The Alkyne Moiety as a Latent Electrophile in Irreversible Covalent Small Molecule Inhibitors of Cathepsin K

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ABSTRACT: Irreversible covalent inhibitors can have a beneficial pharmacokinetic/pharmacodynamics profile but are still often avoided due to the risk of indiscriminate covalent reactivity and the resulting adverse effects. To overcome this potential liability, we introduced an alkyne moiety as a latent electrophile into small molecule inhibitors of cathepsin K (CatK). Alkyne-based inhibitors do not show indiscriminate thiol reactivity but potently inhibit CatK protease activity by formation of an irreversible covalent bond with the catalytic cysteine residue, confirmed by crystal structure analysis. The rate of covalent bond formation (kob) does not correlate with electrophilicity of the alkyne moiety, indicative of a proximity-driven reactivity. Inhibition of CatK-mediated bone resorption is validated in human osteoclasts. Together, this work illustrates the potential of alkynes as latent electrophiles in small molecule inhibitors, enabling the development of irreversible covalent inhibitors with an improved safety profile.

INTRODUCTION
Irreversible covalent inhibition of a target protein minimizes the required systemic drug exposure as protein activity can only be restored by de novo protein synthesis, resulting in a prolonged therapeutic effect long after the compound is cleared from the blood.12 Strategically placing an electrophilic moiety on the inhibitor will allow it to undergo attack by a nucleophilic amino acid residue upon binding to the target protein, forming an (ir)reversible bond that is much stronger than typical noncovalent interactions. However, the ability to form a covalent bond with the target enzyme has raised concerns about indiscriminate reactivity with off-target proteins.3–5 even though some of the most prescribed drugs are covalent irreversible binders.6,7 This led to the disfavor of covalent modifiers as drug candidates until the recent successful development of irreversible covalent kinase inhibitors ibrutinib and afatinib, which form an irreversible covalent bond between an acrylamide warhead and a nonconserved cysteine residue on the ATP-binding site but also with nontargeted cellular thiols.11 The ability to form covalent adducts with off-target proteins has been linked to an increased risk of unpredictable idiosyncratic toxicity along with the daily drug dose administered to patients.11–14 This risk can be reduced by incorporating less reactive electrophilic moieties into irreversible covalent inhibitors.

Terminal alkynes are generally considered “inert” toward cellular components in the absence of radical initiators and are therefore often used in bioorthogonal approaches as chemoselective “Click” handles.15,16 However, our group has shown a C-terminal propargyl moiety on ubiquitin to react in an activity-based manner with the catalytic cysteine residue in...
Markovnikov-type thiovinyl product,23 here we show that driving the formation of the thermodynamically unfavored properties of CatK or because of o whether this is due to inhibition of nonskeletal degradation.

We therefore assessed the potency of our inhibitors in an in vitro inhibition assay on recombinant human cathepsins (Table 2). As reported, ODN is selective for hCatK with an IC₅₀ below 1 nM. Noncovalent interactions have been optimized for ODN, and we anticipated that replacing the polarized nitrile moiety by a nonpolarized alkyne moiety would reduce metabolic liabilities.26 Alkyne electrophilicity increases if an electron-withdrawing substituent is introduced on the terminal position,19,35,36 while remaining less electrophilic than acrylamides, and therefore, electron-deficient alkyne 6 was taken along to investigate the effect of electrophilicity on the inhibitor selectivity. Conjugate addition of cysteine to electron-deficient internal alkyne in aqueous medium has been reported and has recently been utilized in the irreversible covalent kinase inhibitor acalabrutinib.15,35,36

Indiscriminate Thiol Reactivity. Indiscriminate thiol reactivity was assessed following an established protocol in which nitrile-based inhibitors form an irreversible covalent adduct with cysteine37 (Table 1, Figure S2). Briefly, compounds were incubated with cysteine for 23 h after which they were analyzed by LC−MS. The adduct formation was quantified from the UV trace. ODN and nitrile 2 show adduct formation that increases upon increasing the pH of the buffer, as do acrylamide-based inhibitors ibrutinib and afatinib and irreversible pan-cathepsin inhibitor E-64.38 Adduct formation was not detected for alkyne-based inhibitors 3−5, which supports our hypothesis that the unactivated alkyne is not reactive toward cysteine residues in nontargeted proteins. As expected,36 adduct formation with electron-deficient alkyne 6 and acalabrutinib was observed, underlining the importance of alkyn electrophilicity in indiscriminate thiol reactivity.

Glutathione (GSH), a tripeptide with a cellular concentration of 0.5−10 mM,39 is a commonly used biological thiol to assess the risk of iodosyncratic toxicity. GSH adduct was observed for acrylamides and electron-deficient alkyne, as reported,11,36 but not for inhibitor 4 upon incubation with 5 mM GSH.

In Vitro Inhibition. A recurring issue in CatK drug development is the difference in amino acids at the active-site for rodentCatK compared to humanCatK, thus reducing the apparent potency of ODN up to 182-fold in mice and rats.30 We therefore assessed the potency of our inhibitors in an in vitro inhibition assay on recombinant human cathepsins (Table 2). As reported, ODN is selective for hCatK with an IC₅₀ below 1 nM. Noncovalent interactions have been optimized for ODN, and we anticipated that replacing the polarized nitrile moiety by a nonpolarized alkyne moiety would decrease the interaction with active-site residues, reducing the noncovalent complex formation (kₘₐₓ). This is indeed reflected in increased IC₅₀ values for all alkyne-based inhibitors.
Table 1. Indiscriminate Thiol Reactivity

| Compound | Cysteine adduct | GSH adduct |
|----------|----------------|------------|
|          | pH 5.5         | pH 7.5     | pH 8.0     | pH 7.5     |
| ODN      | <1%            | 17%        | 48%        | 0%         |
| 2        | 5%             | 91%        | 98%        | <1%        |
| 3        | <1%            | 0%         | 0%         | 0%         |
| 4        | <1%            | 0%         | <1%        | <1%        |
| 5        | <1%            | <1%        | 0%         | 2%         |
| 6        | <1%            | 55%        | 66%        | 47%        |
| E-64     | <1%            | 68%        | 79%        | 64%        |
| Afatinib | 92%            | 98%        | 95%        | 94%        |
| Ibrutinib| 9%             | 99%        | 96%        | 91%        |
| Acalabrutinib | 19% | 98% | 97% | 95% |

Adduct formation quantified from LC−MS UV trace after 23 h incubation with 10 mM cysteine or 5 mM GSH at 37 °C in buffer at different pH values. Reversible adduct formation.

Selectivity for CatK over related human cathepsins was conserved for alkynes 4 and 5, while all selectivity is lost for electron-deficient alkyne 6. Inhibition of hCatK activity was validated in a gel-based probe labeling experiment with quenched activity-based probe BMV109 (Figure S3).

Binding Mode of Alkynes Is Irreversible and Covalent. Reversibility of hCatK inhibition was assessed in a jump dilution assay. Recombinant hCatK was incubated with inhibitors at high concentration to allow full active-site occupation and subsequently diluted 300-fold into fluorogenic substrate (Z-FR-AMC) solution resulting in an increase of substrate hydrolysis in case of reversible inhibition (Figure 1). The progress curve shows that ODN is a (fast) reversible inhibitor, while inhibition by alkynes 4−6 is irreversible.

The nature of the inhibitor−cathepsin interaction was elucidated by LC−MS analysis of intact CatK and intact CatK−inhibitor complexes (Figure 2). Recombinant hCatK was incubated with inhibitor for 6 h to allow full covalent bond formation and submitted for measurement. An increase in the deconvoluted mass corresponding to addition of the inhibitor complex was observed for alkynes 4−6, concomitant with inhibition of hCatK proteolytic activity after dilution in Z-FR-AMC. Control: E-64 is an irreversible pan-cathepsin inhibitor.

The apparent potency of irreversible covalent inhibitors increases upon longer incubation with the enzyme, since the interaction of inhibitor with enzyme is not at equilibrium. As a result, the potency of these compounds can better be assessed by comparison of the $k_{\text{inact}}/K_i$ ratio, which can be derived from the progress curve of substrate hydrolysis when the reaction is initiated by addition of the enzyme (Table 3; more details in the SI). Interestingly, the maximum rate of covalent bond formation ($k_{\text{inact}}$) did not correlate with reactivity of the alkyne, as $k_{\text{inact}}$ for alkynes 4 and 5 is faster than for electron-deficient alkyne 6. We hypothesize that halogen bonding by the terminal bromine with the thiol moiety on hCatK positions the alkyne less optimal relative to the cysteine residue thus reducing the rate of proximity-driven C=S bond formation.

The rate of covalent bond formation for ODN ($k_i$) is faster than for the alkynes, also when correcting for the reverse reaction ($k_f$).

Alkynes Form a Covalent Thiovinyl Bond with Catalytic Cysteine Residue. Covalent CatK−alkyne 4 complex was submitted to bottom-up proteomic analysis to identify which amino acid residue is modified. In the tryptic digestion of unreacted CatK, the various length variant peptides containing the NQGQWAFSSVGALEGQLKKK indicates inhibitor 4 is on the second cysteine residue (Cys25, Figure S4C). Together, this clearly shows that one of these cysteine residues is labeled, most likely catalytic Cys25.

The formation of a vinyl thiether linkage between catalytic Cys25 on hCatK and the internal carbon of the alkyne moiety was confirmed by solving the crystal structure of CatK−inhibitor 7 complex (Figure 3). Mature CatK was inactivated

Table 2. In Vitro IC$_{50}$ Values (M) against Proteolytic Activity of Cysteine Proteases

| Compd | hCatK | hCatL | hCatS | hCatV | hCatB | Papain |
|-------|-------|-------|-------|-------|-------|--------|
| ODN   | 5.6×10$^{-10}$ ± 2.2×10$^{-12}$ | 5.8×10$^{-6}$ ± 7.2×10$^{-7}$ | 2.4×10$^{-4}$ ± 5.7×10$^{-10}$ | 6.0×10$^{-7}$ ± 4.0×10$^{-8}$ | 6.3×10$^{-6}$ ± 2.5×10$^{-6}$ | 6.9×10$^{-7}$ ± 5.8×10$^{-8}$ |
| 2     | 5.7×10$^{-10}$ ± 9.0×10$^{-12}$ | >1.0×10$^{-6}$ | 1.8×10$^{-6}$ ± 3.0×10$^{-10}$ | 9.1×10$^{-7}$ ± 1.1×10$^{-7}$ | 2.1×10$^{-6}$ ± 6.6×10$^{-8}$ | 2.3×10$^{-7}$ ± 1.2×10$^{-8}$ |
| 3     | 2.6×10$^{-12}$ ± 2.2×10$^{-10}$ | >1.0×10$^{-4}$ | >1.0×10$^{-4}$ | 1.1×10$^{-8}$ ± 8.4×10$^{-7}$ | 2.4×10$^{-5}$ ± 1.4×10$^{-4}$ | 9.4×10$^{-6}$ ± 3.6×10$^{-5}$ | 1.4×10$^{-5}$ ± 1.8×10$^{-6}$ |
| 4     | 2.9×10$^{-7}$ ± 8.7×10$^{-9}$ | >1.0×10$^{-4}$ | >1.0×10$^{-4}$ | 1.6×10$^{-5}$ ± 1.8×10$^{-6}$ | 4.6×10$^{-5}$ ± 2.3×10$^{-4}$ | 4.0×10$^{-5}$ ± 4.4×10$^{-5}$ | 2.2×10$^{-7}$ ± 3.9×10$^{-6}$ |
| 5     | 3.5×10$^{-7}$ ± 1.2×10$^{-8}$ | >1.0×10$^{-4}$ | 1.0×10$^{-7}$ ± 4.6×10$^{-9}$ | 5.5×10$^{-7}$ ± 2.9×10$^{-9}$ | 1.6×10$^{-6}$ ± 8.8×10$^{-10}$ | 9.9×10$^{-6}$ ± 5.9×10$^{-7}$ | 6.3×10$^{-7}$ ± 7.3×10$^{-7}$ |
| 6     | 4.7×10$^{-8}$ ± 1.8×10$^{-9}$ | 1.0×10$^{-7}$ ± 4.6×10$^{-9}$ | 5.5×10$^{-9}$ ± 2.9×10$^{-9}$ | 1.6×10$^{-6}$ ± 8.8×10$^{-10}$ | 9.9×10$^{-6}$ ± 5.9×10$^{-7}$ | 6.3×10$^{-7}$ ± 7.3×10$^{-7}$ | 6.3×10$^{-7}$ ± 7.3×10$^{-7}$ |
| E-64  | 1.9×10$^{-9}$ ± 3.2×10$^{-11}$ | 3.4×10$^{-9}$ ± 1.8×10$^{-10}$ | NA | NA | NA | 2.4×10$^{-9}$ ± 8.2×10$^{-10}$ |

Incubation of cysteine protease and inhibitor for 30 min prior to addition of fluorogenic substrate. Protease concentrations: hCatK (150 pM), hCatL (5 pM), hCatS (1 nM), hCatV (25 pM), hCatB (1 nM), and papain (3 nM). Mean ± SD for a single representative experiment (triplicate measurement). NA = not available. More details are available in the SI.
with MMTS for purification and storage and reactivated with DTT in the presence of alkyne inhibitors at high concentration (200 μM) to prevent self-degradation of CatK. Solubility of alkyne 4 and 5 was not sufficient, which can be contributed to the fluoroleucine moiety. We therefore synthesized alkyne 7, a closely related derivative in which the fluorine on the L-leucine building block was replaced by a proton to improve solubility (Scheme S2). The resulting CatK−inhibitor 7 complex was crystallized using a sitting drop method, and the structure could be solved at 1.7 Å resolution using maximum-likelihood free-kick (ML FK) electron density map47 (Figure S5). The refined structure unambiguously revealed the presence of a bond between the thiol atom of Cys25 and the internal carbon in alkyne 7, with a C−S distance of 1.8 Å.

Inhibition of Bone Resorption Activity in Osteoclasts. Having established the covalent, irreversible inhibition of CatK on purified recombinant enzyme, we decided to test the inhibitory properties in a biologically relevant setting; inhibition of bone resorption by osteoclasts (OCs). OCs are the cells that degrade the bone matrix by secretion of acid and CatK into the resorption lacunae, resulting in the cleavage of collagen type I. OCs are essential in bone repair, and aberrant activity is observed in numerous diseases including osteoporosis, rheumatoid arthritis, giant cell tumor of the bone, and bone metastases.48−50

Table 3. In Vitro Kinetic Evaluation of CatK Inhibition

| Compound | $k_{on}$ (min$^{-1}$) | $K_a$ (μM) | $k_{cat}/K_a$ (μM$^{-1}$min$^{-1}$) |
|----------|----------------------|------------|----------------------------------|
| 4        | 0.011 ± 0.00073      | 0.21 ± 0.047 | 53 × 10³ ± 12 × 10³             |
| 5        | 0.047 ± 0.0037      | 3.3 ± 0.60   | 15 × 10³ ± 2.9 × 10³            |
| 6        | 0.019 ± 0.0027      | 0.19 ± 0.083  | 99 × 10³ ± 45 × 10³             |
| E−64     | 0.081 ± 0.0052      | 0.011 ± 0.0016 | 71 × 10⁴ ± 11 × 10⁴           |

Inhibition of osteoclastic CatK was studied by culturing OCs on cortical bone slices in the presence of inhibitor. Mature OCs were obtained by treatment of CD14+ monocytes with M-CSF (macrophage colony stimulating factor) and RANKL (receptor activator of nuclear factor κB ligand) to stimulate differentiation to mature OCs (Figure 4).51 Mature OCs are formed by merging of mononuclear osteoclast precursors to form large multinucleated cells, a process that continued until the end of the culturing period. When the culturing medium

Figure 3. Crystal structure of alkyne 7 bound covalent to catalytic Cys25 in CatK. (A) Structure of inhibitor 7 before and after covalent bond formation with CatK. (B) X-ray structure of inhibitor 7 bound to Cys25 in CatK. PDB: 6QBS.
Inhibition of CatK activity in human osteoclasts (OCs). (A) Maturation of OCs from monocytes. (B) CD14+ monocytes on bone slices were treated with M-CSF (day 0) and RANKL (day 3) to stimulate differentiation to mature OCs. Medium containing either inhibitor or DMSO was refreshed on day 7, 10, 13, and 16. At day 21, OCs were washed away and lysed, and bone slices were stained to visualize bone resorption. (C) Bone resorption visualized by staining of resorption pits with Coomassie Brilliant Blue. More staining means more resorption pits and, thus, more bone resorption activity. Normal OCs predominantly form deep trenches (paths), while OCs lacking CatK form small pits (circular dots). (D) Schematic overview of CatK activation. (E) CatK activity and expression in OC lysates. Top: fluorescence scan of CatK bound to irreversible activity-based probe BMV109 shows mature, active CatK. Middle/bottom: Western blotting against CatK shows total amount of CatK present in OC lysates. Darker bands indicate more CatK activity/expression.

was refreshed (every 3 days), inhibitor was freshly added to make sure there always is inhibitor present to inhibit CatK in the newly formed mature OCs. The OCs were cultured on bone slices for sufficient time to clearly observe bone resorption. After culturing for 21 days, the OCs were washed off the bone slices and the resorption pits were stained to visualize bone resorption activity. OCs with normal CatK activity form trenches, resorbing the bone while they move over the surface of the bone. Previously published observations in OCs from CatK−/− mice show that OCs lacking CatK are still able to form shallow pits, but unable to form trenches, with accumulation of collagen I fragments in the lysosomes.55 Staining of bone slices for bone resorption showed formation of deep trenches for samples treated with 3 nM ODN, while 15 nM ODN resulted in the formation of shallow pits (Figure 4C), corresponding to an effective dose of around 15 nM.53 Treatment with 4 successfully inhibited bone resorption at concentrations from 80 nM, while inhibition with 5 was nonconclusive; we observed trenches as well as pits at all tested concentrations. Quantification of the total resorption area confirmed these observations, even though it is not possible to distinguish between shallow pits and deep trenches (Figure S6). From this experiment, we concluded that alkynes 4 and 5 are inhibitors of bone resorption with a higher potency than expected based on their potency to inhibit recombinant CatK.

Next, we treated the OC lysates with activity-based cathepsin probe BMV109 to assess whether the observed inhibition of bone resorption could be correlated with CatK activity (Figure 4D,E). CatK activity for OCs treated with DMSO is low, which is expected because mature CatK in its uninhibited form is self-degrading.54 and the observed bone resorption is the result of secreted mature CatK activity. Additionally, we expect that intracellular CatK is predominantly catalytic inactive pro-CatK, which is activated by cleavage of the activation peptide, an autoproteolytic event that requires an environment with a low pH for example lysosomes and the resorption lacunae.55 Interestingly, we observe a strong increase of mature CatK activity in all samples treated with ODN, while samples with inhibitor 4 or 5 do not show any CatK activity. The observed increase in mature CatK activity for ODN-treated samples does not reflect the actual intracellular proteolytic activity, but is the result of displacement of reversibly bound ODN by excess of irreversible probe BMV109. Alkynes 4 and 5 form an irreversible covalent bond with CatK, and can thus not be displaced by BMV109. Western blotting for CatK revealed an increase in the intracellular levels of mature CatK for OCs that were treated with high concentration of any inhibitor, which could be the result of inhibition of proteolytic CatK activity, which would normally degrade mature CatK.

Counting OCs that were cultured on plastic revealed an increase in the number of OCs for the highest concentrations of ODN, 4 and 5 (Figure S7). This is in agreement with previous reports that observed an increase of OC maturation as a response to CatK activity loss; the same number of bone marrow cells from CatK−/− mice led to a greater number of active OCs compared to bone marrow cells from the control mice.52 A significant increase in CatK expression upon 100 nM ODN treatment has been reported, without an increase in the number of OCs.56 We hypothesize that complete inhibition of CatK activity stimulates the maturation of OCs, and further investigations to identify the feedback mechanism are ongoing.

CONCLUSION

To conclude, alkynes are not only suitable as latent electrophiles in (large) peptides but also in small molecule inhibitors, as shown here for inhibition of cysteine protease cathepsin K (CatK). Alkyne-based covalent inhibitors do not show indiscriminate thiol reactivity but do form an irreversible
covalent bond formation with CatK, as confirmed by MS analysis of the (intact) CatK–inhibitor complexes. X-ray crystallography confirmed the formation of the Markovnikov-type product between the active-site cysteine thiol and the internal carbon of the alkylene moiety. Kinetic evaluation shows that the rate of covalent bond formation ($k_{\text{act}}$) does not correlate with electrophilicity of the alkylene, supporting our hypothesis of proximity-driven reactivity. Optimization of the alkylene position relative to the cysteine residue could result in more potent compounds with faster covalent bond formation while not compromising on indiscriminate thiol reactivity. Treatment of human osteoclasts (OCs) with alkylones 4 and 5 showed a potent inhibition of CatK-mediated bone resorption activity, with only a 5-fold difference in effective dose between ODN and inhibitor 4. Further investigations into the biological effect of irreversible inhibition of CatK are ongoing.

Finally, we urge everyone using the alkylene moiety as a Click handle to be careful with the assumption that the alkylene is truly bioorthogonal; the binding of a small molecule inhibitor can be strong enough to initiate a thiol–alkylene reaction when the alkylene moiety is positioned in close proximity to a cysteine residue. More importantly, based on the proof-of-concept studies described herein, we foresee latent electrophiles such as the alkylene to be of great value in future development of cysteine-targeting covalent inhibitory drugs with a reduced risk of idiosyncratic toxicity.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b11027.

Detailed experimental procedures, figures, and crystallographic data (PDF)

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**Notes**

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**REFERENCES**

(1) Singh, J.; Petter, R. C.; Baillie, T. A.; Whitty, A. The resurgence of covalent drugs. *Nat. Rev. Drug Discovery* 2011, 10 (4), 307–317.

(2) Bauer, R. A. Covalent inhibitors in drug discovery: from accidental discoveries to avoided liabilities and designed therapies. *Drug Discovery Today* 2015, 20 (9), 1061–1073.

(3) Silva, D. G.; Ribeiro, J. F. R.; De Vita, D.; Cianni, L.; Franco, C. H.; Freitas-Junior, L. H.; Moraes, C. B.; Rocha, J. R.; Burtoloso, A. C. B.; Kenny, P. W.; Leitão, A.; Montanari, C. A. A comparative study of warheads for design of cysteine protease inhibitors. *Bioorg. Med. Chem. Lett.* 2017, 27 (22), 5031–5035.

(4) Lonsdale, R.; Burgess, J.; Colclough, N.; Davies, N. L.; Lenz, E. M.; Orton, A. L.; Ward, R. A. Expanding the Armory: Predicting and Tuning Covalent Warhead Reactivity. *J. Chem. Inf. Model.* 2017, 57 (12), 3124–3137.

(5) Barf, T.; Kaptein, A. Irreversible Protein Kinase Inhibitors: Balancing the Benefits and Risks. *J. Med. Chem.* 2012, 55 (14), 6243–6262.

(6) Baillie, T. A. Targeted Covalent Inhibitors for Drug Design. *Angew. Chem., Int. Ed.* 2016, 55 (43), 13408–13421.

(7) González-Bello, C. Designing Irreversible Inhibitors—Worth the Effort? *ChemMedChem* 2016, 11 (1), 22–30.

(8) Solca, F.; Dahl, G.; Zoephe, A.; Bader, G.; Sanderson, M.; Klein, C.; Kraemer, O.; Himmelbach, F.; Haaksma, E.; Adolfs, G. R. Target Binding Properties and Cellular Activity of Alatinib (BBW 2992), an Irreversible ErbB Family Blocker. *J. Pharmacol. Exp. Ther.* 2012, 343 (2), 342.

(9) Pan, Z.; Scheeren, H.; Li, S.-J.; Schultz, B. E.; Sprengeler, P. A.; Burrill, L. C.; Mendonca, R. V.; Sweeney, M. D.; Scott, K. C. K.; Grothaus, P. G.; Jeffery, D. A.; Spoerke, J. M.; Honigberg, L. A.; Young, P. R.; Dalrymple, S. A.; Palmer, J. T. Discovery of Selective Irreversible Inhibitors for Bruton’s Tyrosine Kinase. *ChemMedChem* 2007, 2 (1), 58–61.

(10) Liu, Q.; Sabnis, Y.; Zhao, Z.; Zhang, T.; Buhrlage, S. J.; Jones, L. H.; Gray, N. S. Developing irreversible inhibitors of the protein kinase cysteine. *Chem. Biol.* 2013, 20 (2), 146–159.

(11) Shibata, Y.; Chiba, M. The Role of Extrahepatic Metabolism in the Pharmacokinetics of the Targeted Covalent Inhibitors Afatinib, Ibrutinib, and Neratinib. *Drug Metab. Dispos.* 2015, 43 (3), 375.

(12) Nakayama, S.; Atsumi, R.; Takakusa, H.; Kobayashi, Y.; Kurihara, A.; Nagai, Y.; Nakai, D.; Okazaki, O. A Zone Classification System for Risk Assessment of Idiosyncratic Drug Toxicity Using Daily Dose and Covalent Binding. *Drug Metab. Dispos.* 2009, 37 (9), 1970.

(13) Schwöbel, J. A. H.; Koleva, Y. K.; Enoch, S. J.; Bajot, F.; Hewitt, M.; Madden, J. C.; Roberts, D. W.; Schultz, T. W.; Cronin, M. T. D. Measurement and Estimation of Electrophilic Reactivity for Predictive Toxicology. *Chem. Res.* 2011, 111 (44), 2562–2596.

(14) Zhao, Z.; Bourne, P. E. Progress with covalent small-molecule kinase inhibitors. *Drug Discovery Today* 2018, 23 (3), 727–735.

(15) Wright, M. H.; Sieber, S. A. Chemical proteomics approaches for identifying the cellular targets of natural products. *Nat. Prod. Rep.* 2016, 33 (5), 681–708.

(16) Exceptions are published for MAO inhibitors in which the terminal alkylene acts as a nucleophile and for terminal alkylens that are transformed into more reactive ketene species by CYP450 enzymes. (a) Sadler, N. C.; Nandhikonda, P.; Webb-Robertson, B.-J.; Ansong, C.; Anderson, L. N.; Smith, J. N.; Corley, R. A.; Wright, A. T. Hepatic Cytochrome P450 Activity, Abundance, and Expression Throughout Human Development. *Drug Metab. Dispos.* 2016, 44 (7), 984.  
    (b) Fan, P. W.; Gu, C.; Marsh, S. A.; Stevens, J. C. Mechanism-Based Inactivation of Cytochrome P450 2B6 by a Novel Terminal Acetylene Inhibitor. *Drug Metab. Dispos.* 2003, 31 (1), 28. (c) Borstnar, R.; Repič, M.; Kržan, M.; Mavri, J.; Vianello, R. Irreversible Inhibition of Monoamine Oxidase B by the Antiparkinsonian Medicines Rasagiline and Selegiline: A Computational Study. *Eur. J. Org. Chem.* 2011, 2011 (32), 6419–6433.

(17) Ekkebus, R.; van Kasteren, S. I.; Kulathu, Y.; Scholten, A.; Berlin, I.; Geurink, P. P.; de Jong, A.; Goerdalyal, S.; Neefjes, J.; Heck, A. J. R.; Komander, D.; Ovaa, H. On Terminal Alkynes That Can React with Active-Site Cysteine Nucleophiles in Proteases. *J. Am. Chem. Soc.* 2013, 135 (8), 2867–2870.

(18) Orlov, N. V. Metal Catalysis in Thiolation and Selenylation Reactions of Alkynes Leading to Chalcogen-Substituted Alkenes and Dienes. *ChemistryOpen* 2015, 4 (6), 682–697.
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1824 (1), 68

from structure, function and regulation to new frontiers. Biochim. Biophys. Acta, Proteins Proteomics 2012, 1824 (1), 68–88.

Vizoviček, M.; Fonović, M.; Turk, B. Cysteine cathepsins in extracellular matrix remodeling: Extracellular matrix degradation and beyond. Matrix Biol. 2018, DOI: 10.1016/j.matbio.2018.01.024.

Gauthier, J. Y.; Chauret, N.; Cromlish, W.; Desmarias, S.; Duong, L. T.; Falgueryret, J.-P.; Kimmel, D. B.; Lamontagne, S.; Léger, S.; LeRiche, T.; Li, C. S.; Masse, J.-M.; Resnati, G.; Terraneo, G. The Halogen Bond.

Angew. Chem., Int. Ed. 2013, 52 (32), 8210–8212.

Turk, V.; Stoka, V.; Vasiljeva, O.; Renko, M.; Sun, T.; Turk, B.; Turk, D. Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochim. Biophys. Acta, Proteins Proteomics 2012, 1824 (1), 68–88.

Vizoviček, M.; Fonović, M.; Turk, B. Cysteine cathepsins in extracellular matrix remodeling: Extracellular matrix degradation and beyond. Matrix Biol. 2018, DOI: 10.1016/j.matbio.2018.01.024.

Gauthier, J. Y.; Chauret, N.; Cromlish, W.; Desmarias, S.; Duong, L. T.; Falgueryret, J.-P.; Kimmel, D. B.; Lamontagne, S.; Léger, S.; LeRiche, T.; Li, C. S.; Masse, J.-M.; Resnati, G.; Terraneo, G. The Halogen Bond.

Angew. Chem., Int. Ed. 2013, 52 (32), 8210–8212.

Turk, V.; Stoka, V.; Vasiljeva, O.; Renko, M.; Sun, T.; Turk, B.; Turk, D. Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochim. Biophys. Acta, Proteins Proteomics 2012, 1824 (1), 68–88.

Vizoviček, M.; Fonović, M.; Turk, B. Cysteine cathepsins in extracellular matrix remodeling: Extracellular matrix degradation and beyond. Matrix Biol. 2018, DOI: 10.1016/j.matbio.2018.01.024.

Gauthier, J. Y.; Chauret, N.; Cromlish, W.; Desmarias, S.; Duong, L. T.; Falgueryret, J.-P.; Kimmel, D. B.; Lamontagne, S.; Léger, S.; LeRiche, T.; Li, C. S.; Masse, J.-M.; Resnati, G.; Terraneo, G. The Halogen Bond.

Angew. Chem., Int. Ed. 2013, 52 (32), 8210–8212.

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Vizoviček, M.; Fonović, M.; Turk, B. Cysteine cathepsins in extracellular matrix remodeling: Extracellular matrix degradation and beyond. Matrix Biol. 2018, DOI: 10.1016/j.matbio.2018.01.024.

Gauthier, J. Y.; Chauret, N.; Cromlish, W.; Desmarias, S.; Duong, L. T.; Falgueryret, J.-P.; Kimmel, D. B.; Lamontagne, S.; Léger, S.; LeRiche, T.; Li, C. S.; Masse, J.-M.; Resnati, G.; Terraneo, G. The Halogen Bond.

Angew. Chem., Int. Ed. 2013, 52 (32), 8210–8212.

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Vizoviček, M.; Fonović, M.; Turk, B. Cysteine cathepsins in extracellular matrix remodeling: Extracellular matrix degradation and beyond. Matrix Biol. 2018, DOI: 10.1016/j.matbio.2018.01.024.

Gauthier, J. Y.; Chauret, N.; Cromlish, W.; Desmarias, S.; Duong, L. T.; Falgueryret, J.-P.; Kimmel, D. B.; Lamontagne, S.; Léger, S.; LeRiche, T.; Li, C. S.; Masse, J.-M.; Resnati, G.; Terraneo, G. The Halogen Bond.

Angew. Chem., Int. Ed. 2013, 52 (32), 8210–8212.

Turk, V.; Stoka, V.; Vasiljeva, O.; Renko, M.; Sun, T.; Turk, B.; Turk, D. Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochim. Biophys. Acta, Proteins Proteomics 2012, 1824 (1), 68–88.

Vizoviček, M.; Fonović, M.; Turk, B. Cysteine cathepsins in extracellular matrix remodeling: Extracellular matrix degradation and beyond. Matrix Biol. 2018, DOI: 10.1016/j.matbio.2018.01.024.

Gauthier, J. Y.; Chauret, N.; Cromlish, W.; Desmarias, S.; Duong, L. T.; Falgueryret, J.-P.; Kimmel, D. B.; Lamontagne, S.; Léger, S.; LeRiche, T.; Li, C. S.; Masse, J.-M.; Resnati, G.; Terraneo, G. The Halogen Bond.

Angew. Chem., Int. Ed. 2013, 52 (32), 8210–8212.

Turk, V.; Stoka, V.; Vasiljeva, O.; Renko, M.; Sun, T.; Turk, B.; Turk, D. Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochim. Biophys. Acta, Proteins Proteomics 2012, 1824 (1), 68–88.
(53) Pirapaharan, D. C.; Soe, K.; Panwar, P.; Madsen, J. S.; Bergmann, M. L.; Overgaard, M.; Brömme, D.; Delaisse, J.-M. A Mild Inhibition of Cathepsin K Paradoxically Stimulates the Resorptive Activity of Osteoclasts in Culture. Calci...J. 2019, 104 (1), 92–101.

(54) Thompson, S. K.; Halbert, S. M.; Bossard, M. J.; Tomaszek, T. A.; Levy, M. A.; Zhao, B.; Smith, W. W.; Abdel-Meguid, S. S.; Janson, C. A.; D’Alessio, K. J.; McQueney, M. S.; Amegadzie, B. Y.; Hanning, C. R.; Desjarlais, R. L.; Briand, J.; Sarkar, S. K.; Huddleston, M. J.; Ijames, C. F.; Carr, S. A.; Garnes, K. T.; Shu, A.; Heys, J. R.; Bradbeer, J.; Zembryki, D.; Lee-Rykaczewski, L.; James, I. E.; Lark, M. W.; Drake, F. H.; Gowen, M.; Gleason, J. G.; Veber, D. F. Design of potent and selective human cathepsin K inhibitors that span the active site. Proc. Natl. Acad. Sci. U. S. A. 1997, 94 (26), 14249.

(55) McQueney, M. S.; Amegadzie, B. Y.; D’Alessio, K.; Hanning, C. R.; McLaughlin, M. M.; McNulty, D.; Carr, S. A.; Ijames, C.; Kurdyla, J.; Jones, C. S. Autocatalytic Activation of Human Cathepsin K. J. Biol. Chem. 1997, 272 (21), 13955–13960.

(56) Leung, P.; Pickarski, M.; Zhuo, Y.; Masarachia, P. J.; Duong, L. T. The effects of the cathepsin K inhibitor odanacatib on osteoclastic bone resorption and vesicular trafficking. Bone 2011, 49 (4), 623–635.