyl-dU. This post-PCR modification avoids interference with polymerase activity during amplification while ensuring uniform placement of the reactive handle across the entire library. The alkyne-functionalised DNA was then reacted with 4-(azidomethyl)benzenesulfonyl fluoride using CuAAC, forming a triazole-linked benzyl sulfonyl fluoride moiety at the oligonucleotide terminus. This design places the SuFEx warhead distal to the folded aptamer core, minimising structural perturbation while allowing proximity-driven reactivity upon target engagement. From a chemical standpoint, the work demonstrates that CuAAC-mediated attachment of ASFs to enzymatically modified DNA is robust, scalable, and compatible with iterative selection cycles, further consolidating CuAAC as a successful strategy for installing SuFExable groups onto oligonucleotides.

A related but metal-free strategy, demonstrated by Zhao and Tan,[41] employed SPAAC (Fig. 6A) to attach SuFEx warheads to the 5′ terminus of pre-existing aptamers. Here, a dibenzocyclooctyne (DBCO) group was introduced at the 5′ end during solid-phase synthesis. Reaction with an 4-(azidomethyl)benzenesulfonyl fluoride proceeded rapidly under mild aqueous conditions, avoiding the need for copper catalysts. For an applications-focused perspective on these constructs, see the Applications/Other emerging uses and design principles section.

Alkylation of phosphorothioate linkages with benzyl bromides bearing SuFEx warheads

Phosphorothioate alkylation is a practical route to backbone-displayed ASF/AFS warheads and is especially valuable when multivalent presentation is desired. The key transformation relies on the high nucleophilicity of the phosphorothioate sulfur, which undergoes selective *S*-alkylation with electrophiles that are most commonly benzyl bromides bearing preinstalled SuFEx warheads. Because many proximity-driven systems benefit from higher local electrophile density, this strategy enables installation of multiple SuFEx warheads in a programmable way—either at library scale (*via* PCR incorporation of phosphorothioates) or at user-defined sites (*via* solid-phase synthesis). A further functional advantage is the ''recoverability'' of sequence information: after covalent capture, basic cleavage of phosphorothioate-derived linkages can release the DNA component for PCR amplification and sequencing in selection workflows, while leaving a covalent tag on the protein that can be used to identify the modified residue(s) by mass spectrometry in mapping/proteomic experiments. These features make phosphorothioate alkylation central to covalent aptamer inhibitors and *in vitro* selection platforms discussed in the Applications section. Representative references in which this chemistry is used include ref. 43 and 44.

A second major and increasingly prominent approach for constructing SuFEx-capable oligonucleotides relies on the chemoselective alkylation of phosphorothioate internucleotide linkages (Fig. 6B). Alkylation of sulfur in phosphorothioate backbones is a well-known and widely applied transformation,[45] and it provides an exceptionally direct route to installing SuFEx type electrophiles onto the oligonucleotide scaffold. This strategy proceeds in two steps: first, introducing phosphorothioate linkages into the DNA strand; second, alkylating the sulfur atoms with benzyl bromides functionalised with proper SuFEx reactive groups.

Phosphorothioate linkages can be introduced into oligonucleotides by two complementary strategies, each offering distinct advantages for the design of SuFExable constructs. In the enzymatic route, phosphorothioate linkages are introduced during polymerase chain reaction (PCR) by substituting the canonical deoxyadenosine triphosphate with deoxyadenosine-α-thiotriphosphate.[43] Every incorporation event forms a phosphorothioate at the corresponding internucleotide position. Because PCR can be performed on large randomised pools, this method allows sulfur-containing linkages to be introduced across entire DNA libraries, enabling parallel functionalisation of billions to trillions of sequences. This makes enzymatic phosphorothioate installation uniquely suitable for high-throughput discovery platforms, where backbone-embedded SuFEx handles can be uniformly installed before selection (see section Applications/Aptamers/SuFEx *in vitro* selection).

In the fully synthetic route, phosphorothioate linkages are introduced at selected positions during automated solid-phase oligonucleotide assembly. This approach offers maximal control over the number, spacing, and placement of sulfur atoms within the oligonucleotide chain. As a result, solid-phase synthesis supports sophisticated SuFEx aptamer designs, including positional fine-tuning around binding motifs,[46] multi-site modification patterns,[44,47] and modular incorporation of phosphorothioate-rich segments such as 3′ thymidine tails.[48] This synthetic flexibility makes it possible to precisely direct SuFEx warheads to structurally meaningful loci within aptamers (see section Applications/Aptamers).

Once phosphorothioate linkages are introduced, SuFEx warheads are installed by treating the sulfur-modified oligonucleotide with benzyl bromides bearing ASF or AFS groups. These alkylation reactions typically proceed:

- in mildly acidic aqueous phosphate buffer,
- with acetonitrile as a co-solvent
- at moderate temperatures (25–37 °C).

Across published examples, the stoichiometry of reagent to oligonucleotide is strongly substrate-biased, reflecting the need to drive alkylation to completion. Benzyl bromide reagents bearing SuFEx warheads are commonly used in large excess, typically 100–300 equivalents relative to the oligonucleotide strand, and in some workflows up to millimolar reagent concentrations are applied. When modifying libraries, reagent excess can be even higher to ensure complete conversion across diverse sequences.[43]

Following alkylation, SuFExable oligonucleotides are typically isolated by ethanol precipitation, ultrafiltration, depending on scale and purity requirements. For analytical characterisation or preparative use, reversed-phase HPLC is frequently employed to separate fully modified oligonucleotides from partially derivatised or unreacted species. Because

the alkylation generates a new carbon–sulfur bond and introduces a hydrophobic benzyl substituents, modified strands often exhibit substantial changes in retention time that facilitate their purification. The resulting SuFEx-functionalised backbones are generally stable in aqueous buffers and under storage, with the sulfur(VI) electrophiles remaining intact for extended periods at neutral pH.

An additional feature of this general strategy is that phosphorothioate-derived linkages can be cleaved under alkaline conditions (Fig. 6C). Several studies note that after covalent adduct formation between a SuFEx-bearing oligonucleotide and a protein target, treatment with base removes the DNA portion, that can be separated and amplified, while leaving the protein tagged at the amino acid that took part in cross-link. This capability underpins both iterative PCR cycles (in selection workflows) and proteomic identification of binding sites of covalently labelled proteins.[43,47,48] Discussion of how this approach is used in practice can be found in the Applications/Aptamers section.

DNA Encoded Libraries

Examples of installing SuFEx warheads in DNA-encoded library (DEL) chemistry generally rely on two complementary approaches developed for specific platforms and use cases. When the goal is to generate electrophilic library members for covalent screening, the sulfonyl fluoride warhead is typically introduced at a late stage by coupling chlorosulfonyl–benzenesulfonyl fluorides to an amine on the DNA-linked small-molecule scaffold, thereby installing the SF group in the final library member. In contrast, a distinctive role of AFS is demonstrated in studies where the FS group is used as a pseudohalide-like leaving group in subsequent palladium-catalysed on-DNA cross-coupling reactions, enabling diversification while remaining compatible with the DNA tag. Although these methodologies were developed in the context of specific covalent DEL workflows, the underlying DNA-compatible installation logic and stability constraints are broadly informative and can be translated to the design of other oligonucleotide-based constructs for diverse applications.

DNA encoded libraries (DELs) enable the synthesis and screening of vast numbers of small molecules by linking each compound to a DNA barcode that records its synthetic history.[49,50] A key chemical constraint of this technology is that all transformations must be compatible with the chemical stability of DNA and with aqueous or mixed aqueous–organic reaction conditions. As a result, the incorporation of electrophilic functionalities into DELs has traditionally been limited. In this context, sulfur(VI) fluoride exchange chemistry has emerged as an attractive solution, as ASFs and AFSs combine stability during library synthesis with latent electrophilicity that can be revealed at the screening stage.

DEL synthesis hinges on transformations that proceed at very low substrate concentrations in aqueous or mixed aqueous–organic media while preserving the integrity of the DNA barcode. For this reason, electrophilic warheads are typically introduced in a way that balances two competing requirements: they must be chemically stable throughout multistep on-DNA synthesis and encoding, yet remain competent electrophiles once the final library members are screened. Sulfur(VI) fluoride motifs satisfy this balance particularly well, and a distinctive chemical role for AFSs is showcased in the study by Lerner and Yang, where the $-OSO_2F$ group is explicitly used as a leaving group for subsequent palladium-catalysed on-DNA cross-couplings (Fig. 7A).[51] In that work, the AFS handle was installed directly on a DNA-conjugated phenol (a DNA-linked 4-hydroxybenzoic acid headpiece) by treatment with AISF in the presence of DBU in borate buffer (pH 9.4) at room temperature, delivering the DNA-tagged AFS in high conversion after a short incubation. It is worth to mention that AISF appeared to be superior in terms of yield to other reagents used during the screening of the conditions. With this DNA–AFS in hand, the $-OSO_2F$ group serves as a robust, universal and DNA-compatible pseudohalide in a wide range of cross coupling reactions (Suzuki–Miyaura, Sonogashira, Buchwald–Hartwig and related transformations[52]), enabling general and straightforward diversification platform by palladium catalysis.

In contrast, SF warheads in covalent DEL formats are typically introduced as terminal functionalities after full scaffold assembly, ensuring maximal exposure of the electrophile in the final library members. For example, in activity-based proteome profiling and covalent DEL enrichment (ABPP-CoDEL) workflows, on-DNA library preparation is followed by a final introduction of SuFEx unit using chlorosulfonylbenzenesulfonyl fluorides to install the SF group on the DNA-linked small molecule (Fig. 7B).[53] More recent CoDEL designs broaden the same chemical logic by treating electrophile incorporation as a modular late-stage operation, in which diverse warheads (including sulfur(VI) fluoride electrophiles among others) are appended to DNA-linked scaffolds through DNA-compatible bond-forming steps such as amide coupling, nucleophilic substitution, or click reactions, chosen to preserve both the DNA tag and warhead stability during library synthesis.[54] Collectively, these studies establish two complementary chemical roles for sulfur(VI) fluoride motifs in DEL chemistry: AFSs can function as DNA-compatible pseudohalides for palladium-mediated diversification, whereas ASFs are most often installed as end-stage covalent handles that are poised for subsequent proximity-driven reactivity during screening.

Applications

SuFEx-functionalised nucleotides and nucleosides

Nucleotides and nucleosides serve multiple essential functions in living organisms. They constitute the fundamental building blocks of nucleic acids, while ATP acts as the universal energy donor in biochemical processes. Other nucleotides and their derivatives participate in redox reactions as electron carriers, exemplified by the NAD^+/NADH and $NADP^+$/NADPH pairs, and play central roles in cellular signaling as second messengers, such as cAMP or GTP. In addition, nucleotides and nucleosides function as important extracellular signaling molecules through their interactions with purinergic P1, P2X, and P2Y receptors, and they act as allosteric regulators of numerous enzymes involved in

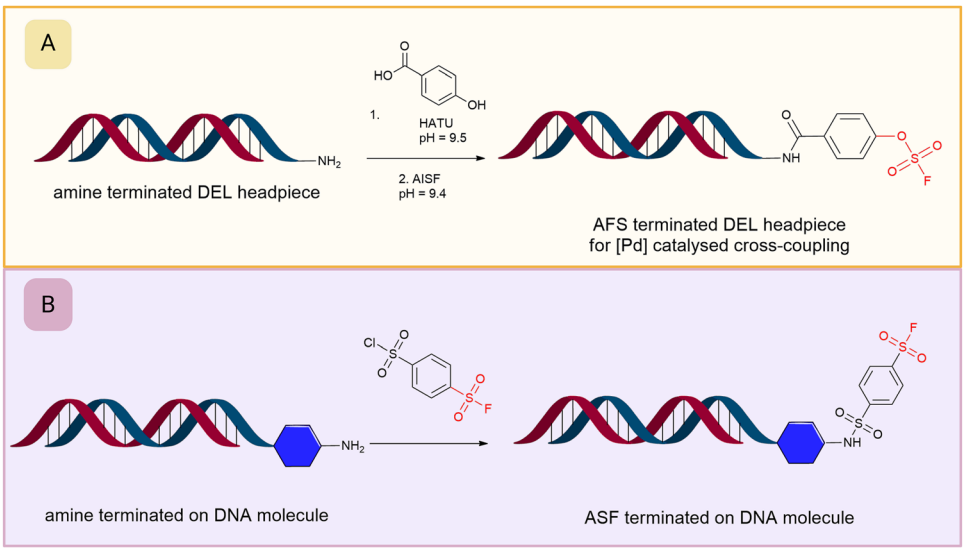

Fig. 7 Installation of sulfonyl fluorides and fluorosulfates in DNA-encoded library (DEL) chemistry. (A) Preparation of DNA-linked aryl fluorosulfates as on-DNA cross-coupling handles; (B) late-stage installation of sulfonyl fluoride warheads on DNA-linked small molecules.

metabolic pathways.[55] The cellular levels of ATP, ADP, and AMP provide a fundamental mechanism for monitoring the cellular energy state and tightly regulate specialized signaling pathways, most notably the AMP-activated protein kinase (AMPK) pathway.[56] A particularly important role of nucleotides is their involvement in phosphorylation. ATP serves as the source of phosphate groups that are transferred to substrates by kinases. The introduction of a negatively charged phosphate group alters the biophysical and functional properties of the modified targets, which is critical for the regulation of diverse metabolic and signaling processes. Consequently, kinases represent well-established and clinically relevant molecular targets.[57]

SuFEx chemistry offers new opportunities for the design and synthesis of reagents suitable for probing these nucleotide- and nucleoside-dependent processes. It enables the development of compounds capable of covalently binding to proteins at their active or allosteric sites. Moreover, the incorporation of SuFEx moieties into DNA and RNA molecules creates new possibilities for covalent protein–nucleic acid crosslinking reactions. In the following section, applications of SuFEx-bearing nucleotides, nucleosides, and nucleic acids are presented, with particular emphasis on their use as chemical tools for investigating biological targets.

FSBA

The first applications of SuFEx nucleoside derivatives date back to 1975, when Pal, Wechter, and Colman synthesized two adenosine-based SuFEx ATP analogues: 5′-*p*-fluorosulfonylbenzoyl adenosine[15] (Fig. 8, compound **1**, 5′-FSBA) and 3′-*p*-fluorosulfonylbenzoyl adenosine[53] (Fig. 9, compound **2**, 3′-FSBA). In the further text FSBA refers exclusively to 5′-FSBA. It was demonstrated that 3′-FSBA acts as a covalent binder of the purine nucleotide–binding site in bovine liver glutamate dehydrogenase (GDH). Interestingly, FSBA binds to a second allosteric site that enables regulation of GDH by NADH. Although FSBA and related nucleotide analogues were developed decades before SuFEx was formalised within the click-chemistry framework, they represent some of the earliest successful examples of installing sulfur(VI)–fluoride electrophiles on nucleotide scaffolds and applying them in aqueous biochemical settings. This body of work established practical principles that remain instructive today, including the balance between stability and conditional reactivity of S(VI)–F motifs and the value of nucleotide scaffolds for positioning electrophiles within preorganised binding pockets. Early examples of FSBA usefulness have been summarized by Colman in 1990,[58] however more recent applications remain scattered across the enzymology and chemical biology literature and, to the best of our knowledge, have not been recently reviewed in a dedicated way; therefore, we consider this review an appropriate place to summarise them. We also aim to highlight how these early experiments anticipate several contemporary themes, including covalent profiling of nucleotide-binding proteins and the design of covalent nucleotide-inspired probes.

The usefulness of FSBA in biochemical research has been demonstrated in numerous studies involving various proteins exhibiting affinity for ADP or ATP. Representative applications reported in the literature are summarized in Fig. 8. One relevant example of FSBA applicability is the elucidation of mechanisms of platelet aggregation. It was shown that the compound binds to the platelet membrane receptor, inhibiting the ADP-induced shape change,[59] as well as ADP-induced aggregation by reducing the exposure of fibrinogen binding sites.[60] FSBA-induced shape change of platelets was further demonstrated by spectrophotometric and scanning electron microscopy assays.[61] Another example of FSBA applicability was its use in rabbit muscle pyruvate kinase (PK) investigation, where the adenosine derivative inactivated the kinase upon incubation[62] and upon incubation modified two distinct amino acid residues (Tyr and Cys) at different nucleotide binding sites.[63] It was also shown, that PK can be modified by fluorescent

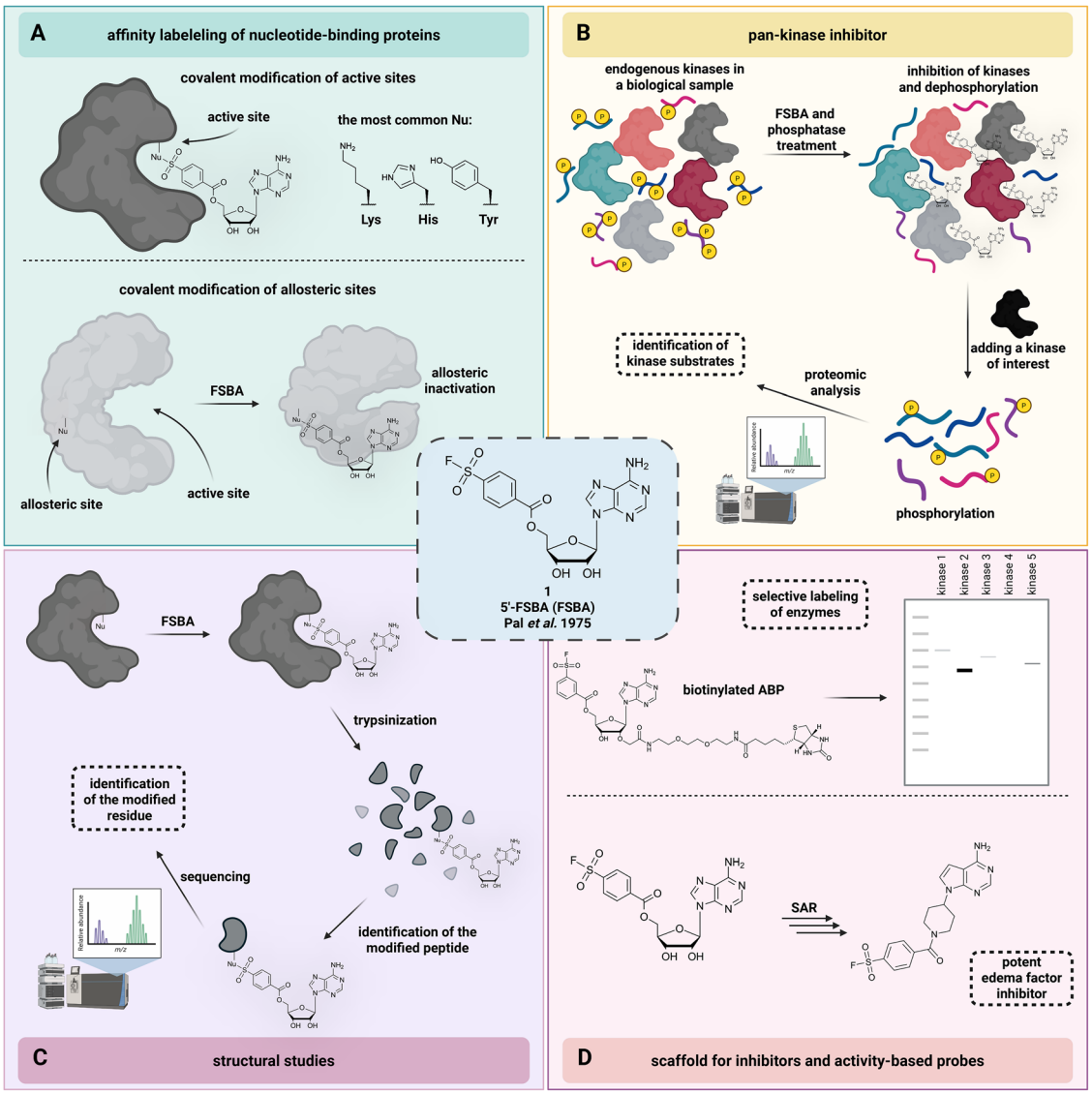

Fig. 8 Applications of FSBA. (A) FSBA has been used as a covalent inhibitor of enzymes and as an affinity label for proteins allosterically regulated by nucleotides; (B) FSBA has been applied in kinase research as a pan-kinase inhibitor; (C) in combination with chromatography or mass spectrometry, FSBA has enabled the identification of nucleotide-binding sites within proteins; (D) FSBA has been used as a scaffold for inhibitor optimization and for the design of activity-based probes. Created with BioRender.com.

FSBA derivative, however the modified residues differ from the amino acids labelled by FSBA.[64] FSBA has also been used for the early elucidation of epidermal growth factor receptor (EGFR) activity as a tyrosine kinase. Examples of this are studies undertaken by Buhrow *et al.* who used ^{14}C-labelled FSBA to label EGFR localized in A431 cell membranes and characterized its kinase activity.[65–67] These studies provided key evidence for the EGFR intrinsic tyrosine kinase activity. Another example of FSBA application in early protein structure and function studies is biochemical characterization of myosin light chain kinase (MLCK). Colburn *et al.* incubated rabbit skeletal muscle MLCK (skMLCK) with FSBA and observed the loss of kinase activity. This inactivation could be prevented by adding ATP and Mg^{2+} ions to the mixture indicating that the inhibitor and the substrate occupy the same binding site.[68] Later, Kennelly and colleagues used FSBA to probe the nucleotide-binding site of skMLCK in its calmodulin-free form and in the active complex with Ca^{2+}-bound calmodulin (CaM) revealing that activation by CaM induces only relatively small conformational changes within the ATP-binding site.[69] Similar results have been obtained for smooth muscle MLCK.[70] Interestingly, Komatsu and Ikebe observed that in the absence of Ca^{2+}, MgATP did not inhibit the affinity labelling of the kinase by FSBA suggesting ATP-insensitive inactivation of skMLCK in a CaM free form.[71]

Historically, FSBA has emerged as the most widely used nucleotide analogue incorporating a SuFEx moiety. It has been applied in studies of proteins from various organisms with more recent examples including: *E. coli* ArsA,[72] *E. coli* asparagine synthetase B,[73] *T. gondii* adenosine kinase,[74] West Nile virus (WNV) NTPase/helicase,[75] hepatitis C virus (HCV) and

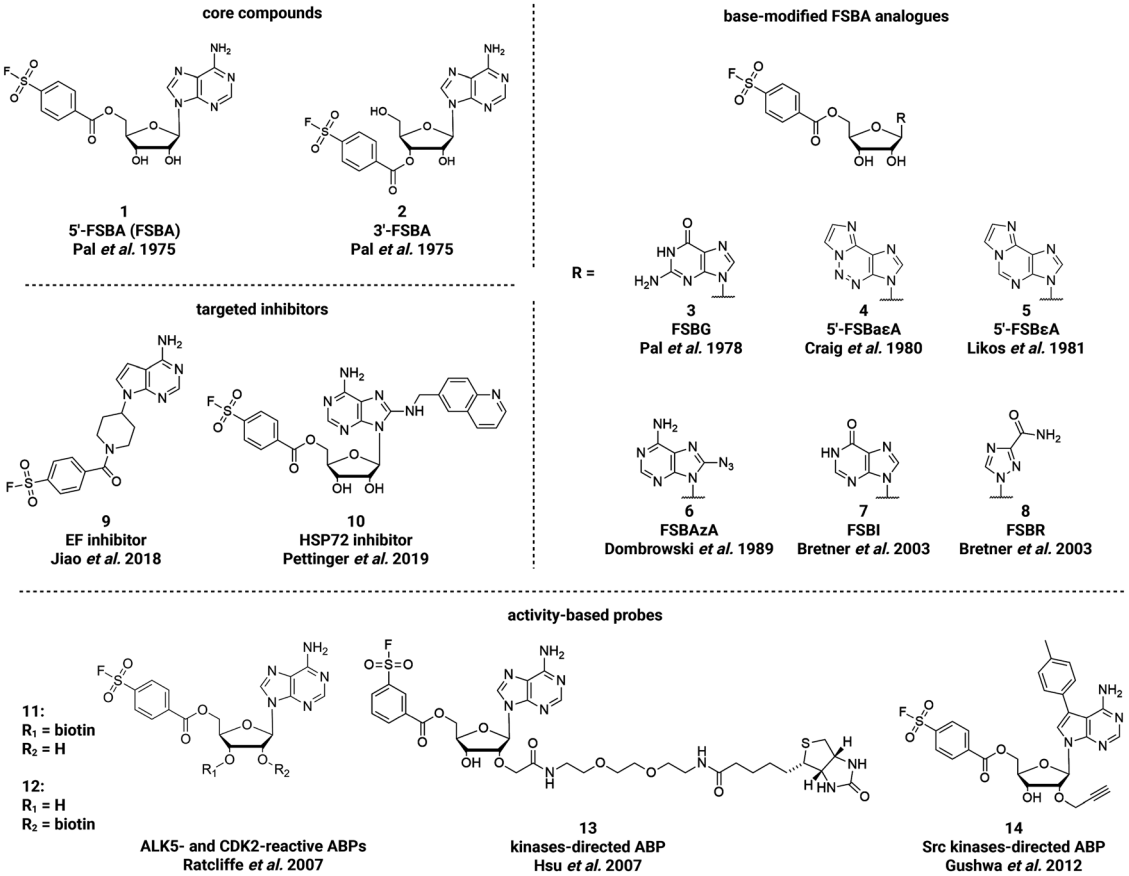

Fig. 9 FSBA-derived chemical tools. FSBA has been employed as a scaffold in the design and synthesis of affinity labels, inhibitors, and activity-based probes.

WNV polymerase,[76] human p38γ kinase, or bovine brain phosphatidylinositol 4-kinase 230.[77] In the work by Knight *et al.* from 2012, whole-cell lysate kinase assay was used to identify substrates of p38α MAPK in differentiating myoblasts.[78] In this approach, lysates were treated with FSBA to eliminate endogenous kinase activity, after which recombinant p38α or p38β was added and the samples were analysed by liquid chromatography tandem mass spectrometry (LC–MS/MS) to identify phosphorylation sites attributable to the added kinases, including seven *in vivo* phosphorylation sites on six substrates. A related approach to identifying kinase substrates was developed by Xue *et al.*[79] who introduced Protein Kinase Assay-Linked Phosphoproteomics (proKALIP), an extension of the earlier KALIP method that employed synthetic peptides as kinase substrates.[80] In this workflow, cells are treated with phosphatase inhibitors to elevate endogenous phosphorylation that enables affinity enrichment of phosphoproteins. To suppress background phosphorylation, the enriched phosphoproteins are pre-incubated with FSBA, which efficiently inactivates endogenous kinases and prevents autophosphorylation or downstream kinase activation. After removal of excess FSBA, the proteins are dephosphorylated with alkaline phosphatase and subsequently re-phosphorylated *in vitro* by a recombinant kinase of interest. Newly phosphorylated proteins are digested, phosphopeptides are identified by LC–MS/MS and quantitatively compared by SILAC, and high-confidence direct substrates are defined by intersection with *in vivo* phosphoproteomics data obtained under kinase inhibition.

FSBA as a chemical tool for structural analysis

The affinity labelling method with FSBA has enabled the understanding and elucidation of the regulatory mechanisms and modes of action of numerous enzymes and proteins. Covalent attachment of the label to the molecular target, however, allows for more in-depth structural analysis, such as the precise identification of amino acid residues involved in the formation of the ATP analogue binding site. Such analyses are typically performed in several steps. First, the selected target is incubated with isotopically or fluorescently labelled FSBA. Next, the protein with the covalent label is subjected to enzymatic digestion with a protease, most commonly trypsin. In the final step, the resulting enzymatic fragmentation peptides are analysed using radiographic, spectroscopic, or mass spectrometry methods which enable determination of the exact location of the nucleotide binding site.

Initially, radiolabelled FSBA was applied to identify and characterize the ATP binding site of the F_1 domain of mitochondrial ATPase.[81] Affinity labelling in conjugation with enzymatic digestion, chromatographic methods, and Edman

degradation revealed the modified sequence with the Tyr residue identified as the site of FSBA modification. Similar workflow was applied by Russo *et al.* who used ^{14}C-labeled FSBA to identify the ATP-binding site of the EGFR in membrane vesicles prepared from A431 cells.[82] The researchers identified a Lys residue within the IPVAIKEL tryptic peptide as the site of modification. The Sefton group adopted a somewhat different approach to identify an ATP binding site of pp60^{src} tyrosine kinase from Rous sarcoma virus.[83] Comparative analysis of 2D TLC results of tryptic peptides derived from the isotopically labelled protein incubated with FSBA and a control sample showed that the modification site is Lys295. The modification site was consistent with previous analyses of active-site residues in RNA tumour virus kinases from six additional classes.

A major advance over paper and thin-layer chromatography methods was the application of liquid chromatography techniques. Knight and McEntee used ^{3}H-labelled FSBA to identify the ATP-binding site of *E. coli* RecA, a multifunctional protein that utilizes ATP both as a substrate and as a regulatory molecule. High-performance liquid chromatography (HPLC), combined with UV detection and radiometric analysis, enabled the identification and purification of a tryptic peptide covalently modified by ^{3}H-FSBA, leading to the assignment of Tyr264 as the site of affinity labelling.[84] Similarly, van Berkel *et al.* identified Tyr38 as the key residue in the NADPH binding site of *Pseudomonas fluorescens p*-hydroxybenzoate hydroxylase.[85] Other relevant examples of radioactivity or fluorescence detection of FSBA-labelled peptides coupled with liquid chromatography include *Salmonella typhimurium* 5-phosphoribosyl-α-1-pyrophosphate (PRPP) synthetase,[86] smooth muscle MLCK,[71] GDH,[87] or rat liver adenosylhomocysteinase.[88] In the latter example FSBA itself was not radiolabelled, but it triggered enzyme inhibition by promoting disulfide bond formation involving a reactive cysteine pair. Radiographic detection relied on labelled iodoacetate, which bound to unmodified Cys side chains which enabled identification of the region containing the key residue.

Mass spectrometry is a powerful platform for the detection of covalent modifications within proteins, allowing such modifications to be identified in a relatively straightforward manner. The development of LC–MS/MS techniques has additionally enabled proteome-wide analyses, providing a comprehensive view of interactions between proteins and designed inhibitors, affinity tags, or other small molecules.[89] Khandekar *et al.* used FSBA as a kinase-reactive ATP analogue to covalently label the ATP-binding sites of CDK2 and ALK5 kinases.[90] The active site Lys modifications were identified by comparing the mass spectra of tryptic peptides from FSBA-treated and untreated control samples. Subsequent LC–MS/MS sequencing confirmed the identity of the labelled peptides and the positions of the modified lysine residues. A similar approach was applied by Manvar *et al.*, who used FSBA to identify major modification sites in HCV NS5B replicase, with LC–MS/MS revealing Tyr382 and Lys491 as the principal FSBA-reactive residues within the nucleotide-binding region of the enzyme.[91] Another example of proteomics strategy used in conjunction with FSBA labelling is Combined Fractional Diagonal Chromatography (COFRADIC).[92] Protein extracts from Jurkat T cells were labelled with FSBA, digested with trypsin, and fractionated. The fractions were treated with a strong base to hydrolyse the nucleoside portion of the FSBA adduct, leaving a sulfonylbenzoate (SB) group covalently attached to modified peptides. Following acidification, the samples were reanalysed under identical chromatographic conditions, and peptides displaying retention time shifts between the two runs were selected for LC–MS/MS analysis. Using this workflow, 209 SB-modified peptides corresponding to 185 distinct purine nucleotide–binding sites in 132 unique proteins were identified, demonstrating the utility of FSBA–COFRADIC for large-scale mapping of nucleotide-binding sites in complex proteomes.

FSBA derivatives and analogues

The versatility of FSBA in protein studies, together with its relatively simple chemical structure, has made this compound a starting point for the design of its derivatives and analogues (Fig. 9). The use of FSBA derivatives has expanded the scope of targetable proteins as well as the range of applicable research techniques. Structural modifications of FSBA include: (a) change of the nucleobase; (b) introduction of a click handle or biotin into the molecule, enabling activity-based probe profiling; (c) differences in structural isomerism as well as structural analogues; and (d) introduction of radioisotopes, primarily ^{14}C and ^{3}H.

Using different nucleobases. A strategy involving the use of different nucleobases or adenine derivatives has been employed in synthesis of multiple FSBA-inspired affinity probes (Fig. 9, compounds **3–8**). Shortly after introducing FSBA, in 1978 Colman Group synthesized 5′-*p*-fluorosulfonylbenzoyl guanosine (FSBG, **3**).[93] Similarly to FSBA, the novel compound has been widely employed for investigations of proteins possessing affinity toward GTP. Examples of FSBG usage have been summarized in a work by Colman.[58] In the original study researchers showed that FSBG reacts covalently with bovine liver GDH at the allosteric site. The researchers also utilized FSBG in rat liver phosphoenolopyruvate carboxykinase (PEPCK) and rabbit muscle PK investigations. While PEPCK was shown to react with the GTP analog at the active site,[94] FSBG bound to PK at two Cys residues localized in close proximity to the metal-nucleotide binding site.[95] Other notable examples of studies employing FSBG include identification of GTP-binding sites in human platelet membranes[96] or transducing – a G protein that is activated by photoexcited rhodopsin through GDP–GTP exchange and subsequent dissociation of the GTP-bound α-subunit.[97] In a work by Bretner and colleagues, a small collection of 4-fluorosulfonylbenzoyl nucleotide analogs, including FSBA, FSBG, 5′-*p*-fluorosulfonylbenzoyl inosine (FSBI, **7**), as well as the nucleoside analog 5′-*p*-fluorosulfonylbenzoyl ribavirin (FSBR, **8**), was screened against polymerases from WNV and HCV.[76] In the assays with and without enzyme-inhibitor preincubation, among these compounds, FSBI and FSBR displayed the strongest, although still relatively weak, inhibitory activity, with IC_{50} values in the high micromolar range.

Despite their relatively simple chemical structures, FSBA and FSBG enabled proteome-level interrogation of nucleotide-binding proteins in complex biological samples. In a study by the Geahlen Group, FSBA and FSBG were used to covalently label proteins in membrane fractions and whole-cell lysates from DG75, Jurkat, and Lck-deficient Jurkat cell lines.[98] Following labelling and protein blotting, base treatment hydrolysed the nucleoside portion of the affinity label, leaving a covalently attached SB tag on modified proteins that could be subsequently probed with a rabbit anti-SB serum, enabling selective detection of labelled proteins by immunoblotting. Selected immunoreactive proteins were then identified by mass spectrometry, demonstrating the general applicability of nucleoside fluorosulfonylbenzoate derivatives for functional profiling of nucleotide-binding proteins in complex proteomes. An interesting example of FSBA derivatives involving modification of the nucleobase is 5′-*p*-fluorosulfonyIbenzoyl-2-aza-1,N^6-ethenoadenosine (5′-FSBaεA, **4**).[99] This compound exhibits fluorescence, with absorption and emission maxima at 356 nm and 490 nm, respectively. In a study by Craig and Hammes, it was used both as an affinity label and as an energy donor to probe the cAMP-binding site of phosphofructokinase. Another fluorescent FSBA derivative is 5′-*p*-fluorosulfonylbenzoyl-1,N^6-ethenoadenosine (5′-FSBεA, **5**).[64] This nucleotide analog, with excitation and emission maxima at, respectively, 308 nm and 412 nm, enabled spectrofluorimetric quantification of its binding to rabbit muscle PK. The Colman Group designed and synthesized a novel bimodal FSBA derivative, 5′-*p*-fluorosulfonylbenzoyl-8-azidoadenosine (5′-FSBAzA, **6**), which combines a fluorosulfonylbenzoyl covalent labelling moiety with a photoactivatable azido group at the 8-position of adenine that generates a highly reactive nitrene upon UV irradiation.[100] Covalent attachment prior to photoactivation led to increased crosslinking efficiency of the probe to glutamate dehydrogenase (GDH)[101] In a subsequent study, 5′-FSBAzA was employed to map the allosteric nucleotide-binding site of GDH. Tyr190 and Lys143 were identified as sites of covalent modification by the SF group, while the Leu475–Asp476–Leu477–Arg478 region was identified as spatially proximal based on photocrosslinking *via* the azide moiety.[87]

Inhibitors and activity-based probes on the FSBA scaffold. Edema factor (EF), a component of anthrax toxin produced by *Bacillus anthracis*, is a calmodulin-dependent adenylate cyclase toxin that uses ATP to generate cAMP in host cells. Jiao *et al.* employed FSBA as a model scaffold for the rational design and optimization of EF inhibitors.[102] After demonstrating that FSBA covalently modifies EF, a series of structural modifications was introduced to improve inhibitory potency and target engagement. This optimization yielded multiple inhibitors more potent than FSBA. The most potent compound incorporated a 7-deazaadenine recognition motif, a piperidine linker, and an amide bond connecting the linker to the fluorosulfonylbenzoyl warhead (Fig. 9, compound **9**), and exhibited a k_{inact}/K_I value more than 1000-fold higher than that of the reference. In addition, the inhibitor displayed the lowest EC_{50} among the tested compounds in a cellular assay measuring inhibition of intracellular cAMP production in RAW264.7 cells. Pettinger and colleagues optimized the structure of an acrylate derivative of 8-(*p*-chlorobenzyloamino)adenosine as an HSP72 inhibitor.[103] During successive optimization rounds, the largest increase in the k_{inact}/K_I parameter was observed for a compound bearing a fluorosulfonylbenzoyl warhead and an 8-[(quinolin-6-yl)methylamino]adenosine moiety (Fig. 9, compound **10**). Notably, although FSBA was not used as a starting point, the optimized inhibitor architecture converges on key structural features characteristic of FSBA-derived kinase inhibitors, combining an adenosine scaffold with a bulky aromatic substituent at the 8-position and an ASF electrophile at 5′ position.

Activity-based probes (ABPs) are active site reactive probes that provide measurable readout that enables quantification of enzyme activity level. ABPs also enable identification of active enzymes within complex biological matrices.[104,105] Due to FSBA reactivity toward kinases, it has been employed as a scaffold for prototypic kinase-targeting ABPs. Ratcliffe, Yi, and Khandekar synthesized biotinylated derivatives of FSBA by conjugating biotin to hydroxyl groups of the ribose ring, yielding two isomers: 3′-biotinyl-FSBA and 2′-biotinyl-FSBA (Fig. 9, compounds **11** and **12**).[106] Both isomers labelled recombinant ALK5 kinase in an LC/MS experiment and the 3′ isomer **11** was further employed in western blot assays. Western blots with ALK5 and CDK2 revealed that **11** can be used for identification of ATP competitive kinase inhibitors. Hsu and colleagues further developed this concept and synthesized a small collection of four ABPs based on the FSBA scaffold.[107] These probes were equipped with hydrophilic polyethylene glycol (PEG) linker connecting biotin to the ribose at the 2′-OH position and differed in the position of the $-SO_2F$ group (*para*- and *meta*- isomers) as well as the nature of the linkage between the ribose and the fluorosulfonylbenzoyl moiety (ester and amide bonds). In a preliminary characterization, the probes were evaluated for labelling of the tyrosine kinase LCK in comparison with several non-kinase proteins, demonstrating probe selectivity. A probe with an ester bond between the ribose and the *meta*-substituted aryl sulfonyl fluoride (Fig. 9, compound **13**) displayed the most favourable selectivity profile. Subsequently, it was shown to label FAK and Lck (tyrosine kinases) as well as p38 (a serine/threonine kinase), supporting its use as a broad-spectrum kinase activity-based probe. Another example is the work by Gushwa *et al.*, who designed and synthesized two ABPs targeting Src-family tyrosine kinases.[108] The inhibitors' structures were inspired by FSBA. One of the probes incorporated a fluorosulfonylbenzoate electrophile (Fig. 9, compound **14**), whereas the second probe contained a vinylsulfonate warhead. Both probes featured a 2′-propargyl ether moiety on the ribose for CuAAC click chemistry and are based on a 7-deazaadenine scaffold bearing a *p*-tolyl substituent. The SuFEx probe **14** predominantly labelled Src kinase in lysates from ABP-treated, Src-transfected COS-7 cells and was further shown to bind five Src-family kinases (Src, Yes, Lck, Blk, and Fyn) in Jurkat and HeLa cells, enabling their affinity enrichment from complex proteomes. Notably, the ABP binding to Yes, Lck, and Blk was reduced upon pretreatment

with ponatinib, a clinically approved broad-spectrum tyrosine kinase inhibitor, whereas labelling of Src and Fyn was unaffected, demonstrating the utility of fluorosulfonylbenzoyl probe for competitive inhibitor profiling in living cells.

Other SuFEx nucleotide and nucleoside compounds

Despite the important role that FSBA has played in protein research, it is necessary to recognize several limitations associated with its use. First, FSBA lacks a negatively charged triphosphate moiety and therefore is not an optimal ATP analogue, exhibiting relatively low affinity for nucleotide-binding sites in proteins. In addition, it can also function as an ADP analogue, which may complicate data interpretation, particularly in studies of proteins regulated by both ATP and ADP. Another limitation is its susceptibility to hydrolysis of the ester linkage between the sulfonylbenzoyl and the nucleoside moieties, although in some of the studies discussed herein this property was deliberately exploited. FSBA also displays limited aqueous solubility, necessitating the use of organic solvents such as DMSO or DMF, which may adversely affect protein activity and limits its applicability in cell-based assays. Moreover, its generally low affinity for many targets requires relatively high reagent concentrations, increasing the risk of nonspecific labelling. Nevertheless, FSBA has served as an excellent proof-of-concept platform demonstrating the utility of SuFEx chemistry in studies employing nucleotide and nucleoside derivatives. Consequently, there is considerable interest in the development of new reagents in this class that likewise incorporate SuFEx electrophiles in their structures (Fig. 10). On the other hand this area remains largely still emerging.

The Scammells' Group developed a series of novel A1 adenosine receptor (A_1AR) agonists based on an adenosine scaffold modified at the N^6 position, designed as irreversible agonists.[22] The compounds were equipped with different electrophilic warheads, including isothiocyanate, bicyclo[2.2.2]octa-2,5-dien, and fluorosulfonylbenzoate moieties, connected to the adenosine core *via* aliphatic or aromatic linkers (Fig. 10, compound **15**). Initial cellular assays identified several highly efficacious compounds, among which the fluorosulfonylbenzoate derivative with an aromatic linker produced A_1AR responses comparable to the full agonist NECA. In the xCELLigence real-time cellular assay, this compound produced a NECA-level response that persisted after addition of an antagonist, suggesting irreversible receptor engagement. This property was further supported by ^{35}S-GTPγS binding assays. In addition, the fluorosulfonylbenzoate derivative increased the thermal stability of the receptor, as indicated by an elevated melting temperature, highlighting its potential utility for structural biology studies. SuFEx chemistry in combination with nucleotides was applied to create novel tools for kinase-substrate crosslinking. An additional promising application of SuFEx chemistry involves the use of electrophilic warheads to capture enzyme–substrate interactions *via* covalent crosslinking. The Pflum Group developed ATP–aryl fluorosulfate (ATP-AFS), in which a SuFEx electrophile is attached to the γ-phosphate of ATP through a PEG linker (Fig. 10, compound **16**).[36] This design was inspired by earlier kinase-catalyzed photocrosslinking reagents bearing aryl azide and benzophenone moieties developed by the same group.[109,110] Upon kinase-catalyzed transfer of the γ-phosphate to the substrate, the appended AFS is positioned *via* the flexible linker to react with nucleophilic residues on the kinase, resulting in covalent kinase–substrate crosslinking. **16** was shown to induce autocrosslinking of recombinant kinases, including PKA, EGFR, and Src, as well as kinase–substrate crosslinking in cell lysates from HEK293 and RKO cells. In a recent work from the Jemielity Group, SuFEx electrophilic warheads were used for the design of novel cytosolic nucleotidase IIIB (cNIIIB) inhibitors.[20] The inhibitors were GMP derivatives functionalized with fluorosulfonylbenzyl or benzyl fluorosulfate moieties at N^7 nitrogen atom. Inhibitor incubation with the enzyme revealed probable covalent inhibition of the cNIIIB by the *p*-fluorosulfonylbenzyl GMP derivative (Fig. 10, compound **17**). Covalent binding to His110 was further confirmed using mass spectrometry and NMR. This study exemplifies how SuFEx chemistry can be leveraged for the rational design and optimization of inhibitors, enabling the transformation of small-molecule noncovalent inhibitors into covalent counterparts.

A somewhat different application of SuFEx chemistry in nucleoside-based probe design is illustrated by the work of Brighty *et al.*[111] In this study, the authors employed an inverse drug discovery strategy using a library of compounds bearing sulfuramidimidoyl fluoride (SAF) electrophile. The library comprised deoxythymidine derivatives modified at the 3′ position of the deoxyribose sugar and equipped with a propargyl group at the 5′-hydroxyl position. The compound collection was used to identify proteins capable of covalent modification *via* SuFEx reactions. Notably, poly(ADP-ribose) polymerase 1 (PARP1), a clinically validated DNA repair enzyme, was identified as

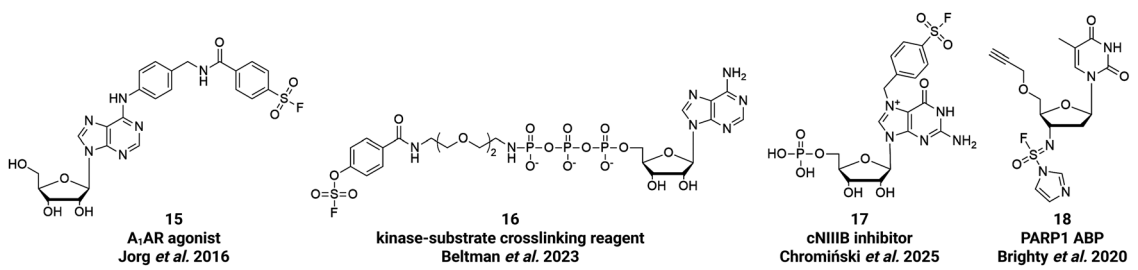

Fig. 10 Nucleotide- and nucleoside-based chemical tools equipped with SuFEx groups. SuFEx chemistry is an emerging platform for the synthesis of novel functionally diverse bioactive molecules.

one of the proteins modified by the deoxythymidine-derived probes. Furthermore, one of these derivatives (Fig. 10, compound **18**) exhibited inhibition of PARP1 in a cellular assay, highlighting its potential as a starting point for further optimization.

Aptamers

The first FDA approval of an aptamer-based therapeutic, pegaptanib (Macugen), occurred more than two decades ago.[112] To date, avacincaptad pegol (Izervay), approved by the FDA in 2023,[113] remains only the second aptamer-based drug to reach the clinic, although it was subsequently withdrawn from use in the European Union in 2024.[114] Nevertheless, a growing number of aptamers are currently undergoing clinical evaluation, underscoring the substantial potential of this class of active pharmaceutical ingredients for clinical translation. As a result, considerable effort has been devoted to strategies aimed at enhancing the therapeutic performance of aptamers. One such approach involves moving beyond traditional reversible binders toward covalent aptamers, incorporating either reactive electrophiles or photoactivatable groups. This transition offers several potential advantages, including prolonged target engagement, reduced dosing requirements, diminished susceptibility to competitive displacement, and increased resistance to nuclease degradation.[14]

SuFEx, as a proximity-driven reaction, is particularly well suited for this application. In recent years, several research groups have incorporated SuFEx moieties into aptamer structures for a variety of purposes, including the development of covalent inhibitors of enzymes and protein–protein interactions (PPI), integration into *in vitro* selection workflows, or targeted protein degradation. In this section we present a current state-of-art for SuFEx-based covalent aptamers.

Enzyme and PPI inhibitors. SuFEx-modified aptamers have enabled the conversion of classical reversible binders into time-dependent covalent inhibitors of enzymes and protein–protein interactions. In these designs, the SuFEx warhead is positioned peripherally on the oligonucleotide scaffold, preserving the native recognition mode and allowing covalent bond formation to occur only after productive binding. In one of the earliest examples, Tabuchi and co-workers converted the thrombin-binding DNA aptamer (TBA)[115] into a covalent inhibitor by installing an ASF at selected thymidine positions (T3 or T12, see section Chemistry/SuFExable oligonucleotides/CuAAC and SPAAC reactions for introducing SuFEx warheads).[40] Guided by the crystal structure of the thrombin–aptamer complex, the electrophile was oriented toward nucleophilic residues in or near the active site. Covalent complex formation resulted in time-dependent inhibition of thrombin activity, with an apparent IC_{50} of 1.2 ± 0.1 nM for the TBA_3 aptamer compared to 3.5 ± 0.1 nM for the noncovalent TBA. Notably, treatment of the aptamer–thrombin complex with a complementary strand antidote diminished inhibition, demonstrating on-demand functional reversibility despite covalent modification.

A complementary strategy was later applied to PPIs. Qin and co-workers[48] developed covalent aptamer inhibitors of the SARS-CoV-2 spike protein receptor-binding domain (RBD) by introducing multiple ASF modifications into two known DNA aptamers 6C3 and A1. These modifications were introduced *via* phosphorothioate sites appended on an 8-thymidine (8T) tail at the aptamers 3′ ends, allowing incorporation of 1, 3, 5, or 7 SuFEx sites for 6C3 and 5 or 7 for A1 (see section Chemistry/SuFExable oligonucleotides/Alkylation of phosphorothioate linkages with benzyl bromides bearing SuFEx warheads). The SuFEx-modified aptamers can form covalent bonds with RBD residues, including Y453 and R408, at the RBD–ACE2 interface. In functional assays, the 6C3-7SF covalent aptamer showed enhanced RBD–ACE2 blocking (lower apparent IC_{50}) and more than 25-fold improvement in pseudovirus neutralization compared with the original aptamer, demonstrating the potential of covalent inhibition to increase potency. This study also shows unique arginine reactivity toward ASF group. The general idea of virus neutralization by covalent aptamers is presented in Fig. 11A.

Taken together, these two studies reveal a common design strategy in which SuFEx warheads are positioned adjacent to the aptamer binding fold and are brought into close proximity with reactive residues, such as tyrosine or other nucleophilic amino acids, at an active site or protein–protein interaction interface upon target binding. Both studies further demonstrate that introduction of covalent reactivity leads to increased potency of the aptamers compared with their non-covalent counterparts.

SuFEx *in vitro* selection. A second major direction has been to integrate SuFEx chemistry directly into aptamer discovery workflows, so that covalent reactivity becomes part of the selection pressure. The Xiang Group[43] exemplified this strategy by proposing a general workflow for discovering potent covalent aptamers using large randomized DNA libraries bearing ASF warheads (Fig. 11B). In this approach, a randomized single-stranded DNA library containing phosphorothioate linkages is first generated and subsequently modified to introduce SuFEx electrophiles (see section Chemistry/SuFExable oligonucleotides/Alkylation of phosphorothioate linkages with benzyl bromides bearing SuFEx warheads). Upon incubation with a protein target, aptamers that both bind and form covalent adducts with the protein are selectively retained under stringent washing and denaturing conditions. The susceptibility of the phosphorothioate linkage to basic cleavage enables recovery of DNA sequence information from protein-bound complexes, followed by PCR amplification. Thus, enrichment is driven not only by binding affinity but also by the ability to form a stable covalent complex. As a proof of concept, the authors identified covalent aptamers targeting the SARS-CoV-2 spike protein that neutralized pseudovirus infection *in vitro*, and further applied the strategy to discover covalent aptamer inhibitors of human complement component C5, demonstrating the versatility of the approach. Within this framework, aptamers against therapeutically relevant proteins are enriched based on their covalent capture efficiency rather than purely equilibrium affinity. By designing the selection so that SuFEx-based covalent capture is required for retention, these library

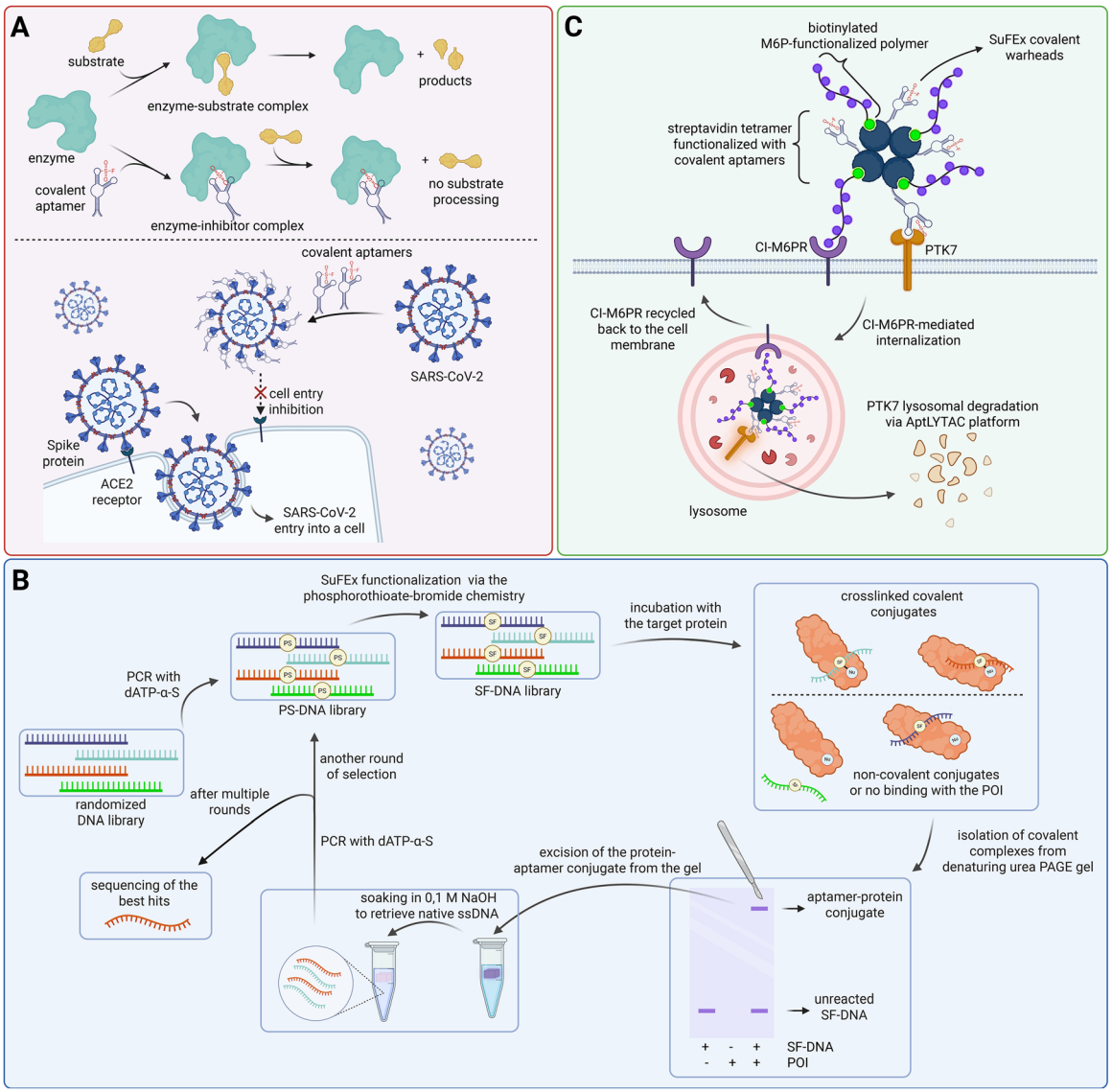

Fig. 11 Examples of applications of SuFEx-modified covalent aptamers. (A) Covalent aptamer as an enzyme inhibitor and virus cell-entry inhibitor; (B) SuFEx covalent aptamer *in vitro* selection (adapted from ref. 43); (C) Targeted protein degradation exemplified by the AptLYTAC platform for selective degradation of membrane proteins (adapted from ref. 44). Created with BioRender.com.

approaches implicitly privilege sequences that position the warhead in a productive geometry, providing a discovery route that is complementary to the more rational, structure-guided designs discussed above.

In a more recent work from the same group,[47] the authors explored the use of less reactive SuFEx electrophiles to develop a workflow for identifying highly target-selective covalent aptamers. The study employed a series of AFS warheads, including "bare" aryl fluorosulfate (bAFS), fluorine-substituted AFS (fAFS), and carboxamide-substituted AFS (cAFS), to systematically tune electrophile reactivity. Compared to ASF, AFS warheads exhibit greater resistance to hydrolysis and reduced nonspecific reactivity under physiological conditions. The proposed *in vitro* selection workflow involves: (1) preparation of a phosphorothioate-modified DNA (PS-DNA) library; (2) conversion to an AFS-DNA library *via* reaction with AFS-substituted benzyl bromides (see section Chemistry/SuFExable oligonucleotides/Alkylation of phosphorothioate linkages with benzyl bromides bearing SuFEx warheads); (3) incubation with the protein target followed by SDS-PAGE isolation of covalent aptamer–protein conjugates; and (4) recovery of aptamer sequences through alkaline cleavage of PS linkages and PCR amplification. This strategy was applied to the extracellular domain of human epidermal growth factor receptor 3 (HER3-ECD) and vascular endothelial growth factor 165 (VEGF165). From the selection, fAFS-Seq1-HER3 was identified as the best hit. The covalent reaction with HER3-ECD proceeded rapidly, reaching half-maximal labelling within approximately 1 min. In contrast, the corresponding PS-Seq1-HER3 aptamer lacking the fAFS warhead showed no detectable target binding and

adopted a distinct fold, indicating that the fAFS modification is an integral structural element of the aptamer–protein interface and is essential for both productive binding and proximity-driven SuFEx cross-linking. The identified covalent aptamers were further shown to inhibit the HER3–EGFR and VEGF–VEGFR protein–protein interactions, demonstrating the utility of reactivity-tuned SuFEx chemistry for the discovery of functional covalent aptamers.

In a study by Bian *et al.*, covalent cross-linking chemistry was directly incorporated into the selection process to identify cross-linking aptamers for the membrane protein labelling.[39] This strategy ensures that the resulting aptamers are not only high-affinity binders but are also geometrically positioned to form stable covalent bonds with their targets. In this work, DNA libraries bearing a defined sulfonyl fluoride warhead at the 3′ terminus (see section Chemistry/SuFExable oligonucleotides/CuAAC and SPAAC reactions for introducing SuFEx warheads), were subjected to iterative rounds of selection against recombinant proteins followed by selection using cell-surface proteins, thereby enriching sequences capable of efficient covalent capture. In contrast to phosphorothioate or internally modified aptamer strategies, this design preserves full nucleobase diversity. The selected aptamers showed sustained, wash-resistant target labelling and durable cellular signals compared with noncovalent counterparts, reflecting evolutionary optimization of aptamer folding and binding geometry that enables efficient proximity-driven SuFEx cross-linking.

A unique example of SuFEx-assisted covalent aptamer selection is the study by Taki *et al.*, who introduced a novel approach termed ARCaDia.[42] In this work, the authors propose a workflow based on single-round selection, eliminating the need for multiple iterative enrichment cycles characteristic for conventional SELEX. A library of 25-nt randomized DNA sequences containing a single 5-octadiynyl-dU residue was generated. The library was subsequently functionalized *via* CuAAC click chemistry with AFS and SF warheads, as well as a control nonreactive benzyl group. To minimize nonspecific interactions, a preselection step using BSA-functionalized beads was performed prior to incubation with the protein of interest (thrombin). The remaining library was then incubated with thrombin immobilized on nanobeads and subjected to high-stringency, denaturing washing conditions to retain only covalently bound sequences. Following recovery and PCR amplification, only the AFS modified library was selected for further analysis. Enriched sequences were analysed by high-throughput sequencing (HTS) and bioinformatic processing using AptaSuite and Tallymer, enabling *k*-mer-based motif analysis. Rather than focusing on the most abundant full-length sequences, the authors identified enriched sequence motifs and positional trends. This analysis revealed a dominant 15-nt motif forming a G-quadruplex structure closely resembling the thrombin-binding aptamer (TBA), as well as a strong preference for warhead placement at position 4 of the sequence. The leading candidate, AFS-TBA_4, was experimentally validated and shown to form a highly specific covalent complex with thrombin, with no detectable covalent modification of human serum albumin under the tested conditions. Collectively, this study demonstrates that single-round covalent aptamer screening combined with motif-based HTS analysis enables simultaneous identification of both binding sequence and optimal warhead positioning, providing an alternative to conventional trial-and-error strategies in covalent aptamer design.

Degraders of proteins. A newer but rapidly growing application area is the use of SuFEx-modified aptamers as components of targeted protein degradation systems. Here, the covalent bond is used to prolong the residence time of the aptamer on its target, thereby stabilising ternary complexes that recruit cellular degradation machinery.

Shi and co-workers[46] engineered a new class of membrane protein degraders termed covalent aptamer-based autophagosome-tethering chimeras (CApTEC), designed to induce autophagic degradation of membrane proteins. As proof of concept, two cancer-associated membrane proteins with established DNA aptamers were selected: transferrin receptor 1 (TfR1) and nucleolin (NCL). For TfR1, molecular docking analysis of the XQ-2d aptamer (termed Ap_{TfR1} in the study) was combined with experimental screening to identify backbone positions suitable for SF installation. Among four candidate sites, positions 37 and 56 were selected based on SDS-PAGE crosslinking assays, and dual modification yielded the optimised covalent aptamer CAp_{TfR1}. Under the tested conditions, CAp_{TfR1} exhibited minimal crosslinking to off-target proteins such as BSA and HSA and selectively labelled live HeLa cells when fluorescently tagged. To generate a functional degrader, the LC3-binding ligand 5,7-dihydroxy-4-phenylcoumarin (DP) was conjugated to the 5′ terminus of CAp_{TfR1}, affording the covalent aptamer chimera $CApTEC_{TfR1}$. Cellular imaging and colocalization studies demonstrated that $CApTEC_{TfR1}$ trafficked through early and recycling endosomes and efficiently recruited LC3, consistent with formation of a TfR1–aptamer–LC3 ternary complex. Subsequent biochemical and imaging experiments confirmed robust, time- and dose-dependent degradation of cell-surface TfR1 *via* the autophagy–lysosomal pathway. This strategy was successfully extended to NCL, for which a covalent aptamer chimera ($CApTEC_{NCL}$) was designed using the AS1411 aptamer, demonstrating comparable crosslinking efficiency and targeted degradation, thereby highlighting the modularity of the approach. Finally, $CApTEC_{TfR1}$ exhibited pronounced synergistic antitumor effects in combination with 5-fluorouracil (5-FU), both *in vitro* in HeLa cells and *in vivo* in a HeLa xenograft mouse model, resulting in approximately 93% reduction in tumor volume and 88% reduction in tumor weight. Collectively, this work represents the first demonstration of SuFEx-enabled covalent aptamer chimeras driving autophagic degradation of endogenous membrane proteins and establishes covalent aptamers as a viable modality for targeted protein degradation.

The Tan Group further explored the targeted-protein degradation *via* covalent aptamers by introducing the concept of ''double covalent aptamers'', which combine a SuFEx-based aryl sulfonyl fluoride warhead with a photoaffinity diazirine tag.[44] First, structural *in silico* analyses were employed to

identify the most promising modification sites within the known sgc8c aptamer that targets protein tyrosine kinase 7 (PTK7). In an initial optimization step, deoxyuridine derivatives functionalized at the 5-position with a trifluoromethyl phenyl diazirine (U^{diaz}) were systematically introduced at different sites within the aptamer. Screening of these variants led to the identification of several optimised constructs, including $3U^{diaz}$, $3U^{diaz}\Delta$, and $5U^{diaz}$, with $3U^{diaz}\Delta$ displaying the highest photocrosslinking efficiency. In a subsequent step, optimal positions for ASF installation were determined, enabling the design of a dual-labelled aptamer bearing both SuFEx and diazirine functionalities. This double covalent aptamer outperformed both single-modified variant and non-covalent variant. To assess biological activity, the dual covalent sgc8c aptamer (DC-sgc8c) was incorporated into an AptLYTAC platform *via* streptavidin-mediated assembly with a biotinylated mannose-6-phosphate polymer (bpM6P), enabling simultaneous engagement of PTK7 and the cation-independent mannose-6-phosphate receptor (CI-M6PR) and promoting lysosomal degradation (Fig. 11C). DC-sgc8c–based AptLYTACs induced more efficient and sustained membrane-bound PTK7 degradation than their non-covalent counterparts. Moreover, NK cells functionalized with membrane-anchored dual covalent aptamers exhibited enhanced cytotoxicity, highlighting the versatility of this multivalent covalent aptamer design.

Other emerging uses and design principles. Beyond inhibition, SuFEx *in vitro* selection, and targeted protein degradation assays, SuFEx-modified aptamers have been evaluated as potential reagents for constructing aptamer-based analytical and diagnostic platforms, where irreversible capture and high tolerance to stringent washing conditions are highly desirable. The Tan Group[41] employed previously reported DNA aptamers targeting the SARS-CoV-2 nucleocapsid protein (NP) and receptor-binding domain (RBD) and functionalized them with different electrophilic warheads, including ASF, *N*-hydroxysuccinimide (NHS) ester, and acrylamide (Acr). Initial reducing SDS-PAGE experiments revealed that ASF-modified aptamers exhibited the highest level of off-target labelling toward human serum albumin (HSA) under the tested conditions. At a 1 : 10 aptamer-to-HSA ratio, the off-target crosslinking efficiency was approximately 9% for the ASF warhead, whereas no detectable binding was observed for the NHS- and Acr-modified aptamers. Consistent with its higher intrinsic reactivity, the ASF warhead also displayed the fastest reaction kinetics among the tested electrophiles. However, the difference in target-binding kinetics between ASF- and NHS-modified aptamers was relatively modest. At a 1 : 6 NP-to-aptamer ratio, the time required to reach half-maximal crosslinking ($t_{1/2}$) was 10.2 min for ASF-NApt, 11.2 min for NHS-NApt, and 200.6 min for Acr-NApt. At a 1 : 2 ratio, the corresponding $t_{1/2}$ values were 12.7 min, 22.8 min, and >240 min, respectively. Taken together, these results led the authors to prioritize the NHS warhead for further platform development, owing to its comparable target-binding kinetics combined with a reduced propensity for off-target labelling under the examined conditions. Importantly, these findings indicate that while SuFEx electrophiles offer rapid and durable covalent capture, their application in aptamer-based systems may require careful optimization and off-target profiling to balance reactivity and selectivity.

In summary, the biological studies to date show that SuFEx-modified aptamers can be deployed in a wide variety of contexts, yet they all exploit the same core principle: DNA scaffolds are used to pre-organize a weakly reactive electrophile within a precise three-dimensional environment, such that a slow but highly selective covalent step converts a transient recognition event into a durable biological effect. Importantly, several studies further suggest that the SuFEx moiety itself can participate in molecular recognition, in some cases contributing directly to the aptamer–protein interface and influencing binding affinity. At the same time, comparative analyses using AFS warheads demonstrate that intrinsic electrophile reactivity is often secondary to accurate spatial alignment of the warhead relative to a reactive amino acid residue, as even comparatively less reactive SuFEx species can undergo rapid covalent bond formation when properly positioned.

Outlooks and perspectives

Taken together, the literature reviewed here shows that sulfur(VI) fluoride exchange chemistry is a highly promising but still early-stage platform for modifying nucleosides, nucleotides, and nucleic acids. Notably, the most advanced examples of SuFEx chemistry applied to nucleic acids have largely emerged within the past three years. This strong recency underscores that the field is still in its formative, method-building phase, while also highlighting the rapid acceleration and clear momentum toward more sophisticated nucleic acid applications. Progress is currently limited by a shortage of strictly methodological studies that develop general, transferable approaches for installing sulfur(VI)–F electrophiles on nucleoside/nucleotide scaffolds; yet such foundational chemistry is precisely what enables broader adoption and accelerates downstream applications. At present, incorporation of sulfur(VI)–F motifs into oligonucleotides remains largely constrained to post-synthetic modification because suitable solid-phase-compatible building blocks are scarce and warhead stability under standard coupling and deprotection conditions is not broadly established. Equivalent limitations exist for enzymatic workflows: there are no widely adopted deoxynucleotide or ribonucleotide triphosphates that encode sulfur(VI)–F functionality for PCR or *in vitro* transcription, which likely contributes to the near absence of RNA constructs that inherently bear SuFEx-type electrophiles. Beyond access, design remains a bottleneck: across covalent aptamer and related studies, productive covalent bond formation depends strongly on three-dimensional pre-organisation and microenvironmental activation, meaning that general rules for warhead positioning are still underdeveloped and many constructs converge on architectures dictated by what is synthetically accessible (termini, selected backbone sites, appended tails). However, in recent works, selection of the best binders follows the library modification with SuFEx groups, which enables direct participation of the SuFEx

moieties in target recognition, making them integral components of the aptamer–protein binding interface and equally important as canonical nucleobases for optimal aptamer recognition and affinity.[39,42,43,47] The frequent use of multivalent designs—multiple warheads distributed along a backbone or clustered within a tail—highlights a practical response to this uncertainty, but systematic principles for when multivalency is required and how best to implement it are still emerging.

Looking forward, the field could accelerate substantially if these limitations are addressed through dedicated methods development. In our opinion, key priorities include creating solid-phase-compatible strategies for incorporating sulfur(VI)–F motifs (*via* properly designed building blocks, masked electrophiles that can be unmasked under mild conditions, or robust on-resin late-stage transformations, application of non-obvious protecting groups and/or tailored solid supports), establishing RNA-specific installation methods that account for the 2′-hydroxyl and RNA handling constraints, and developing enzyme-compatible triphosphates that either bear sulfur(VI)–F groups directly or incorporate stable precursors convertible to sulfur(VI)–F electrophiles after polymerase synthesis. Another underexplored opportunity lies in the broader adoption of aryl fluorosulfates (AFS) as covalent warheads. While generally regarded as less reactive than sulfonyl fluorides, emerging evidence from aptamer-based systems (and other contexts, such as proteins) indicates that AFS can achieve comparable on-target reactivity when properly positioned within a preorganized binding interface.[47] Importantly, their lower intrinsic electrophilicity translates into improved stability and reduced off-target modification, suggesting that AFS may be particularly well suited for proximity-driven covalent strategies. As structure-guided design principles mature, warheads such as AFS—whose reactivity can be strongly amplified by optimal spatial arrangement—are likely to play an increasingly prominent role in SuFEx-enabled chemical biology. Expanding the reagent toolbox will also matter: broader adoption of iminosulfur oxydifluoride derivatives could be enabled by practical, stable ''equivalents'' of thionyl tetrafluoride analogous to recent advances for sulfuryl fluoride, and newer exchange manifolds such as sulfur–triazole exchange (SuTEx)[116] or phosphorus fluoride exchange (PFEx)[117,118] may provide alternative entry points with improved synthesis compatibility or tunable reactivity. These developments are particularly consequential for small nucleotide/nucleotide-like covalent inhibitors, where nucleotide-binding pockets offer an inherently preorganized environment for proximity induced chemistry and where progress may be driven by identifying reliable attachment sites on nucleoside/nucleotide scaffolds, optimizing linker type and geometry and warhead class to match the binding-site microenvironment. In this context, sulfur(VI)–F electrophiles offer a realistic route to covalent engagement of non-cysteine residues that are common in nucleotide-binding sites, and they enable a pragmatic reversible-to-covalent progression starting from established nucleotide binders. An additional and often underappreciated limitation is the narrow structural diversity of SuFEx warheads currently in use. ASFs and AFSs dominate the field, largely by historical precedence and reagent availability rather than optimal fit for nucleic acid or nucleotide-derived scaffolds. Recent work demonstrates that alternative, non-aryl SuFEx architectures can be viable,[119] underscoring the need to systematically explore, characterize, and validate new sulfur(VI)–F electrophile designs beyond the aromatic paradigm. Overall, once programmable incorporation strategies, broader building-block availability, and clearer warhead-positioning principles are in place, sulfur(VI)-fluoride chemistry should become a far more general and enabling tool for covalent probes and inhibitors across both nucleic acid and nucleotide binding protein systems, where geometry often matters more than bulk electrophilicity.

Conflicts of interest

There are no conflicts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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