Applied Research

PERSPECTIVE OPEN ACCESS

Accelerating Drug Discovery With High-Throughput Crystallographic Fragment Screening and Structural Enablement

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Received: 6 August 2024 | **Revised:** 27 September 2024 | **Accepted:** 22 October 2024

ABSTRACT

Fragment-based drug discovery is a well-established method for the identification of chemical starting points for development into clinical candidates. Historically, crystallographic fragment screening was perceived to be low-throughput and time consuming. However, thanks to advances in synchrotron capabilities and the introduction of dedicated facilities, such as the XChem platform at Diamond Light Source, there have been substantial improvements in throughput and integration between sample preparation, data collection and hit identification. Herein we share our experiences of establishing a crystallographic fragment screening facility, our learnings from operating a user programme for ten years and our perspective on applying structural enablement to rapidly progress initial fragment hits to lead-like molecules.

1 | Introduction

Since its emergence over 25 years ago, fragment-based drug discovery (FBDD) has become a pivotal technique in the identification of promising starting points for drug development [1–3]. Unlike traditional high-throughput screening (HTS), which tests large libraries of lead or drug-like molecules to identify potent hits that can be optimised using well-established medicinal chemistry processes, FBDD involves the identification of low molecular weight small molecules (fragments) that bind to a target protein. These fragments are then optimised into potent and selective clinical candidates. FBDD offers unique advantages over HTS, including more efficient sampling of chemical space and detailed mapping of molecular interactions [1]. Although developing potent inhibitors from fragments often requires innovative medicinal chemistry approaches, and robust biophysical and biochemical assays, this can lead to the development of superior drug candidates [1].

A primary method for fragment screening is X-ray crystallography due to its high sensitivity and information rich readout, with an ability to directly yield structural information relating to molecular interactions and protein dynamics, which is not directly available with other biophysical techniques [4–9]. This enables the identification of putative allosteric binding sites and cryptic pockets, facilitates the immediate elimination of artefactual binding of fragments away from the site of

interest, and prevents discarding authentic binders as false positives [7, 9, 10].

Crystallographic fragment screening (CFS) has historically been underutilised due to perceptions that it is tedious, low-throughput and requires considerable time investment in comparison to other biophysical fragment screening methods such as nuclear magnetic resonance (NMR) and surface plasmon resonance (SPR) [4, 7, 8]. However, fragment chemical space can be effectively sampled with as few as 1000 fragments and advances in the throughput and automation of X-ray data collection and analysis has made collection of such data sets routine [7, 8, 11–16]. Therefore, screening by X-ray is an effective and complementary technique to other fragment screening methodologies, HTS and DNA encoded library (DEL) screening, that augments the initial pool of starting points for hit-to-lead development [8, 17, 18].

Furthermore, combining structural enablement with HTS and other biophysical fragment screening approaches can provide a more comprehensive understanding of ligand binding and enhance the overall success rate of drug discovery projects. A recent perspective of successful fragment-to-lead campaigns published between 2015 and 2022 attributed X-ray screening as the primary fragment hit identification technique in only 8% of case studies in 2022 [19]. However, 83% of campaigns subsequently generated a structure for the fragment hit, 78% generated a structure for the lead compound, and 100% of entries used structural information during lead optimisation. Similarly, an independent analysis of successful hit-to-clinical candidate pairs published in the Journal of Medicinal Chemistry between 2018 and 2021 reported that 65% of campaigns reported the use of structure-based drug design as a "key enabling technology" [20]; this increased to 100% when starting with fragment hits. These case-studies illustrate that structural enablement is critical for efficient hit-to-lead and lead optimisation, and therefore developing the required tools for crystallographic fragment screening at the conception of a project can prove advantageous in the latter stages.

This aligns with our own experiences from the COVID Moonshot, a large spontaneous collaboration with the collective mission to leverage open science principles to develop potent, orally bioavailable inhibitors of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Main protease (Mpro) with equitable access, which emerged directly from the crystallographic fragment screen performed at XChem in February 2020 [21–23]. In this project, high-throughput structural biology was used to solve more than five hundred liganded complexes of Mpro, helping to deliver preclinical candidates from a crystallographic fragment screen in under 2 years (Figure 1) [21, 22].

2 | Crystallographic Fragment Screening at Synchrotron Facilities

Fragment-based drug discovery has been revolutionised by rapid advancements in synchrotron capabilities and the rise of high-throughput, automated platforms. Notable examples include the XChem facility at Diamond Light Source, as well as other advanced platforms at synchrotrons such as the F2X facility at BESSY II in Germany, the Fast Fragment and Compound Screening (FFCS) platform at the Swiss Light Source, the HTX lab at ESRF/EMBL in France and FragMAX at MAXIV synchrotron in Sweden [8, 13, 14, 16, 24–26]. These advancements have not only increased the efficiency and speed of the crystallographic screening process but also enhanced the quality of the data obtained, thereby accelerating the development of new drugs for clinical use. Key improvements that have enabled entire screening campaigns to be conducted in a matter of weeks include:

- Integration and automation of sample preparation. The development of high-throughput crystal soaking and harvesting platforms for fragment screening dramatically reduce the manual effort and time required to prepare samples while ensuring data integrity throughout the process [13, 14, 16, 25, 27, 28].
- Enhanced throughput in data collection and processing. Order of magnitude improvements in robotics, automation, detector technology, computing power, and pipelines for data processing mean that complete diffraction data sets can be measured completely unattended in seconds [29–31].
- Efficient hit identification and contextualisation. Improved software tools and computational methods for analysing entire crystallographic fragment screening campaigns have been developed. These tools, such as the PanDDA 2 algorithm and XChemAlign, facilitate the rapid identification and placement of fragment hits, from which crucial insights into their binding modes can be deduced [15, 32–36].

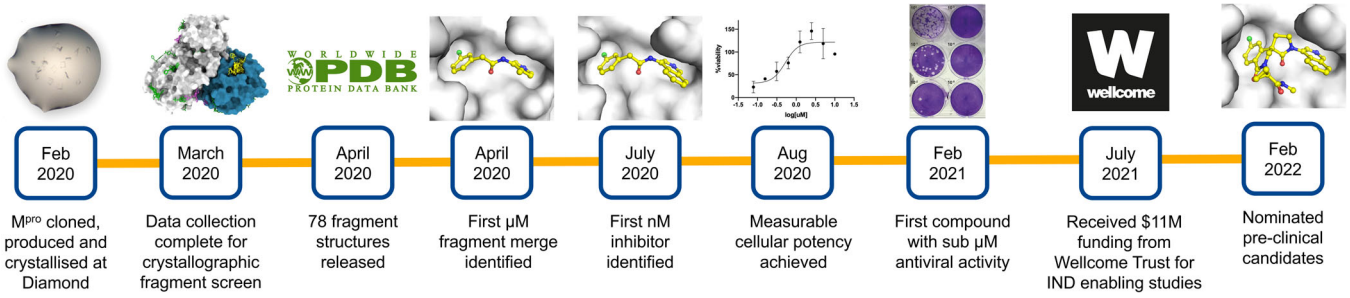

FIGURE 1 | Progress of COVID Moonshot from crystallographic fragment screen to nomination of preclinical candidates, supported by the elucidation of over 500 liganded X-ray structures.

The XChem platform at Diamond Light Source was a pioneer in integrating the various steps of the crystallographic fragment screening process at a synchrotron facility. The XChem laboratory is colocated with its associated beamline, I04-1, directly on the synchrotron experimental floor, and makes extensive use of the Crystallisation Facility at the Research Complex at Harwell for initial crystal sample preparation, although users also have the option of crystallising at "home" and bringing crystal trays on-site.

XChem welcomed its first collaborative users in 2014 and formally established a peer-reviewed user programme in 2015. Calls for academic access proposals are issued twice a year with an independent panel of industry and academic fragment screening experts evaluating projects on technical feasibility, scientific merit, and the users' credentials in fragment progression [37]. These criteria have carefully evolved over several years, based on feedback from experienced drug discovery crystallographers, to maximise the impact of approved projects [38].

Initially, a significant challenge for the platform, and its users, was establishing crystallisation systems which were sufficiently robust, reproducible, and well-diffracting, as these are the key limitations of crystallographic fragment screening. Subsequently, we have extensively documented the challenges and formalised the onboarding process for user experiments to defray the risks and ensure users are aware of mitigations involved [13, 37, 39, 40]. Every experiment is allocated a dedicated local contact to facilitate a successful screening experiment, all the way from sample preparation to data dissemination.

Since establishing the platform, XChem has performed nearly 300 academic user and grant-funded experiments, collecting more than 240,000 data sets (Figure 2), in parallel to a substantial and highly successful industrial user programme that has completed XChem campaigns against 150 targets as of August 2024. As a result, XChem has contributed significantly to the global repository of protein structures, depositing over 3750 models in the protein data bank (PDB), facilitating further research and drug discovery efforts and technological developments [41–45].

The efficacy of fragment-based methods facilitated by platforms like XChem is well illustrated by its role in the discovery of inhibitors targeting Notum, a carboxylesterase involved in the Wnt signalling pathway [46]. Crystallographic screening at the XChem facility identified 60 fragment hits targeting Notum. From these hits, two fragments were selected as highly promising starting points for a drug discovery campaign and hit-to-lead development culminated in a potent (nanomolar), selective, and brain-penetrant inhibitor of Notum activity. Furthermore, this inhibitor proved suitable for oral dosing in rodent models of disease.

Owing to the maturity and resilience of the XChem platform, and the dedicated team operating it, when the coronavirus pandemic was declared in 2020, the facility was well poised to turn its attention to screening SARS-CoV-2 viral proteins in collaboration with our international colleagues and collaborators (Figure 3) [21, 47–50]. Despite a temporary suspension of the XChem user programme due to multiple lockdowns in the UK preventing users from accessing their own laboratories and travelling to Diamond, XChem operations continued uninterrupted, and we contributed to eight distinct crystallographic fragment screens targeting nine SARS-CoV-2 proteins/protein complexes over 2 years.

3 | Lessons From the XChem User Programme and Antiviral Research

With 10 years of experience in crystallographic fragment screening, we have delineated the key characteristics of an ideal "XChem ready" crystal system. These crystals are typically chunky rather than needle-like, do not adhere to the plate, and avoid forming a skin on the drop. They grow reproducibly, with over 50% of drops yielding crystals in required plate formats. Additionally, they consistently

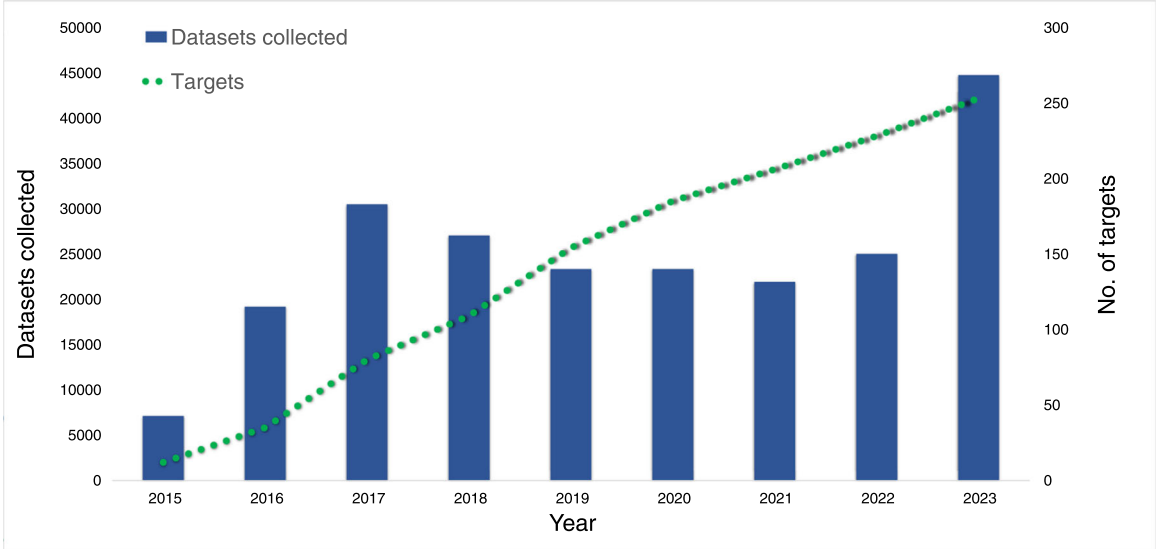

FIGURE 2 | Throughput of academic and grant-funded projects at the XChem facility.

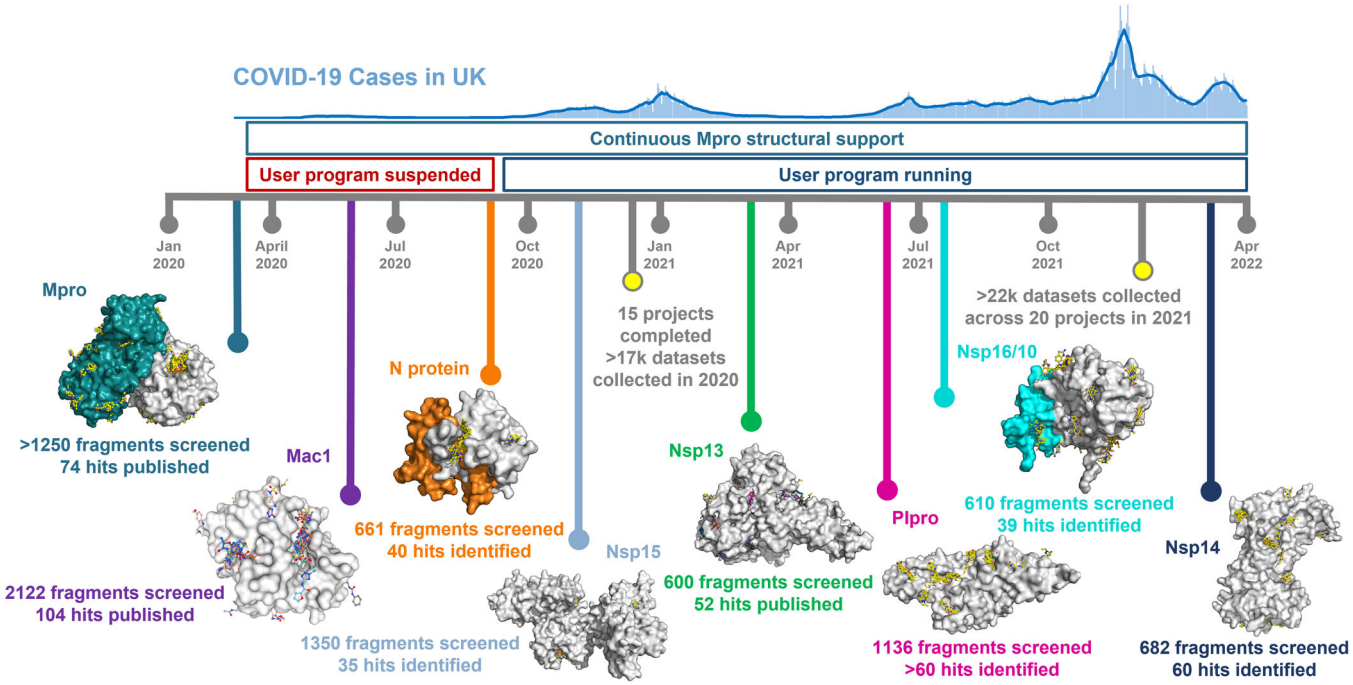

FIGURE 3 | Timeline of crystallographic fragment screens targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) proteins performed at XChem.

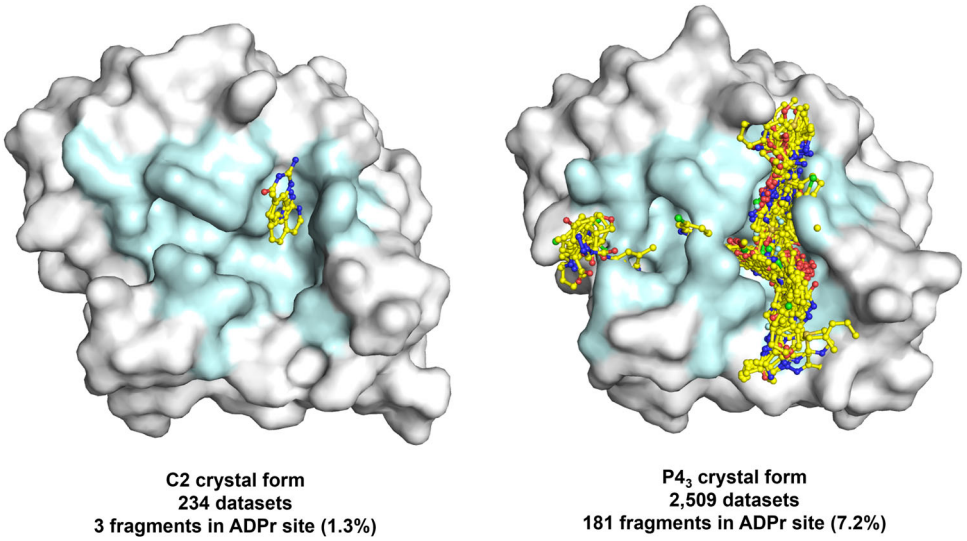

FIGURE 4 | Results from crystallographic fragment screen of two different crystal forms of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Nsp3 macrodomain highlights the importance of finding the "right" crystal form [50]. While both crystal forms had comparable resolution, a higher hit rate was observed in the adenosine diphosphate ribose (ADPr) binding site (surface coloured in pale cyan with fragments shown as yellow sticks) in the $P4_3$ crystal form in comparison to the C2 crystal form.

diffract to high resolution (better than 2.5 Å), tolerate high organic solvent concentrations (e.g., dimethyl sulfoxide or ethylene glycol, due to availability of fragment libraries at high concentration in such solvents), and do not require complicated cryoprotection [13, 27]. While many of these factors do not preclude the successful completion of a screen, mitigating them early in the process can dramatically alleviate potential issues downstream. The practicalities of generating crystallographic fragment screen ready crystals have been discussed elsewhere [13, 14, 16, 40] and are here repeated to emphasise their value:

- Exploring multiple protein constructs to find the "right" crystal form [50] and considering surrogate systems [51–53], but ensure you are working with a biologically relevant protein construct (Figure 4). Construct design is relatively inexpensive, but the time and effort required afterward is not, so it is important to avoid sunk costs.

- Performing quality control for all your protein batches and crystal trays. Determine life span of crystals and when a robust system is established, keep things consistent.

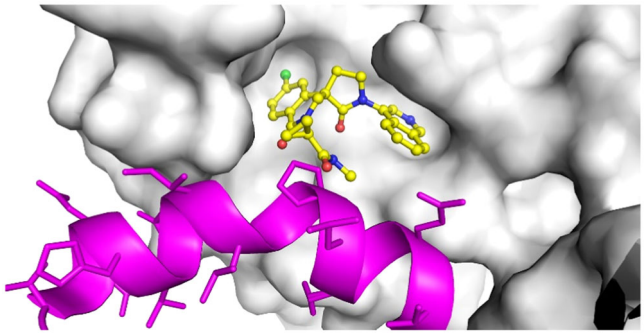

FIGURE 5 | Identification of an alternate crystal form ($P2_12_12_1$) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Mpro proved essential to structurally enable the COVID Moonshot lead series due to crystal packing occluding ligand binding in the previous crystal form. Helix formed by residues 242-257 in symmetry-related molecule from C2 crystal form shown in magenta with COVID Moonshot clinical candidate DNDI-6510 shown in yellow.

- Identifying multiple crystal forms for the apo-protein. Although a crystal system may appear suitable for initial fragment screening, alternate crystal forms may be critical for effective hit-to-lead development (Figure 5) [21, 22, 50, 54].
- Preparing and optimising seed stocks, even when initial crystallisation seems robust. Seeding decouples nucleation from crystal growth, can improve crystal quality and reproducibility, reduce crystallisation time, and help identify more useful crystal forms [13, 14, 39, 50]. This can be critical in transferring protocols between facilities and protein/buffer batches.
- Confirming that crystal packing and/or crystallisation artefacts do not occlude ligand binding. Validate the "soakability" of your site of interest with tool compounds wherever possible.

The COVID Moonshot consortium brought together a global network of medicinal chemists, computational chemists, biochemists, virologists, clinicians, and structural biologists. that sought to rapidly turn the promising initial findings from our crystallographic fragment screen into effective treatments for coronavirus disease 2019 (COVID-19) [21–23]. Consequently, the consortium's efforts identified various learnings that can be applied to similar fragment-to-lead campaigns, potentially benefiting future drug discovery endeavours. One important lesson was the necessity of selecting an appropriate crystal form to test our specific hypothesis as the choice of crystal form can significantly influence the outcome of an experiment. Although the monoclinic crystal form utilised for our crystallographic fragment screen of SARS-CoV-2 Mpro yielded a large number of hits with good coverage of the active site, to continuously provide structural support for the Moonshot's lead series, we had to identify an alternative, orthorhombic, crystal form which would accommodate larger molecules whose binding was sterically occluded by the crystal packing in the initial crystal form (Figure 5). This crystal form was established by cross-seeding with crystals of an "immature" Mpro protein construct with additional N-terminal residues that occupied the enzyme active site [55]. Independently, scientists at the Swiss Light Source performed a crystallographic fragment screen with the same orthorhombic crystal form and identified fragments which bind with a pose that was incompatible with the monoclinic crystal form [54].

3.1 | Fragment Progression Is the Rate Determining Step

In our experience, the rate-determining step in FBDD is advancing fragment hits to on-scale potency, not fragment screening. However, expanding the size and scope of fragment screens can create more opportunities for hit-to-lead progression by increasing the diversity of chemical space explored and improving the chances of identifying promising candidates for further development. A recent poll by the popular drug discovery blog, Practical Fragments, identified that the most common fragment library size in 2023 was between 1000 and 2000 compounds [56]. Conversely, crystallographic fragment screens performed at XChem and elsewhere have historically encompassed fewer than 1000 compounds [8]. Due to the advances in synchrotron throughput described previously and the significant global threat posed by Covid-19, we conducted a larger screen of more than 1250 distinct fragments for SARS-CoV-2 Mpro [21]. This extension enabled the identification of 71 fragments that collectively displayed broad coverage of the Mpro active site, providing substantially more opportunities for fragment merging, and linking. Similarly, independent but complementary crystallographic screens for the SARS-CoV-2 Nsp3 macrodomain performed at the XChem facility and at the University of California San Francisco tested 2593 diverse fragments, identifying 234 binders, again with excellent coverage of the substrate binding site [50].

Additionally, increasing the number of conditions utilised in a crystallographic fragment screen (temperature, buffer composition, solvent concentration, different crystal forms) can identify unique fragment binders or alternative binding conformations which may be productive for hit-to-lead development [16, 54, 57, 58].

3.2 | Real Time Data Dissemination Is Key

One goal before, and throughout, the COVID Moonshot project was to endeavour to disseminate well-annotated structural data as rapidly as feasible. Both to help rationalise structure–activity relationships and allow the rational design of compounds in real time but also to maximise the impact to the wider scientific community.

The wider impact of our rapid dissemination was evidenced when data from our SARS-CoV-2 Mpro fragment screen was utilised by academic researchers developing potent, broad-spectrum coronavirus Mpro inhibitors [42]. Furthermore, structural data from our subsequent hit-to-lead campaign was utilised by Shionogi to define pharmacophore filters for virtual screening of Mpro. This screen led to the discovery of the antiviral Ensitrelvir, which received emergency regulatory approval in Japan in November 2022 [41].

Another metric of impact was the real-time data usage and engagement through our open web platform Fragalysis (Figure 6) [59]. In addition to rapidly publishing preprints describing the details of our fragment screens and hit-to-lead process, and depositing all our structures in the PDB, we provided ligand-centric models of our aligned structures. By capturing the number of instances when any user generated a shareable link (total number of snapshots) from Fragalysis to illustrate a particular protein-ligand interaction or overlay of multiple fragments, we could monitor the real-time usage of the data. This included observable spikes which corresponded to manuscript publications and data usage in training

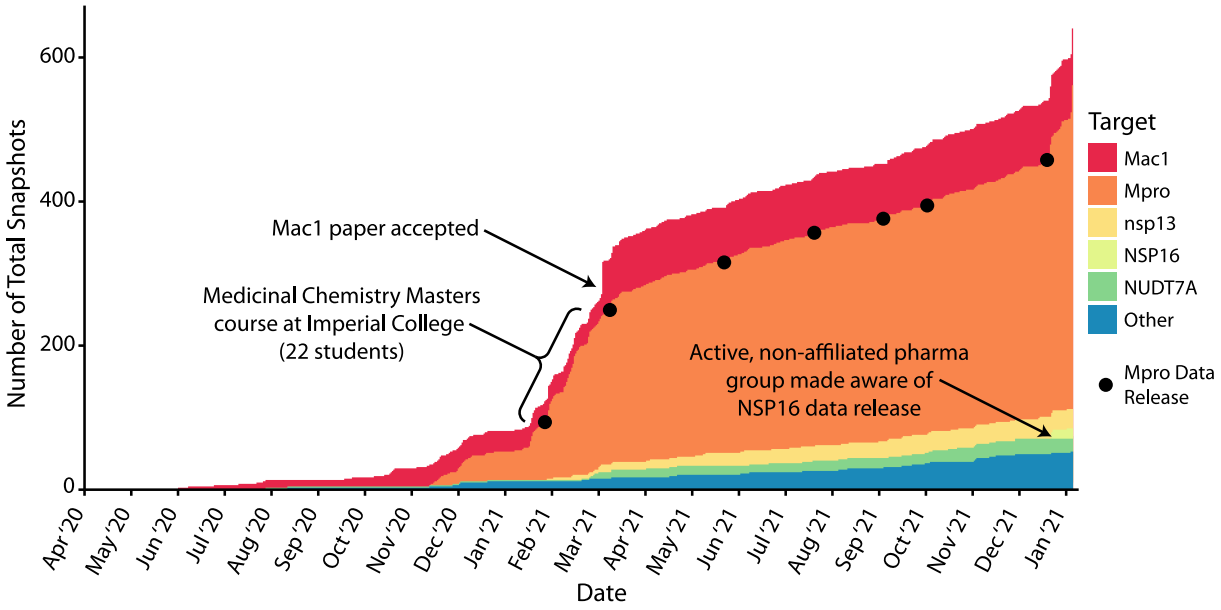

FIGURE 6 | Benefit of real-time open science as illustrated through usage of Fragalysis.

exercises such as the Medicinal Chemistry Masters course at Imperial College, London.

3.3 | Fragment Merging Is the Most Rapid Route to On-Scale Potency

One criticism often levelled at crystallographic fragment screening is that it's extremely high sensitivity results in the identification of weak binders, which can be particularly challenging to develop into potent inhibitors. However, we have demonstrated that it is not worth throwing the baby out with the bathwater, and that even fragments which bind with affinities beyond the detection limits of other biophysical techniques, or that can be directly observed in conventional crystallographic difference maps, can be successfully merged to deliver on-scale potency in a single step (Figure 7) [22, 44, 60]. Furthermore, designing such merges can be accomplished algorithmically and searching commercial catalogues provides a quick and cheap route to follow-up compounds [43, 45]. In our experience by "looking under the lamp posts" and aiming to recapitulate the observed fragment shape and colour (i.e. the specific interactions observed from crystallographic fragment screens) in the initial hit-to-lead development, you can better ensure rapid structural enablement, which in turn improves the efficiency of the early design-make-test (DMT) cycles.

4 | Future of Crystallographic Fragment Screening and Accelerating Fragment-Hit-to-Lead Development

4.1 | Ongoing Innovations at Diamond Light Source

The crystallographic fragment screening landscape is evolving rapidly, with significant reduction in barriers over the past decade. While this technique is now commonly used for primary hit identification, it still faces challenges in routine and efficient fragment hit-to-lead progression. To maximise the impact of these screens, the development of formulaic approaches and integrated pipelines enabling rapid and low-cost fragment progression through iterative DMT cycles is essential. Additionally, increasing the scale of crystallographic fragment screens will provide more opportunities for algorithmic fragment merging and linking.

The Diamond-II project, which will deliver a new synchrotron machine and new beamlines with upgrades to optics, detectors, sample environments, sample delivery capabilities and computing, presents a unique opportunity to shift the scientific scope of crystallographic fragment screening experiments [61]. One of the flagship beamline projects for Diamond-II, K04, promises a significant increase in flux and brilliance, alongside an order-of-magnitude increase in throughput. Building on the success of the XChem programme, the K04 beamline and the associated Automated XChem Lab (AXL) aims to offer an unparalleled structure-based drug design platform with high levels of automation [62]. These improvements are expected to make extremely large fragment screens routine and enable the screening of previously intractable targets, such as membrane proteins and large protein complexes (Figure 8).

Additionally, the development of automated and integrated pipelines for effective room temperature ligand screening represents a significant advancement in structural biology and drug discovery. The VMXi beamline at Diamond Light Source is at the forefront of this innovation, enhancing the identification of biologically relevant fragment binding events and conformations while eliminating artefactual observations associated with cryo-cooling [58, 63, 64]. This will complement the data produced by the K04 beamline, providing a comprehensive and robust platform for fragment screening and structure-based drug design [58].

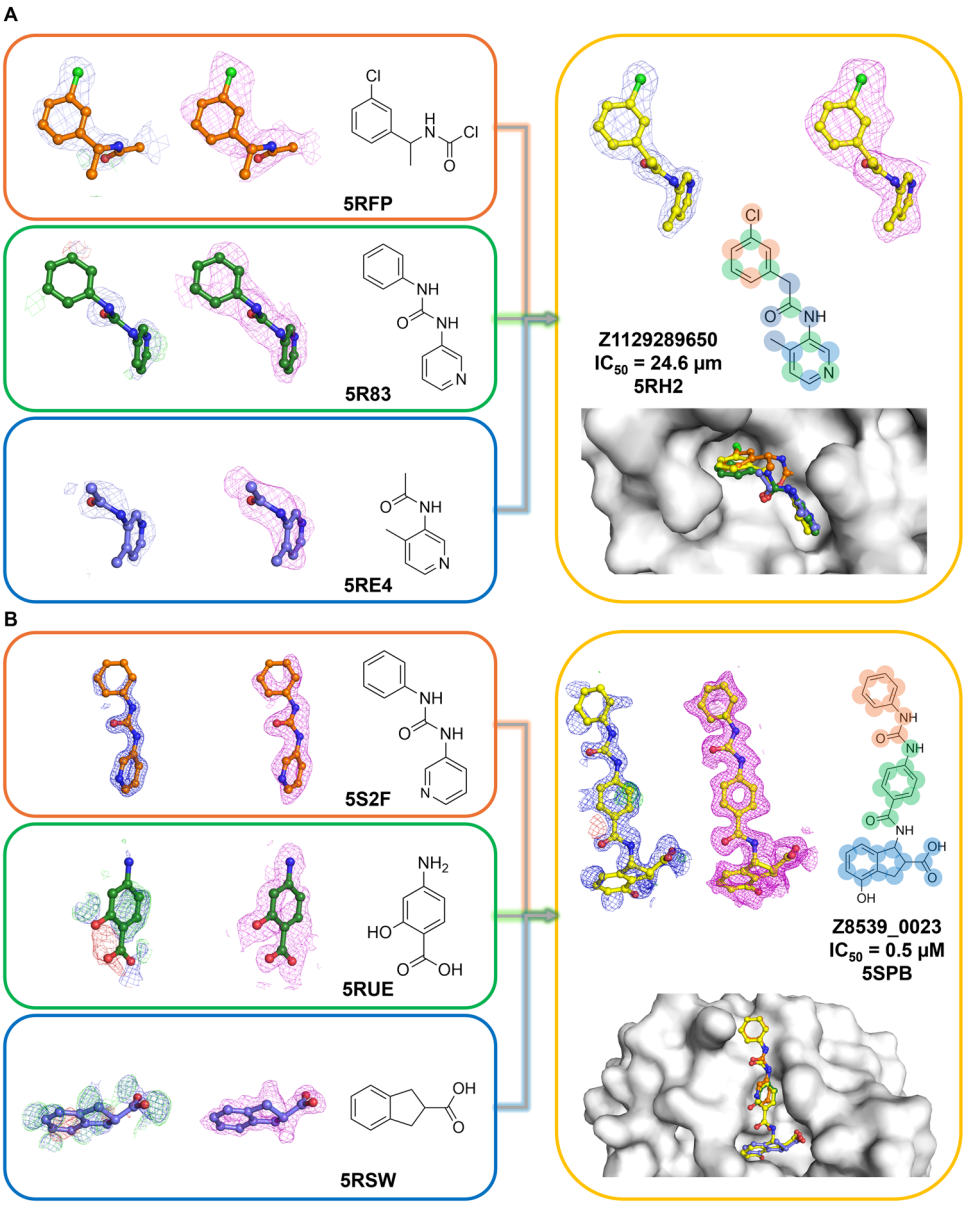

FIGURE 7 | Fragment merging for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Mpro (A) and Nsp3 macrodomain (B) identified high quality leads in a single step despite no significant activity in biochemical assays or significant density in conventional crystallographic maps for majority of fragment hits ($2mF_oF_c$ maps shown in blue at 1 σ, mF_oF_c maps shown in green/red at 3 σ, and PanDDA event maps shown at 1 σ in magenta) [21, 22, 50, 60]. The colour of the atoms in the chemical structures on the right corresponds to the border colour of the fragment progenitors on the left.

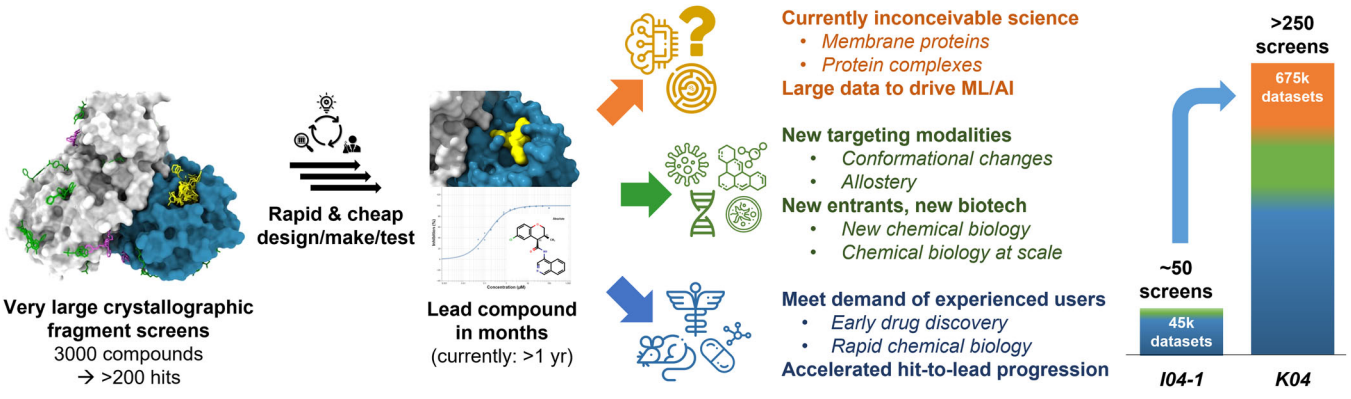

FIGURE 8 | Anticipated impact of delivery of new K04 beamline and associated Automated XChem Lab (AXL) [62].

4.2 | Present Challenges Confronting the Field

The increasing volume of data generated from large-scale crystallographic fragment screening experiments and subsequent structure-enabled lead development necessitates the development and refinement of processes for disseminating both positive and negative data. It is currently possible to deposit a collection of related crystal structures in the PDB under a group deposition and we have integrated tools to help prepare the necessary files into our pipelines. However, this process remains inefficient [65, 66]. The data are not standardised across different facilities, and the structures are not adequately flagged in the PDB, which can lead to misinterpretation by the structural biology and drug discovery communities [66]. Additionally, the current mmCIF format inadequately captures the conformational and compositional heterogeneity frequently observed in crystallographic fragment screening data sets [67, 68].

While there are existing mechanisms for disseminating hits from these screening experiments, no public-facing repository currently exists for negative data—specifically, fragments that were screened but no binding was observed despite sufficient quality data being collected. Collaborative efforts between various crystallographic fragment screening facilities and the Protein Data Bank in Europe (PDBe) are ongoing as part of the iNEXT Discovery and Fragment Screen projects [69, 70]. These initiatives aim to enable the deposition of investigational Crystallographic Information Files (CIFs) detailing the various parameters of crystallographic fragment screens. However, they are not the final endpoint for transferring such data to sustainable repositories that can be utilised for machine learning and improving binding prediction models. Making the data available through public databases, like ChEMBL, would be highly beneficial and support better utilisation of the data. Curating data in such informatic databases will require significant infrastructure and commensurate effort from the scientific community.

The future of crystallographic fragment screening and the acceleration of fragment-hit-to-lead development are poised for transformative advancements and paradigm shifts that could revolutionise the efficiency and scope of structure-based drug design. The evolution of automated and integrated pipelines represents a significant leap forward in structural biology and drug discovery. These advancements not only enhance the identification of biologically relevant ligand binding events but also improve the reliability of the data. Additionally, the substantial volume of structural data generated will drive the next generation of binding prediction models, facilitating more efficient structure-based drug design.

To fully realise the potential of these advancements, the development of standardised and efficient data dissemination processes is crucial. Such processes will support better utilisation of the data, enhancing machine learning models and binding predictions. Researchers, facilities, and stakeholders are encouraged to embrace these innovations and collaborate on developing comprehensive, standardised pipelines. By doing so, we can deepen our understanding of molecular interactions formed by fragment molecules and accelerate the discovery of new therapeutics.

Acknowledgements

The authors would like to extend their thanks to all users of the XChem platform and beamline I04-1, as well as colleagues from the MX village at Diamond Light Source—especially Dave Hall for his critical review of this manuscript. Additional thanks go to our colleagues and collaborators from other crystallographic fragment screening facilities, the COVID Moonshot project, the ASAP and READDI AViDD centres, the EUbOpen Consortium, iNEXT Discovery and Instruct ERIC.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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