**Promise and challenge of β-lactone electrophiles to target Asp12 of mutant KRASG12D**

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**ABSTRACT** After decades of drug discovery research on KRAS inhibitors with the potential to achieve a transformative cancer treatment for some of the most prevalent cancer types, there is still an unmet need to identify KRASG12D mutant selective inhibitors. The early clinical success of a covalent targeting strategy for KRASG12C inhibition prompts further expansion of the concept to target non-cysteine oncogenic mutation sites as in KRASG12D. This endeavor was hampered by the lack of suitable electrophiles for selective, covalent engagement of aspartate. Thanks to the recent discovery of b-lactone-bearing covalent inhibitors of KRASG12D new opportunities for drug discovery are emerging. Based on structural insights from X-ray crystallography and quantum mechanical considerations, we herein describe our work on elucidation of structure-activity and structure-stability correlations to further advance such electrophiles for drug discovery. Guided by predictions of transition state barrier heights for attack of aspartate 12 at the β-lactone electrophile as well as by structure-based design, we generated substituted b-lactones with the aim to achieve a balance of specific reactivity, chemical and metabolic stability. Our optimization strategy was driven by MS-based and cellular covalent target occupancy assays and PD marker analysis, as well as proteome-wide profiling and synthetic chemistry. With an improved understanding of structure and reactivity in biological systems, we wish to expand the use of β-lactones as chemoselective electrophiles and catalyze further applications of covalent carboxylate targeting in medicinal chemistry and drug discovery.

**TABLE OF CONTENT GRAPHIC (Draft)**

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## INTRODUCTION

RAS is among the most frequently mutated oncogenes with the KRAS G12D mutation being of highest occurrence (pancreatic ductal adenocarcinoma - PDAC 35%, colorectal cancer - CRC 14%, non-small cell lung cancer - NSCLC 4%).1 Several direct RAS inhibitors have entered clinical trials to address this high unmet medical need. While first generation oral mutant selective KRASG12C inhibitors2–11 have been approved for the treatment of *KRAS G12C* mutated cancers and many other KRASG12C inhibitors are following, also panRAS inhibitors12 and mutant selective KRASG12D inhibitors (RMC-980513, HRS464214,15, ASP-308216, MRTX113317 and others) have entered clinical trials, and some reported first clinical data. Based on early clinical data, direct inhibition of the driver RAS oncogene (e.g. RMC-623612 / RMC-9805 second line treatment in PDAC with KRAS G12X mutation) yields high disease control rates but depth and duration of response are so far limited indicating that combination therapies with potent and combinable RAS anchor drugs might be needed. Mutant selective RAS inhibitors might be preferred in this regard as they have a more favorable safety profile over panRAS inhibitors given that they are sparing on target RAS side effects such as skin rash induction.18 Mutant selective KRASG12D approaches were spearheaded by Mirati and are switch II pocket (SW-II)-based.  MRTX1133 is a potent, selective, noncovalent, and GDP-biased (OFF-state) KRASG12D inhibitor that achieves picomolar reversible binding affinity to the SW-II pocket.17 The molecule is efficacious in KRASG12D driven cancer cell lines and xenograft mouse models but lacks passive permeability possessing two basic amines that interact with aspartate 12 (D12) and glutamine 62 (E62), key residues to confer mutant selectivity and selectivity over N/HRAS, respectively19. Nevertheless, MRTX1133 is being evaluated in phase I clinical trials using an oral route of administration. Many other companies are also in the race to balance potency, selectivity, and oral exposure despite the necessity of the Asp12 interacting basic amine.20

Covalent modification of Asp12 represents an interesting avenue to achieve mutant selectivity and oral bioavailability. While RMC-9805 achieves covalent targeting of KRASG12D via a cyclophilin A glue, trapping the GTP-bound (ON-state) of KRASG12D, covalent and selective inhibition of KRASG12D targeting the SW-II pocket has remained elusive.21 Recently Shokat *et al.* reported the discovery of strain-release alkylation of Asp12 by β-lactones leading to covalent modification and thereby selective targeting of KRASG12D.22 In general, the challenges of covalent Asp targeting are two-fold: The carboxylic acid functionality of the Asp side chain, and similarly of Glu, is one of the least reactive nucleophiles in all amino acid side chains, and secondly, the reactivity of any electrophile capable of reacting with weak nucleophiles, like the carboxylic acid side chains of Asp or Glu, needs to be considered in biological systems which contain water and other nucleophiles such as glutathione (GSH).23 Structural insights from X-ray crystallography and careful consideration of stereoelectronic requirements for a covalent reaction with Asp12 under the constraints of the presumed binding pocket allowed the development of a di-methyl substituted β-lactone warhead attached via an amide bond to the MRTX1133 KRASG12D scaffold. This electrophile is described to exhibit good chemical stability under assay conditions and rapidly cross-links with Asp12 of KRAS in both GDP- and GTP-states. Compound (*R*)-**1** containing this disubstituted electrophile was found to be selective towards KRASG12D and showed cell growth inhibition in 3D-spheroid suspensions.22

Based on this seminal work of Shokat *et al.*, we continued the optimization β-lactone electrophiles to covalently target Asp12 of mutant KRAS. Herein, we report the mechanism- and structure-guided optimization relying on transition state modelling of the β-lactone warhead in the context of the MRTX1133 core scaffold towards improved potency, covalent target occupancy, and specific reactivity with the goal to enable an oral route of administration of such KRASG12D inhibitors.

## RESULTS AND DISCUSSION

With the foundation laid by researchers in Kevan Shokat’s team, we were interested in further exploring this concept of covalency by employing substituted b-lactone electrophiles to directly target Asp12 through a stereospecific SN2 mechanism leading to β-lactone ring opening.22 Initially, we aimed towards a deeper understanding of compound`s (*R*)-**1** *in vitro* profile in KRASG12D mutant selective target modulation and its chemical properties with regard to drug-likeness. In a side-by-side comparison of cell potencies of the clinical compound MRTX1133 and (*R*)-**1** in our functional cellular assay system, where we measure the inhibition of ERK phosphorylation (pERK) as a distal PD marker of the RAS/MAPK pathway in the KRASG12D mutant cell line SW1990, we determined IC50 values of 0.007 µM for MRTX1133 and 0.14 µM for (*R*)-**1** after 24 h of incubation time (Figure 1a). The cellular selectivity profile towards wild type (WT) KRAS was assessed with the KRASWT-dependent cell line MKN1 in an otherwise comparable set up. A 36-fold selectivity was determined for (*R*)-**1** in contrast to more than 70-fold for MRTX1133, reflecting a reduced potency and selectivity for the β-lactone compound. These data imply that the covalent reaction of (*R*)-**1** with KRASG12D does not compensate for the loss of potency and selectivity due to the presence of a salt-bridge interaction of the MRTX1133 basic amine with Asp12.17 The inherent KRASG12D specific reactivity of (*R*)-**1** was determined using an intact protein MS-based assay, measuring covalent modification of protein after 4 h at an initial 10 mM concentration of compound and 1 mM protein concentration in GDP- and GTP-states of KRASG12D. Under these conditions we observed almost complete covalent modification by (*R*)-**1** of GDP-bound KRASG12D and slightly reduced covalent labelling efficiency of GTP-bound KRASG12D (95% and 83%). The intrinsic chemical reactivity of the β-lactone electrophile of (*R*)-**1** was assessed by monitoring the disappearance of compound in the presence of large excess of reduced glutathione (GSH)24 which represents a concentration close to cellular levels. In this setting we obtained a half-life (t1/2) of only 9 min for (*R*)-**1**. This contrasts with clinically approved covalent acrylamide-based KRASG12C inhibitors like Sotorasib (t1/2 = 173 min) or RMC-9805 (t1/2 > 120 min13)13.

Taken together our preliminary observations raised the question whether a covalent β-lactone KRASG12D inhibitor could compensate the important contribution of MRTX1133’s basic amine, which serves as the major potency and selectivity driver, and after appropriate optimization, could provide a therapeutic benefit *in vivo* by achieving a more sustained target coverage leading to a profound clinical response. In addition, such compound profile should be accompanied by an appropriate selectivity window over wild-type RAS, associated with a favorable safety margin and ultimately allow for oral dosing by improvement of druglike properties, which is a challenge for MRTX1133.

**a.**

|  |  |  |  |
| --- | --- | --- | --- |
|  |  |  |  |
| Compound ID | MRTX1133 | (*R*)-**1** | 4-methyl **2** |
| pERK SW1990 IC50 at 4 h / 24 h [µM] | 0.004 ± 0.002 (n=45) / 0.007 ± 0.005 (n=48) | 0.37 ± 0.13 (n=4) /  0.14 ± 0.09 (n=5) | 0.053 ± 0.033 (n=3) /  0.016 ± 0.008 (n=4) |
| pERK MKN1 IC50 at 4 h / 24 h [µM] | 0.38 ± 0.15 (n=48) /  0.54 ± 0.16 (n=48) | 2.56 ± 1.38 (n=5) /  5.15 ± 2.48 (n=5) | 0.061 ± 0.005 (n=3) /  0.120 ± 0.054 (n=34 |
| Covalent modification [%] KRASG12D GDP / GTP | 0 / 0 | 95 ± 6 (n=46) /  83 ± 10 (n=44) | 0 / 0 |
| Half-life t1/2 in aqueous GSH [min] | >480 | 9 ± 2 (n=2) | >480 |
| Loss compound [%] in cell media at 4 h / 8 h | n.d. | 35 / 60 | 2 / 6 |

**b.**

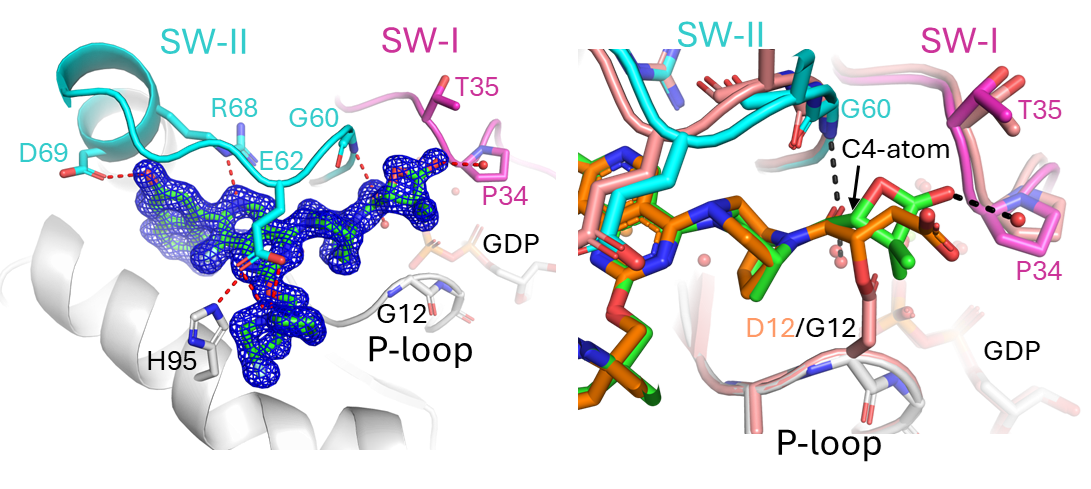
*KRASG12D-specific ring opening via SN2:* *β-Lactone ring opening by GSH:*



***Figure 1.******a.*** *Comparison of in vitro profiles of MRTX1133, (R)-****1****, and 4-methyl* ***2****. All measurements were taken from distinct samples. Arithmetic means and number of repeats are given where appropriate. n.d. not determined.* ***b.*** *Reaction schemes to depict dual reactivity of β-lactone ring opening (left upon reaction with Asp12 in blue via C4; right upon reaction with GSH orange via C2).*

To start our structure-activity relationship (SAR) investigations, we aimed towards balancing specific reactivity towards KRASG12D with intrinsic chemical stability of the warhead toward non-specific nucleophilic attack. For a better understanding of specific Asp reactivity versus non-specific events, we conducted NMR studies on a β-lactone tool compound (see extended data and figures in SI) and investigated the dual reactivity of ring opening after reaction with either GSH or water as nucleophiles (Figure 1b). Our findings are consistent with literature reports25 where non-specific solvolysis and GSH attack seem to occur predominantly at the C2 carbon leading to ring opened b-hydroxy acid products. This result is also in line with the structural and mechanistic work reported earlier, which describes the regiochemical distinction from covalent adduct formation with Asp12 that does occur via a SN2 mechanism under strict stereo-control in the binding cavity under SW-II at the C4 of the β-lactone ring.22 To further investigate this observation, we prepared as a molecular matched pair 4-methyl compound **2** with an additional methyl substituent at the reaction center. As expected, 4-methyl **2** as well as its diastereomer (data not shown) did not undergo any detectable covalent reaction with KRASG12D in our intact protein MS-based assay. Interestingly, 4-methyl **2** is more potent than (*R*)-**1** with an IC50 value of 0.016 µM (after 24 h) in our functional cellular assay although the compound shows no covalent modification of Asp12. In addition, the compound remained intact in the GSH assay after 8 h, in contrast to the short t1/2 for (*R*)-**1**. We therefore hypothesized that the functional cellular activity for these β-lactone compounds might not only result from covalent modification of KRASG12D but also from classical reversible binding contributions of the parent compounds or their potential degradation products which could emerge from nonspecific nucleophilic attack or via solvolysis. This hypothesis arises from the observed degradation of (*R*)-**1** in the cellular assay medium as well as the lack of a correlation of the covalent labelling capacity of compound **2** and the cellular IC50 measured in the SW1990 assay. As we were aiming to examine the covalent contribution to cellular potency, we decided to develop a cellular target occupancy (TO) assay based on Western blot to guide our development of SAR. We investigated whether an additional mass spectrometry-based TO assay might offer superior quantitative accuracy, and we therefore set up a TO-MS assay compatible with the covalent β-lactone-aspartate reaction. Although both TO formats are consistent, we found that the TO-MS assay may slightly underpredict occupancy (see extended data and figures in SI), therefore we determined TO for our compounds by Western blot for this study. With these assays as well as the functional cellular readout and the intact protein MS assay we had suitable *in vitro* assays in place to dissect cellular effects driven by a covalent mode of action and support our SAR investigation. To gain deeper insight in the mechanism and structural prerequisites of the potential transition state of the SN2 reaction of the covalent adduct formation we furthermore invested heavily in X-ray crystallography.

**X-ray crystallography reveals reversible binding mode of β-lactones in the SW-II pocket of KRAS.** To better understand the non-covalent binding mode of (*R*)-**1** to KRAS, we solved a 1.31 Å resolution X-ray crystal structure of KRASWT in complex with GDP and (*R*)-**1** (Figure 2). Clear electron density was observed for (*R*)-**1** non-covalently bound in the SW-II pocket, adopting a similar conformation and binding mode to the previously reported X-ray co-crystal structure of the covalent adduct of KRASG12D(GDP)‑(*RS*)-G12Di-1 (PDB code 8T4V22) which contains a ring-opened β-lactone covalently bound to Asp12 of KRASG12D. The pyridopyrimidine cores, pyrrolizine side chains, and naphthalene moieties of both inhibitors superimpose nicely, making conserved hydrogen bonding and ionic interactions with the side chains of His95, Arg68, and Glu62. The hydroxyl group of the naphthol in (*R*)-**1** forms an additional hydrogen bond with the side chain of Asp69. Interestingly, the amide carbonyl between the bridged piperazine and the β-lactone of (*R*)-**1** makes hydrogen bonding interactions with the backbone NH of Gly60 and a water molecule, thereby positioning the β-lactone between the SW-I, SW-II, and P-loop of the protein. The analogous amide carbonyl of (*RS*)-G12Di-1 of the reported covalent complex makes the same hydrogen bonding interactions with KRAS despite being covalently bound to Asp12 and having a ring opened β-lactone. The orientation of the electrophilic C4 carbon atom of the β-lactone in the non-covalent complex is very close to the position of the same carbon atom in the covalent complex.



***Figure 2.*** *Left: X-ray co-crystal structure of KRASWT in complex with GDP and (R)-****1****. The SW-I and SW-II residues are shown in magenta and cyan, respectively. H-bonding interactions are depicted as dotted lines. The 2Fo-Fc electron density map for (R)-****1*** *non-covalently bound to KRASWT is depicted in blue mesh at 1.4 σ and 1.31 Å resolution. Right: Overlay with the crystal structure of the covalent adduct of KRASG12D(GDP) (RS)-G12Di-1 (PDB code 8T4V*22) *with KRASG12D shown in salmon and (RS)-G12Di in orange. H-bonding interactions are depicted as dotted lines.*

We hypothesized that the conformation of the β-lactone in the (*R*)-**1**-KRASWT(GDP) X-ray co-crystal structure is close to the transition state geometry of the covalent bond forming event. Therefore, we seized the opportunity to develop an *in silico* workflow for transition state scanning calculations of the SN2 ring opening of β-lactones by Asp12. The objective was to develop a deeper mechanistic understanding of the reaction and to guide synthesis prioritization of novel β-lactones which would require more complex and longer synthetic sequences.

**Mechanistic study of Asp12 attack by quantum mechanics.** We developed transition state energy calculations of the ring opening of β-lactones by Asp12 to gain a mechanistic understanding of the covalent reaction and to develop an *in silico* workflow to help prioritize novel β-lactone electrophiles, in particular due to the foreseen synthetic complexity of substituted β-lactones (Figure 3). Transition state (TS) energy calculations have been applied in a variety of reaction mechanism studies, including SN2 reactions.26,27 To obtain the starting coordinates for a transition state scan, we used the X-ray co-crystal structure of (*R*)-**1**-KRASWT(GDP) described above, which provides the position of the bridged amine with β-lactone warhead in combination with the Asp12 positioning from the reported X-ray co-crystal structure from Shokat *et al.* of the covalently bound KRASG12D(GDP)‑(*RS*)-G12Di-1 (Shokat’s compound; unsubstituted β-lactone).22 We then performed transition state energy calculations on the reported unsubstituted β-lactone (*RS*)-G12Di-1 and the gem-dimethyl compound (*R*)-**1**. This resulted in transition state energies (ΔETS) of 6.4 kcal/mol for the (*RS*)-G12Di-1 compound and 7.7 kcal/mol for (*R*)-**1**. This is in agreement with the higher reactivity of the unsubstituted β-lactone, which fully modifies KRASG12D (GDP) in less than 15 min as compared to (*R*)-**1**, which fully modifies in 30 min.22 These data suggest that in addition to the electrophilic character of the C4 position, steric hinderance via substituents at the C3 position may also play a key role in tuning reactivity, as observed by Shokat *et al*.

With this finding in hand, we continued to use transition state energy calculations on novel β-lactones considered for synthesis to screen out proposals predicted to either be unreactive or having too high reactivity and instability. We combined these predictions with a conformational analysis procedure (ReSCoSS)28 to ensure fit into the pocket and the ability to suitably position the β-lactone C4 atom for nucleophilic attack by Asp12. This *in silico* workflow allowed us to prioritize key compounds for synthesis and was broadly applicable for general SAR generation.

A diagram of a reaction

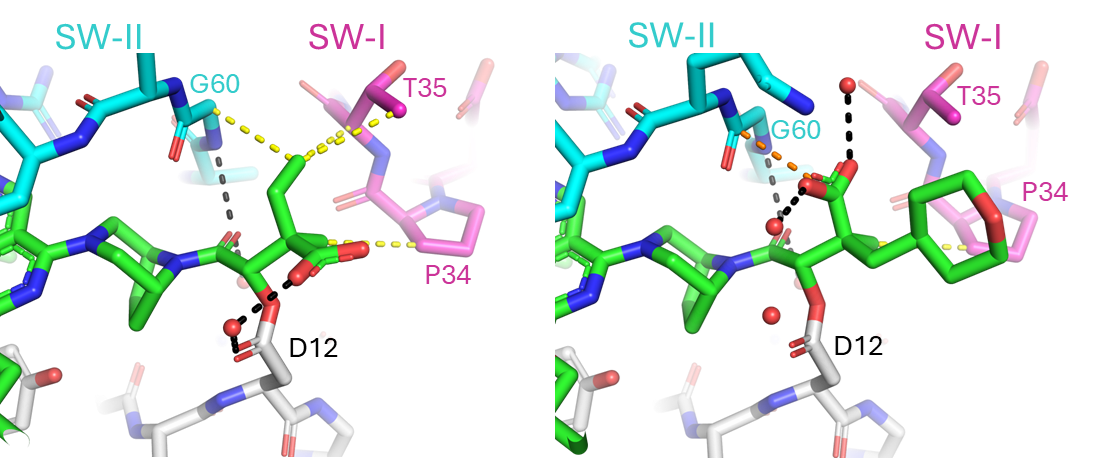
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***Figure 3.*** *Potential energy diagram for the SN2 nucleophilic attack of Asp12 at β-lactone C4 position for unsubstituted and di-methyl warheads.*

**SAR investigation on substituted β-lactones.** We started our SAR investigations by preparing the simplest possible matched pair of *cis* (3*R*,4*R*)-**3** and *trans* isomers (3*S*,4*R*)-**4** (Table 1, entries 2 and 3). The *in silico* calculation of the transition state energy barriers predicted a higher value of 8.1 kcal/mol for the *cis* isomer (3*R*,4*R*)-**3** versus 7.6 kcal/mol for the *trans* isomer (3*S*,4*R*)-**4**. The primary profiling data reflected this rank order of reactivity. In our intact protein MS-based assay (3*S*,4*R*)-**4** displayed a more efficient reaction achieving complete labelling of KRASG12D after 24 h incubation whereas (3*R*,4*R*)-**3** achieved a slightly lower level of 85%. Noteworthy, the reactivity difference regarding covalent modification of the GTP-loaded protein was even more pronounced. Here (3*S*,4*R*)-**4** seemed to have a substantial benefit (about 71% modification) over (3*R*,4*R*)-**3** (33% modification). On the cellular level in KRASG12D-dependent SW1990 cells the observed potencies were comparable for both compounds, again supporting the observation that the cellular readout might not solely be driven by the covalent modification of KRASG12D. The outcome of the TO assay reflected the results of the MS based assay and revealed better target occupancy of the *trans* isomer (3*S*,4*R*)-**4** achieving 59% TO in cells versus 24% for (3*R*,4*R*)-**3**, while both compounds did display comparable stability in the GSH incubation (t1/2 of 23 min 25 min, respectively). Both values represent a slight improvement over (*R*)-**1**. Aiming towards a deeper structural biology understanding, the obtained X-ray crystal structure of (3*R*,4*R*)-**3** in complex with KRASG12D (GDP) (Figure 4) shows that the ligand adopts a similar conformation to that observed in the X-ray of KRASG12D(GDP)‑(*RS*)-G12Di-1. The exposed carboxylic acid needs to rotate 120° around the C3-C4 bond to adopt a minimum energy conformation and thereby exposes the carboxylic acid to solvent and accommodates the ethyl and methyl substituents between the SW-I and SW-II loops. The ethyl group forms Van-der-Waals interactions with Thr35 and Gly60 and the methyl group with Pro34 while maintaining the direct H-bonding interaction of the C2 carbonyl with backbone NH of Gly60. From this first matched pair of *cis* versus *trans* substituted β-lactones we concluded that larger substituents with relative *cis* orientation are less favorable for the directional covalent reaction with Asp12. *Trans* substituted β-lactones, however, are better tolerated in the binding site.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Entry | Compound | β-lactone | Covalent mod. [%]  GDP/GTP | SW1990 IC50 24 h [µM] | MKN1 IC50 24 h [µM] | TO [%] | Half-life t1/2 in aq. GSH [min] | ΔETS [kcal/mol] |
| 1 | (*R*)-**1** |  | 95 / 83 | 0.14 | 5.15 | 68 | 9 | 7.7 |
| 2 | (3*R*,4*R*)-**3** |  | 85 / 33 | 0.02 | 3.41 | 24 | 23 | 8.1 |
| 3 | (3*S*,4*R*)-**4** |  | 99 / 71 | 0.08 | 4.66 | 59 | 25 | 7.6 |
| 4 | (3*S*,4*R*)-**5** |  | 100 / 83 | 0.04 | 3.16 | 91 | 32 | 7.7 |
| 5 | (3*S*,4*R*)-**6** |  | 37 / 30 | 0.03 | 0.08 | 44 | >480 | 9.8 |
| 6 | (3*S*,4*R*)-**7** |  | 83 / 57 | 0.05 | 0.13 | 48 | 54 | 7.5 |
| 7 | (3*S*,4*R*)-**8** |  | 100 / 92 | 0.04 | 0.75 | 90 | 25 | 7.8 |

***Table 1.*** *Initial SAR of β-lactone warhead optimization. Chemical structures of compounds (3S,4R)-****3****-****8****, with covalent modification, cellular assay, and target occupancy data. Chemical stability was determined in GSH incubations. All measurements were taken from distinct samples. Arithmetic means are given for covalent modification and pERK assays (see SI for data with standard deviations). Transition state energies (ΔETS) were determined by quantum mechanics. n.d. not determined.*



***Figure 4.*** *X-ray co-crystal structures of KRASG12D in complex with GDP and (3R,4R)-****3*** *(left) or (3S,4R)-****8*** *(right), solved to 1.55 Å and 1.30 Å resolution, respectively. The inhibitors covalently bound to Asp12 are shown in green. The SW-I and SW-II residues are shown in magenta and cyan, respectively. H-bonding interactions are depicted as black dotted lines, Van-der-Waals contacts as yellow dotted lines, and π-π stacking interactions as orange dotted lines.*

Taking a stepwise approach, the elongation to a *trans* n-propyl substituent resulted in compound (3*S*,4*R*)-**5** (Table 1, entry 4) which was predicted to have a comparable energy barrier of 7.7 kcal/mol to (3*S*,4*R*)-**4** and (*R*)-**1**. Accordingly, (3*S*,4*R*)-**5** completely modified KRASG12D (GDP) covalently after 24 h incubation, in line with a favorable TO (91%) and an IC50 of 0.04 µM in the functional pERK readout, while maintaining favorable selectivity versus KRASWT (MKN1 IC50 = 3.16 µM). The slight increase of steric demand of the C3 substituent in (3*S*,4*R*)-**5** trends towards a longer t1/2 of 32 min in the GSH incubation hinting at the positive impact of steric factors on the nonspecific nucleophilic attack on C2. Building on this observation, we aimed to further increase steric congestion on the β-lactone moiety by incorporation of branched motifs. The first example in this regard was isopropyl derivative (3*S*,4*R*)-**6** (Table 1, entry 5). As predicted by the calculated energy barrier for the covalent reaction of 9.8 kcal/mol, we indeed observed significantly reduced labelling capacity in the covalent MS assay (37% GDP / 30% GTP), which was, however, accompanied by a clear shift toward longer half-life in the GSH assay (t1/2 >480 min).

Moving the branching point from the α- to the β-carbon as exemplified in (3*S*,4*R*)-**7** and (3*S*,4*R*)-**8** (Table 1, entries 6 and 7), energy barrier calculations predicted a reduced transition state barrier of 7.5 kcal/mol for (3*S*,4*R*)-**7** versus 7.8 kcal/mol for (3*S*,4*R*)-**8**. Intriguingly, we observed more efficient covalent labeling of both nucleotide states for the tetrahydropyran derivative (3*S*,4*R*)-**8** versus the isobutyl derivative (3*S*,4*R*)-**7**. This is consistent with the higher TO achieved by (3*S*,4*R*)-**8** versus (3*S*,4*R*)-**7** (90% and 48% respectively) and is also reflected in the reactivity towards GSH with t1/2 values of 25 min for (3*S*,4*R*)-**8** versus 54 min for (3*S*,4*R*)-**7**. To better understand these differences, we obtained an X-ray co-crystal structure of (3*S*,4*R*)-**8** in complex with KRASG12D (GDP) (Figure 4). The structure of the covalent adduct of (3*S*,4*R*)-**8** resembles the transition state immediately after covalent bond formation with Asp12. The tetrahydropyran side chain points towards the solvent and is poorly defined by the electron density, indicating conformational flexibility. The methyl group makes Van-der-Waals contacts with the side chain of Pro34, and the carboxylic acid engages in hydrogen bonding interactions with water molecules. It may also form a weak π-π stacking with the backbone of Gly60. To adopt a minimum energy conformation with the carboxylic acid exposed to solvent, the tetrahydropyran ring requires rotational movement around the C3-C4 bond. However, this is not possible due to the steric bulk of the tetrahydropyran, which cannot be accommodated below Asp12, adjacent to GDP. The pocket environment may explain the discrepancies between the calculated and observed relative reactivities of (3*S*,4*R*)-**7** and (3*S*,4*R*)-**8**, which the QM calculations do not take into account. It is more challenging to fully rationalize the differential ring opening reactivity towards GSH, for which we do not have an approximation of the transition state. GSH reactivities follow the same trend as seen for specific reactivities, however, it could be hypothesized that electronic factors may play a paramount role in driving reactivity due to the polarized C=O bond. To test this hypothesis and understand the impact of electronic effects on the reactivity pattern, we attempted the synthesis of a variety of β-lactones with heteroatom-containing C3 substituents. Those efforts, however, were mostly unsuccessful due to the increased chemical reactivity and thereby stability challenges of the intermediates during synthesis. Hence, we centered our following explorations on branched alkyl substituents without additional heteroatoms.

**Proteome-wide reactivity profiling of β-lactone warheads guides optimization.** Having achieved improved chemical stability with a first set of compounds, we wanted to understand the proteome-wide selectivity of compounds (3*S*,4*R*)-**4** and (3*S*,4*R*)-**7** as compared to (*R*)-**1** (Figure 5 and SI). Although β-lactones are of interest for their ability to label Asp12, they can also react with cysteine residues.22 Chemoproteomics techniques capable of measuring proteome-wide cysteine labeling are well established.29 We employed the isobaric tagging and reactivity-dependent acid cleavable enrichment (iTRACE) method to quantify the extent of cysteine modification across the proteome and to compare the resulting profiles to that of Adagrasib. At 2-fold competition (50% occupancy) Adagrasib treatment results in competition of 49 cysteinyl peptides, whereas (*R*)-**1**, (3*S*,4*R*)-**4**, and (3*S*,4*R*)-**7** compete 245, 331, and 368 sites, respectively. For these β-lactones, we observed a negative correlation between GSH reactivity and number of competed cysteines, suggesting that increasing chemical stability might result in a higher apparent concentration of the reactive compound, yielding a larger number of off-target labeling events. Moreover, the β-lactones showed >5-fold more off-target labeling events at 50% occupancy than Adagrasib. Together, these data suggested that our β-lactones require further optimization and that monitoring of proteome-wide selectivity for such warheads early on helps guide SAR exploration and should be considered during electrophile optimizations in general.

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***Figure 5.*** *Proteome-wide reactivity profiling by iTRACE of β-lactones (R)-****1****, (3S,4R)-****4****, and (3S,4R)-****7****, compared to Adagrasib. H358 cells were incubated with 10 µM compound concentration for 6 h prior to lysis and treatment with a cysteine-reactive biotinylated probe. After tryptic digest, enrichment, and isobaric tagging using a tandem mass tag system, reactive peptides were identified and quantified by LC-MS/MS.*

Based on the data of the proteome-wide reactivity profiling we decided to further explore the impact of steric factors on compound stability as adequate chemical stability is an indispensable property of a drug-like molecule. We concluded that alpha-branched motifs as in (3*S*,4*R*)-**6** could be a privileged handle to exert strong impact on the electrophilic properties and hence prioritized those for the next SAR iteration. In addition, we wanted to probe if (stereo-)electronic factors could be utilized to re-gain Asp12 specific reactivity of the branched β-lactones. We therefore prepared (3*S*,4*R*)-**9** (Table 2, entry 1) as a direct sp2-hybridized matched pair to (3*S*,4*R*)-**6**. With this modification it was possible to fully re-gain covalent labelling capacity (97% GDP / 78% GTP) associated with high cellular potency of 0.03 µM in the pERK assay and cellular TO of 69%. Once again, we observed a strong correlation of specific and nonspecific reactivity, as reflected in the substantially shortened t1/2 in the GSH assay (t1/2 = 30 min).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Entry | Compound | β-lactone | Covalent mod. [%]  GDP/GTP | SW1990 IC50 24 h [µM] | MKN1 IC50 24 h [µM] | TO [%] | Half-life t1/2 in aq. GSH [min] | ΔETS [kcal/mol] |
| 1 | (3*S,*4*R*)-**9** |  | 97 / 78 | 0.03 | 2.24 | 69 | 30 | 9.0 |
| 2 | (3*R*,4*R*)-**10** |  | 97 / 90 | 0.05 | 3.6 | 64 | 14 | 7.0 |
| 3 | (3*S*,4*R*)-**11** |  | 98 / 63 | 0.03 | 0.46 | 59 | 132 | 7.6 |
| 4 | (3*S*,4*R*)-**12** |  | 68 / 35 | 0.03 | 0.08 | 41 | >480 | 7.9 |
| 5 | (3*S*,4*R*)-**13** |  | 97 / 82 | 0.01 | 0.36 | n.d. | n.d. | 8.2 |
| 6 | (3*S*,4*R*)-**14** |  | 100 / 95 | 0.01 | 0.32 | 91 | 101 | 7.5 |
| 7 | (3*S*,4*R*)-**15** |  | 100 / 95 | 0.01 | 0.37 | 78 | 125 | 8.1 |

***Table 2.*** *Continued β-lactone warhead optimization. Chemical structures of compounds (3S,4R)-****9****-****15****, with covalent modification, cellular assay, and target occupancy data. Chemical stability was determined in GSH incubations. All measurements were taken from distinct samples. Arithmetic means are given for covalent modification and pERK assays (see SI for data with standard deviations). Transition state energies (ΔETS) were determined by quantum mechanics. n.d. not determined.*

We next turned our attention to cyclic derivatives (3*S*,4*R*)-**10**, (3*S*,4*R*)-**11**, and (3*S*,4*R*)-**12** (Table 2, entries 2-4) comprising 3-, 4- and 5-membered cycloalkyl substituents. Cyclopropyl derivative (3*S*,4*R*)-**10** displayed improved reactivity (TO = 64%) but also limited stability in the GSH incubation (t1/2 = 14 min), in line with the predicted transition state energy barrier of only 7.0 kcal/mol. We were pleased to observe that the cyclobutyl derivative (3*S*,4*R*)-**11** provided for the first time a good balance of cellular potency (pERK IC50 = 0.02 µM), specific reactivity (TO = 59%), and GSH stability (t1/2 = 132 min). Conversely, cyclopentyl derivate (3*S*,4*R*)-**12** displayed excellent stability in GSH incubations (t1/2 > 480 min) associated with reduced labelling capacity in the cellular system (TO = 41%). Overall, these cyclic analogs confirmed our observation of the SAR being driven by steric factors of the β-lactone substitution. These experimental data reflected the trends of the calculated barrier heights (Table 1 and 2) with efficient labelling in both GDP- and GTP-states achieved for compounds with lower predicted transition state barriers. The transition state barrier calculations can therefore be used as a qualitative guide to rule out warheads predicted non-reactive (see extended data and figures in SI for the analysis of ΔETS for 45 β-lactones synthesized in this work).

**Transition state conformations aid interpretation of covalent labelling.** An interesting matched molecular pair from the perspective of Asp12 covalent labelling and transition state energy calculations is isopropyl β-lactone (3*S*,4*R*)-**6** and cyclopropyl β-lactone (3*S*,4*R*)-**10**. Despite this subtle structural difference, (3*S*,4*R*)-**10** displays almost 100% covalent labelling of KRASG12D (GDP) after 4 h yet (3*S*,4*R*)-**6** achieved only 30% labelling in the same setting. Inspection of the reactant state electrophile conformation versus the transition state complex indicates that the isopropyl group of (3*S*,4*R*)-**6** must rotate away from a global minimum energy conformation to allow Asp12 attack, whereas the cyclopropyl of (3*S*,4*R*)-**10** can adopt the same global minimum energy conformation in both the reactant state and transition state (no movement is required to allow Asp12 attack). The requirement of the isopropyl of (3*S*,4*R*)-**6** to rotate away from the global minimum energy conformation in the transition state contributes to a large difference in transition state energies (9.8 kcal/mol for (3*S*,4*R*)-**6** versus 7.0 kcal/mol for (3*S*,4*R*)-**10**) and therefore may also contribute to the difference in covalent labelling of the warheads by Asp12 (Figure 6).

A graph of a graph of a function

Description automatically generated with medium confidence

***Figure 6.*** *Transition state energy conformations explain the observed covalent labelling difference between (3S,4R)-****6*** *and (3S,4R)-****10****. Left: The cyclopropyl of (3S,4R)-****10*** *has a global minimum energy conformation at a dihedral angle of -68º (indicated by atom indices 1-4) which is maintained during Asp12 attack (green marker on energy scan). Right: The isopropyl of (3S,4R)-****6*** *has a global minimum energy conformation at a dihedral angle of -160º (indicated by atom indices 1-4, green marker on energy scan), yet must rotate to a local minimum at -50º (red marker) to accommodate Asp12 attack.*

Overall, the incorporation of cyclic motifs especially small rings with a partial sp2-like character resulted in compounds with a more balanced profile of stability and specific reactivity. Intrigued by the good stability of (3*S*,4*R*)-**12** we decided to follow up on 5-membered ring systems as this seemed to be an effective motif to sterically shield the β-lactone while maintaining Asp12 specific reactivity. To evaluate heterocyclic and sp2-hybridized derivatives of (3*S*,4*R*)-**12** we synthesized (3*S*,4*R*)-**13** and (3*S*,4*R*)-**14** (Table 2, entries 5 and 6). These substitutions had a positive impact on specific reactivity reflected in almost complete labelling of KRASG12D in both nucleotide states, as compared to (3*S*,4*R*)-**12**, while cellular activity as well as stability towards GSH (t1/2 = 101 min for (3*S*,4*R*)-**13**) remained unchanged. Overall, the profile of (3*S*,4*R*)-**14** appeared promising in terms of covalent labelling, cellular potency, and stability, however, the increased synthetic complexity by the incorporation of three stereogenic centers on the β-lactone hampered fast SAR expansion. To further build on the cyclobutyl motif of (3*S*,4*R*)-**11** we embarked on the synthesis of a bicyclo[2.2.2]octane substituted β-lactone (3*S*,4*R*)-**15** (Table 2, entry 7). (3*S*,4*R*)-**15** displayed high levels of covalent labelling of KRASG12D in both GDP- and GTP-bound states (100% and 95%) with almost equal efficiencies which translated in good TO at the cellular level (78%) and cellular potency (IC50 = 0.01 µM) while maintaining reduced reactivity towards GSH (t1/2 = 125 min), comparable to (3*S*,4*R*)-**11**. These data lead to the hypothesis that with (3*S*,4*R*)-**11** and (3*S*,4*R*)-**15** a nuanced combination of steric and electronic factors is favorably synergizing towards an improved and balanced overall profile.

***In vivo* studies with (3*S*,4*R*)-11.** The overall profile of (3*S*,4*R*)-**11** prompted us to further characterize this compound *in vitro* and *in vivo* more in depth (Tables 3 and 4). Compound (3*S*,4*R*)-**11** displays rather high lipophilicity (LogD = 4.1) which likely contributes to the low aqueous solubility of 0.003 mM at pH 6.8. (3*S*,4*R*)-**11** also has low permeability in a low-efflux MDCK assay (PappAtoB = 2.1 x 10-6 cm/s) but good stability in the presence of GSH(t1/2 = 132 min). However, the half-life of the compound in mouse and human S9 liver fractions with GSH was 30 min and 86 min, respectively. A short half-life of 18 min was also observed in mouse plasma which prevented determination of plasma protein binding for the compound. Despite the limited *in vitro* stability, we conducted a PK study after intravenous (i.v.) and oral (p.o.) administration to mice and a PK/PD study.

Initially, the PK of (3*S*,4*R*)-**11** was studied after single i.v. (0.3 mg/kg) and oral administration (1 mg/kg from suspension) to mice. Blood PK parameters are shown in Table 3. (3*S*,4*R*)-**11** showed medium CL of 57% of mouse liver blood flow (71 mL/min/kg). Following oral administration, only minimal exposure was observed, resulting in low oral bioavailability which is likely attributable to limited permeability and low solubility.

|  |  |  |
| --- | --- | --- |
|  | (3*S*,4*R*)-11 | |
| LogD pH 7.4 | 4.1 | |
| High-throughput solubility pH 6.7 [mM] | 0.003 | |
| LE-MDCK PappAtoB = 2.1 x 10-6 cm/s | 2.1 | |
| S9 liver fraction with GSH mouse / human [min] | 30 / 86 | |
| Half-life t1/2 in mouse plasma [min] | 18 | |
| Liver microsomal CL mouse / human [µL/min/mg] | 70 / 105 | |
| *In vivo* mouse PK parameters: | i.v. 0.3 mg/kg | p.o. 1 mg/kg |
| Formulation | NMP / BSA | MC /Tween |
| CL [mL/min/kg] / liver blood flow [%] | 71 / 57 |  |
| T1/2 [h] | 1.2 |  |
| AUC [nmol h] | 89 | 8 |
| Vss [L] | 7.2 |  |
| Bioavailability [%] |  | <1 |

***Table 3.*** *Advanced in vitro and in vivo characterization of (3S,4R)-****11****. Mouse PK studies were performed after i.v. and p.o. dosing.*

Due to the low exposure after oral dosing, we thereafter decided to conduct the PK/PD study in PK-59 tumor-bearing mice with (3*S*,4*R*)-**11** at a higher dose of 30 mg/kg administered intraperitoneal (i.p.), in comparison to MRTX1133 (Table 4). In this study, (3*S*,4*R*)-**11** did not show any DUSP6 PD modulation in tumors, in contrast to MRTX1133. Assuming equal blood binding (in-house determined for MRTX1133: mouse fu,p 7.5% and blood-to-plasma partitioning 1:1), the average unbound concentration (Cav,u calculated as AUC/24h x fu,b) corrected for cellular potency (pERK IC50) was much higher for MRTX1133 as compared to (3*S*,4*R*)-**11**, in agreement with the lack of observed PD response. The free exposure/potency ratio of (3*S*,4*R*)-**11** requires further optimization to obtain a pharmacodynamic readout comparable to MRTX1133. Additionally, physical and chemical properties such as permeability and solubility need improvement to enable oral dosing.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | i.p. dose [mg/kg] | AUC in blood [µM h] | pERK IC50 [µM] | Cav,u/pERK IC50 | DUSP6 decrease  at 3 h | DUSP6 decrease at 10 h | DUSP6 decrease at 24 h |
| (3*S*,4*R*)-11 | 30 | 8.1 | 20 | 1.2 | 0% | 0% | 0% |
| MRTX1133 | 10 | 8.9 | 0.5 | 51.9 | 97% | 97% | n.d. |
| MRTX1133 | 30 | 32 | 0.5 | 189.6 | 97% | 99% | 93% |

***Table 4.*** *PK/PD results for (3S,4R)-****11*** *and MRTX1133 after single i.p. doses in mice xenografted with PK-59 tumor cells.*

**Synthesis of 3,3-disubstituted β-lactones.** The unique distinctive of β-lactones makes them versatile synthetic intermediates and widely used tools in chemical biology. Moreover, they hold promise as structural elements of pharmaceutical agents and are common structural features in natural products.30 They exhibit ambident electrophilic reactivity at both the C2 and C4 carbons driven by strain release upon ring opening.22,31 The application and use of multi-substituted β-lactones in the above context is hampered by their limited synthetic access.32–34 Here we describe stereoselective and racemic strategies to access 3,3,4-trisubstituted β-lactones used to support our structure-activity and structure-stability studies. The full experimental details for the preparation of compounds **2** to (3*S*,4*R*)-**15** are outlined in the supporting information. Representative syntheses of 3,3,4-trisubstituted β-lactones are shown in the following schemes.



***Scheme 1.*** *Stereoselective route to 3,3,4-trisubstituted β-lactones (3R,4R)-****3****, (3S,4R)-****4****, (3S,4R)-****5****,**and (3S,4R)-****8****.*

β-Lactones with moderate steric bulk at the C3 position could be accessed enantioselectively via a chiral pool strategy.35 The general synthetic sequence for these target molecules started from commercial diethyl (2*R*)-2-hydroxy-3-methylsuccinate (**16**) (respectively diethyl (2*S*)-2-hydroxy-3-methylsuccinate for the *cis* isomer (3*R*,4*R*)-**3**. Adjustment of lactonization conditions by applying Mitsunobu conditions to (2*S*,3*R*)-4-(benzyloxy)-2-ethyl-3-hydroxy-2-methyl-4-oxobutanoic acid allowed us to obtain the desired absolute stereochemistry for the product β-lactone (benzyl (2*R*,3*S*)-3-ethyl-3-methyl-4-oxooxetane-2-carboxylate) leading to (3*R*,4*R*)-**3**. Final amide formation with MRTX1133 was then promoted under mild conditions with COMU as coupling reagent and 2,6-lutidine as base (Scheme 1).

Accessing 3,3,4-trisubstituted β-lactones with one of the substituents at C3 containing a branched α-carbon poses a challenge to synthesis. We developed a robust sequence that allowed us to access a variety of sterically hindered, and therefore chemically stabilized, β-lactones (Scheme 2). The sequence commences with appropriately substituted ethyl-2-alkyl propanoates (**20**) which are accessible in one step from commercial starting materials (Scheme 2). They were generally prepared either by a-methylation of the corresponding commercial esters or starting form cyclic ketones by Wittig-Horner olefination followed by hydrogenation of the double bond. Aldol reaction with ethyl-2-oxoacetate furnished the desired diethyl-2-alkyl-3-hydroxy-2-methylsuccinates (**21**). Hydrolysis to diacids (**22**) followed by regioselective esterification provided the corresponding 4-(benzyloxy)-2-alkyl-3-hydroxy-2-methyl-4-oxobutanoic acids (**23**), which were converted to the β-lactone benzyl esters (**24**) by treatment with PyBOP. This sequence yielded the esters **(24)** as racemic mixtures of *cis* (minor) and *trans* (major) geometric isomers in varying ratios. After chromatographic separation of the diastereoisomers and 2D NMR analysis, the desired *trans* isomers were subjected to chiral SFC separation to obtain the single enantiomers from which we liberated the free β-lactone acids (**26)**. At this point, we tentatively assigned the absolute stereochemistry of new compounds based on the empirical correlation of optical rotation, covalent activity in biochemical assay, and the known absolute stereochemistry of key compounds as obtained by X-ray crystallography.



***Scheme 2.*** *Synthetic routes to 3,3,4-trisubstituted β-lactones with cyclic substituents at C3,* (3*S*,4*R*)-**10** to *(3S,4R)-****15****.*

A complementary route to access C3-α-branched and C3-α-sp2 hybridized β-lactones is depicted in Scheme 3. Starting from benzyl 2-methyl-3-oxobutanoate (**27**) we prepared the common lactone precursor **29** in two steps. **31** (*trans* isomer) was obtained via base-promoted lactonization of the bromide **30**. The subsequent exchange of the *tert*-butyl to benzyl ester protecting group facilitated separation of the enantiomers by chiral chromatography. Debenzylation of single enantiomers of intermediate **32** provided the free acid **33** for which the measurement of optical rotation (OR) allowed the tentative assignment of absolute configuration as (3*S*,4*R*). The isomer with negative sense of rotation was carried forward to the final COMU mediated coupling with MRTX1133 to furnish (3*S*,4*R*)-**6** in 38% yield over two steps. Towards (3*S*,4*R*)-**9**, the common intermediate **29** was subjected to Davis oxidation36 and subsequent debenzylation by treatment with triethylsilane to provide 2-(2-(tert-butoxy)-1-hydroxy-2-oxoethyl)-2,3-dimethylbut-3-enoic acid. Mitsunobu lactonization and chiral separation of the obtained stereoisomers delivered the desired *trans* β-lactone as first eluting isomer (**35**). TFA mediated deprotection to acid **36** and COMU mediated coupling to MRTX1133 furnished (3*S*,4*R*)-**9** in 24% yield over two steps.



***Scheme 3.*** *Synthetic route to 3,3,4-trisubstituted β-lactones with alpha-branching, (3S,4R)-****6*** *and (3S,4R)-****9****.*

# CONCLUSIONS

Herein, we describe our structure-guided SAR studies on novel β-lactone electrophiles targeting Asp12 of KRASG12D expanding the use of this electrophile in medicinal chemistry discovered earlier by Shokat *et al*.22 Initial *in vitro* characterization of the first generation of β-lactone electrophile attached to Mirati’s MRTX1133 core scaffold supported by mechanistic studies let us hypothesize that the functional cellular activity for the described β-lactone compounds stem from both covalent modification of KRASG12D and reversible binding contributions of the compounds and/or their potential degradation products. Guided by the structural information of covalent (with KRASG12D) and non-covalent (with KRASWT) X-ray co-crystal complexes we developed an *in silico* workflow to predict specific reactivities and low energy conformations of the ligand-protein complexes of novel b-lactones. Calculations of transition state energy barriers supported us to prioritize design ideas for substitutions in the C3 position of new β-lactones prior to synthesis and establish SAR. We invested extensively to further expand the assay toolbox, in particular with regard to covalent target occupancy assays, to dissect covalent from non-covalent contributions to cellular assay readouts and to allow informed decisions on progressing our SAR investigation. Our studies on structure-reactivity and structure-stability reveal a strong and direct correlation between the desired reactivity towards Asp12 and undesired non-specific reaction with GSH and solvent molecules under our assay conditions. Within the chemical scope of the study, this correlation was investigated primarily under the aspect of steric factors. We found that by incorporation cycloalkyl substituents at C3 of the β-lactone moiety we could efficiently balance the window for specific reactivity towards KRASG12D with intrinsic reactivity, presumably due to an interplay of steric and electronic factors. This endeavor resulted in the cyclobutyl derivative (3*S*,4*R*)-**11** which we profiled extensively *in vitro* as well as *in vivo*, giving first insights into classical pharmacokinetic parameters for this type of b-lactone-based inhibitors. Not yet being optimized for oral bioavailability, (3*S*,4*R*)-**11** yielded comparable total exposure when given at 30 mg/kg intraperitoneally to mice as 10 mg/kg of MRTX1133. Given that MRTX1133 demonstrated strong PD modulation in contrast to (3*S*,4*R*)-**11**, we concluded that the free exposure to potency ratio of (3*S*,4*R*)-**11** would need to be further optimized to evaluate whether a therapeutic benefit can be obtained *in vivo* through a covalent mechanism of action compared to very potent non-covalent inhibition of the target. The proteome wide selectivity assessment of selected early SAR compounds suggests that increasing hydrolytic stability is conferred with decreased selectivity for Asp labelling. Follow up studies are needed to gain a deeper understanding of Asp-specific reaction kinetics and identification of discriminating factors to reduce reactivity towards cysteine residues which generally do offer a higher intrinsic nucleophilic reactivity than Asp. Our most advanced inhibitors provide a basis for further rational exploration of this class of electrophiles. The described β-lactones are versatile chemical building blocks in a broad sense for which we have detailed several enantioselective and racemic synthetic strategies. They can be attached to a variety of non-covalent lead scaffolds to serve as a novel class of tunable, electrophilic covalent anchor points. The outlined synthetic strategies are of value for future studies seeking to expand covalent labelling of Asp residues on targets beyond KRASG12D. Overall, our work expands the toolbox to selectively target KRASG12D and provides optimization strategies regarding *in silico* workflow, structural biology, *in vitro* assay approaches, and chemistry to apply covalent aspartic acid targeting for medicinal chemistry more broadly.

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