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Reversible Covalent Reactions of Aldehydes and Salicylaldehydes Using a Lysine-Model Substrate

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Abstract: Covalent modification of lysine residues has gained significant attention due to its potential application in drug development and chemical biology. Lysine is an essential amino acid, abundant in proteins, and plays a critical role in many biological processes. In this study, we investigated aldehydes for imine-based chemistries and their reactivity profiles using a lysine-surrogate. By monitoring reactions of various aldehydes and salicylaldehydes over time, we determined dissociation constants (K_D) for each warhead, reflecting the binding affinity towards the surrogate substrate. Strikingly, our data revealed remarkable differences in affinity depending on the substitution of the warheads. Additionally, we analyzed the kinetic profile of selected aldehydes and salicylaldehydes, which revealed significant disparity in their reaction kinetics. Aldehydes reacted quickly, reaching equilibrium rapidly, whereas salicylaldehydes exhibited considerably slower reaction times, in some cases requiring several hours to reach equilibrium. These differences emphasize how the nature of the warhead structure influences the kinetics of covalent binding to lysine residues. Overall, our study provides valuable insights into the application of reversible covalency to target lysines with reactive warheads that can further inspire development of innovative chemical modifications for drug discovery and chemical biology.

Keywords: Aldehydes · Covalent drugs · Lysine targeting · Salicylaldehydes

The team includes Philip R. Skaanderup and Isabel Kerschgens (top row), Emine Sager, Ulrich Hassiepen, Cécile Delmas and Chrystele Henry (lower row, left to right). All researchers are employees of Novartis.

1. Introduction

Covalent modification of lysine residues has emerged as a promising strategy in drug discovery, offering unique opportunities for targeted chemical modifications and enhanced therapeutic interventions.[1] Lysine, one of the 20 natural amino acids that constitute proteins, plays a crucial role in maintaining protein structure, stability, and function. Its abundance within binding sites makes it an attractive handle for covalent modifications that modulate protein activity, protein-protein interactions, and cellular signaling pathways. At the same time, its abundance also presents challenges for selectivity and off-target cross reactivity.[2] In this regard, covalent reversible inhibitors can provide advantages over irreversible compounds comprising warheads such as sulfonyl fluorides,[3] vinyl sulfones,[4] and Michael acceptors[5] as well as undergoing cyclization reactions using 2-ethynylbenzaldehyde (EBA).[6] Among the reversible covalent inhibitors for lysines, aldehydes and salicylaldehydes represent the most commonly used warheads.[7] Productive covalent binding to a lysine typically depends on the surrounding protein microenvironment, which may either perturb the pK_a of the lysine amino group or support high effective molarity of the reactive compound through tight reversible binding of the scaffold.[8] Buried or surface exposed lysines can form reversible covalent Schiff bases and in the case of salicylaldehydes, the imine bond can be stabilized by the adjacent hydroxy group (Fig. 1). This can lead to increased residence times[9] of a compound on protein up to quasi-irreversible adducts[10] if stabilized by additional interactions.

Compounds containing aldehydes and salicylaldehydes have advanced through clinical trials and been approved for market release.[11] For example, ORIN1001 is a coumarin-based aldehyde currently in PH1 and PHII clinical trials (NCT03950570) as a potential treatment for relapsed, refractory metastatic breast cancer (Fig. 2A).[12] The compound is a reversible covalent, allosteric inhibitor of the inositol-requiring enzyme 1 (IRE1). Interestingly, co-crystallization studies with a similar tool compound revealed that the phenolic OH group is not engaged in the typical stabilization of the imine.[13] Instead, the iminium is stabilized by the coumarin core. In addition, Voxelotor[14,15] is a salicylaldehyde-containing compound previously approved as a drug for sickle cell disease. It targets the N-terminal valine of hemoglobin, increasing the affinity of hemoglobin for oxygen and consequently inhibits

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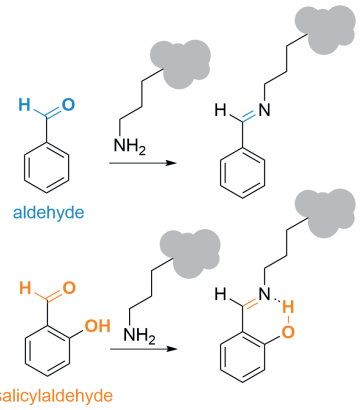


Fig. 1. Aldehydes and salicylaldehydes can form stable Schiff bases with lysine residues.

its polymerization. However, in 2024 it was announced[16] that Voxelotor will be withdrawn from the market. Adverse events in follow-up studies resulted in an increase in vaso-occlusive events indicating that the overall benefits of Voxelotor did not outweigh the risks to people with sickle cell disease.[17]

Salicylaldehydes have also been the subject of numerous academic investigations. For instance, Chen *et al.* reported cell-active covalent inhibitors of protein kinases by targeting a conserved catalytic lysine residue using salicylaldehyde-based imine chemistries.[18] These efforts culminated in the discovery of both irreversible and reversible covalent inhibitors for the BCR-ABL kinase, a key oncogenic driver in chronic myelogenous leukemia (CML). The lead compounds showed high selectivity in biochemical assays, exhibited nanomolar potency against endogenous ABL kinase in cellular assays, and were effective against most drug-resistant ABL mutations. Notably, the reversible, salicylaldehyde-containing covalent inhibitor 'A5' exhibited time-dependent ABL inhibition, prolonged residence time, and a reduced number of cellular off-targets in K562 cells (Fig. 2B). Taunton *et al.* reported on lysine targeting kinase inhibitors and showed that differential

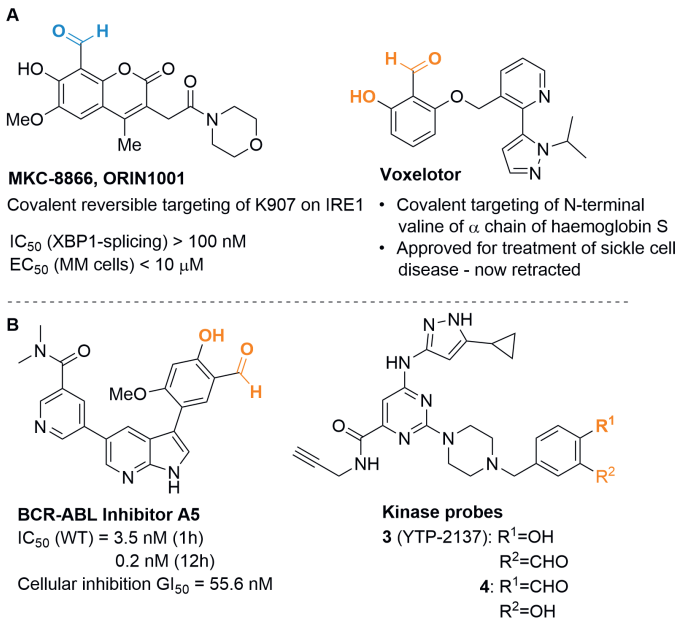


Fig. 2. A) ORIN1001 and Voxelotor are both aldehydes that went into clinical development. Voxelotor was approved for treatment of sickle cell disease but is now withdrawn from the market. B) Salicylaldehydes in the literature: BCR-ABL inhibitor A5 and kinase pull-down probes engaging multiple kinases in a time dependent-manner (>6h: AURKA, AURKB, SGK3, MAST3).

kinetics and residence times can be used to achieve selectivity among kinases.[9] Kinase selectivity was driven by differences in both on-rates and off-rates and could be further improved by linking the salicylaldehyde to a more selective non-covalent recognition scaffold. The precise geometry of the salicylaldehyde, and the resulting imine, played a critical role in the residence time and on-rate, as was evidenced by comparing the salicylaldehyde regioisomers YTP-2137 **(3)** and **4**. Overall, these two studies highlight the potential to develop selective salicylaldehyde-based inhibitors with prolonged residence times for therapeutically relevant kinases, as well as covalent targeting of other protein classes without more established covalent handles such as cysteine.

In addition to the extensive research on tool compounds from academic laboratories, salicylaldehydes are actively being investigated within the biotech and pharmaceutical industries. The biotech company Terremoto, for example, utilizes established compound scaffolds such as the pan-AKT inhibitor Miransertib (ARQ 092)[19,20] to install Schiff base forming warheads that target lysines in proximity of the binding pocket. Through covalent reversible targeting of lysines, different profiles compared to the parent compounds can be achieved *e.g.* increased residence time on target, increased selectivity for particular protein isoforms, and improved pharmacokinetics. Warheads include classical aldehyde and salicylaldehyde motifs[21] but also comprise modified hydrogen bond-donors such as those depicted in Fig. 3.[22]

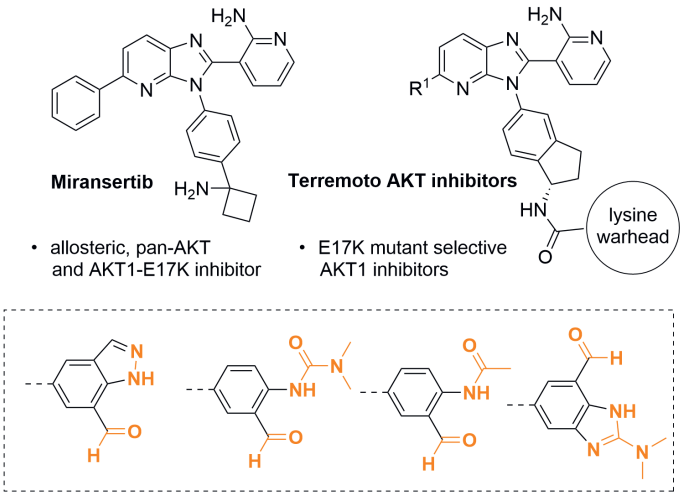


Fig. 3. AKT inhibitor Miransertib and reversible covalent E17K mutant selective analogs reported by Terremoto.

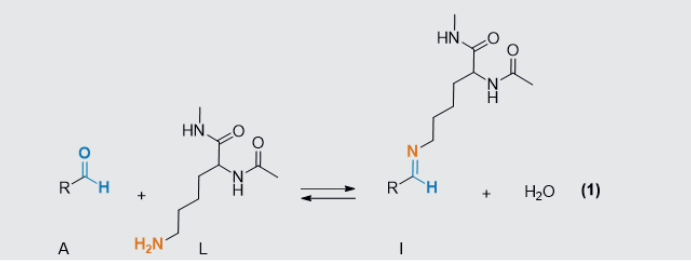
Leveraging lysine as a nucleophilic handle in covalent drug discovery requires a thorough understanding of how amine-reactive warheads interact with specific lysine residues. The selection of appropriate warhead motifs, such as electrophiles or reactive groups, is crucial to ensure high reactivity towards lysine residues while maintaining selectivity to minimize off-target effects. Furthermore, elucidating the binding kinetics and thermodynamics of the covalent interaction between lysine-reactive warheads and lysine residues is integral to fully understanding the mode of action and efficacy of covalent drugs. Determining the dissociation constant (K_D) provides insights into the strength of the interaction, enabling researchers to optimize the binding affinity and develop more potent covalent inhibitors. In this context, our study aims to contribute to the growing body of knowledge on lysine covalency by characterizing the reactivity of different lysine-reactive aldehyde-based warheads and providing insights into their kinetic profiles. These findings can contribute to the design and development of more effective covalent inhibitors.

2. Results

2.1 *NMR-Based Lysine Reactivity Assay*

As part of a program utilizing reversible covalent lysine targeting, we sought to understand the reactivity of different aldehydes and salicylaldehydes towards lysine as a nucleophilic amino acid. Our main focus was to understand the inherent affinity of the warheads for lysine to balance the covalent binding contribution. Additionally, we wanted to explore the time-dependency of the reaction. Therefore, we established an NMR-based (nuclear magnetic resonance) reactivity assay to follow the reaction of different electrophiles with Nα-acetyl-L-lysine methyl (**L**) as a lysine surrogate over time.

The reaction of aldehydes (or salicylaldehydes) (**A**) with **L** is an equilibrium reaction in which water is released upon formation of imine (**I**). We chose the lysine surrogate **L** instead of lysine itself to cap the reactive α-amino group and omit the free carboxylic acid, at the same time mimicking the protein backbone.



The equilibrium in equation **2** can be expressed as a dissociation constant describing the decay of imine (**I**) with addition of water back to aldehyde (**A**) (or salicylaldehyde) and lysine surrogate (**L**) (equation **3**). As water is in large excess in PBS buffer at pH 7.4, and we are working in dilute solution, the concentration of water was omitted in the calculation of the K_D values (Fig. 4).

$$K_D = \frac{c_A \cdot c_L}{c_I} \qquad (3)$$

K_D: *dissociation constant*
c_A: *concentration of Aldehyde*
c_L: *concentration Nα−acetyl−L−lysine methyl*
c_I: *concentration of Imine*

Fig. 4. The K_D of the imine complex is defined by the concentrations of the aldehyde (c_A) and **L** (c_L) divided by the concentration of the imine (c_I).

To monitor the reaction between aldehyde and lysine surrogate **L**, we chose NMR as the method for several reasons: Firstly, product and educts are clearly discriminable and can be directly quantified by integrals. Secondly, the reaction can be followed in real time and no additional work up steps are needed. Finally, any side reactions, in addition to the expected product, are also visible in the NMR spectra.

Stock solutions of each reactant were quantified using an external standard, and the aldehyde or salicyladehyde was mixed with the lysine surrogate in a 1:2 ratio in PBS pH 7.4. Integration and quantification were performed with a Bruker Topspin and a Bruker Eretic module. With the relative peak integrations and the known concentrations of each reactant in the NMR tube, the concentration of aldehyde (c_A), lysine surrogate (c_L) and imine (c_I) can be calculated at each time point (Fig. 5).

$$c_A = \frac{I_A}{I_A + I_I} * c_{A_0} \qquad (4) \qquad c_L = \frac{I_L}{I_L + I_I} * c_{L_0} \qquad (5)$$

$$c_I = \frac{I_I}{I_A + I_I} * c_{A_0} \qquad (6)$$

I_A: *relative peak integration of Aldehyde*
I_I: *relative peak integration of Imine*
c_{A_0}: *starting concentration of Aldehyde at* $t = 0$
I_L: *relative peak integration of Nα−acetyl−L−lysine methyl*
c_{L0}: *starting concentration of Nα−acetyl−L−lysine methyl at* t=0

Fig. 5. Calculation of concentration of aldehyde (c_A), concentration of Nα-acetyl-L-lysine methyl (c_L) and concentration of imine (c_I) from the relative peak integrations of the NMR spectra and the starting concentration of aldehyde and imine at any timepoint during the reaction. Relative peak integration means that the integrations were corrected for their proton counts.

Fig. 6 illustrates the reaction of a salicylaldehyde – here 3-hydroxypyridine-2-carbaldehyde – with lysine surrogate **L**. After a certain time, the reaction reaches equilibrium and only the data at the equilibrium stage were utilized for K_D determination (Fig. 6).

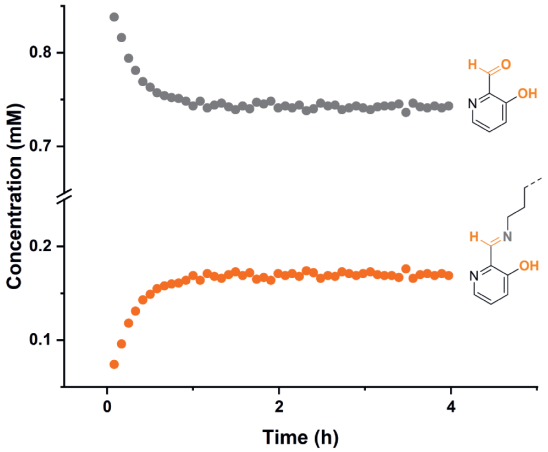


Fig. 6. Concentrations of 3-hydroxypyridine-2-carbaldehyde (SA8) and the corresponding imine over time. Equilibrium was reached after ~1.5 h and only those data were used for K_D calculation.

2.2 *Dissociation Constants*

By following the concentration of the reactants over time, we investigated the reactivity of several lysine-targeting warheads. We were interested in how aldehydes and salicylaldehydes generally compare to each other and how substituents are affecting the K_D of the formed imine as well as the rate of the reaction. The salicylaldehydes and aldehydes tested in this study together with their respective K_D values are shown in Fig. 7.

From the respective calculated K_D results it is clear that substitution patterns had a remarkable influence on the K_D values of the formed imines. Electron withdrawing substituents such as fluorine (**SA2**, **SA3** and **SA4**) or chlorine (**SA7**), led to significant lowering of the dissociation constants of the respective salicylal-

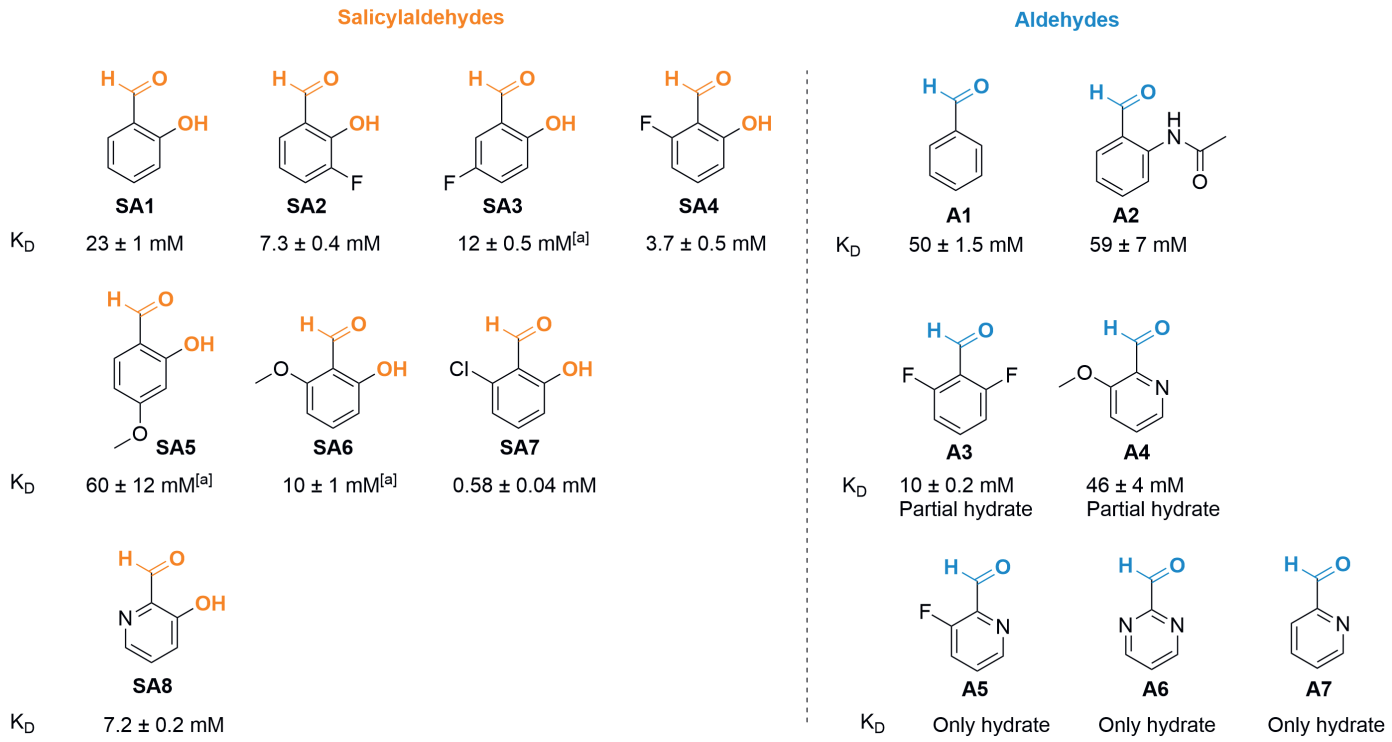


Fig. 7. Dissociation constants for salicylaldehydes and aldehydes [a] no final equilibrium stage was obtained after 6 or 12 h, final data points taken for K_D calculation.

dehydes. An electron donating OMe substituent in the *p*-position to the aldehyde (**SA5**) increased K_D while the same substituent in the *o*-position (**SA6**) lowered K_D relative to the unsubstituted salicylaldehyde **SA1**, indicating that the OMe substituent in the *o*-position might stabilize the formed imine adduct. Overall, our findings are in line with the electronic effects and σ-values of aromatic substituents, where electron-withdrawing substituents are rendering aromatic rings more electrophilic and shifting the equilibrium to the imine product, while the data for the OMe substituted aldehydes are less clear.[23]

When investigating the aldehydes, we obtained similar effects with the difluoro-substituted aldehyde **A3** significantly lowering the K_D value compared to regular benzaldehyde **A1**. For the acetamido-substituted benzaldehyde **A2**, little imine was formed therefore resulting in a high K_D vlue of 59 mM. It is of note that the electron deficient aldehydes all formed hydrates partially or stoichiometrically, and that the pyridine aldehydes **A5**, **A6** and **A7** in particular showed no reaction with **L** at all.

2.3 *Kinetic Profiles of Aldehydes and Salicylaldehydes*

After monitoring the formation of imine over time, we saw remarkable differences in the rates with which the reaction was proceeding. While benzaldehyde reached equilibrium stage immediately and no change was noticeable over time,[24] salicylaldehyde reached equilibrium after ~2.5 h (Fig. 8).

Strikingly, the disparity in binding kinetics was consistent among the aldehydes and salicylaldehydes tested (see supporting information), and for some salicylaldehydes, no definite equilibrium was reached at the end of the 12-hour measurement period as exemplified by 5-fluorosalicylaldehyde (**SA3**) in Fig. 9. The calculated K_D values for **SA3**, **SA5** and **SA6** are therefore an approximation, and the time frame would need to be extended to >12 h measurement time. While studies on K_D value determination for aldehydes towards imines have been published previously,[25] the time-dependent difference between aldehydes and salicylaldehydes to our knowledge has not been described with such consistency among the examples tested.

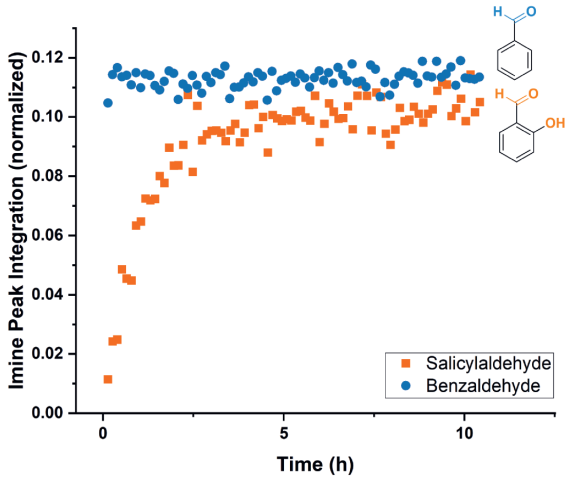


Fig. 8. Kinetic profile of imine formation with benzaldehyde or salicylaldehyde.

One possible reason for the slow reaction rates of salicylaldehydes is the formation of a different – yet clearly disfavored – tautomer, where the electrophilicity of the aldehyde species is reduced. Even slight equilibration between these tautomers may result in slower overall reaction rates with nucleophilic species (Fig. 10). Alternatively, the phenolic OH could influence the rate determining step which has been reported to be the dehydration of the hemiaminal. Further experiments and calculations would be needed to strengthen these hypotheses.

The kinetics of imine formation with aldehydes were too rapid to be tracked using the NMR assay. However, we were able to observe an exponential increase in imine concentration for the salicylaldehydes studied. The rate-determining step of imine formation in aqueous media is dehydration of the hemiaminal intermediate.[26] Therefore, we applied a first-order decay function to model the imine formation (Fig. 11).

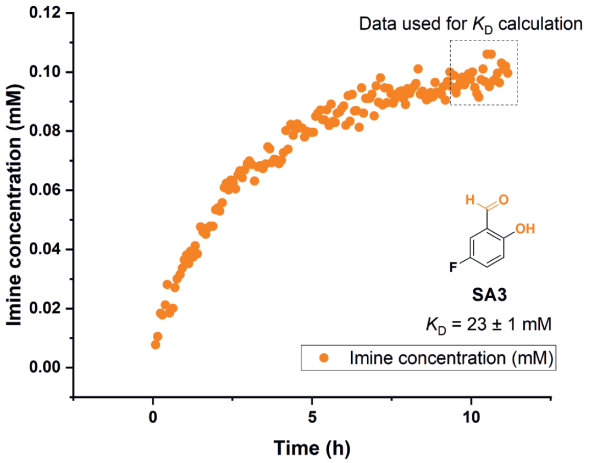


Fig. 9. Time course of imine formation using 5-fluorosalicylaldehyde (SA3).

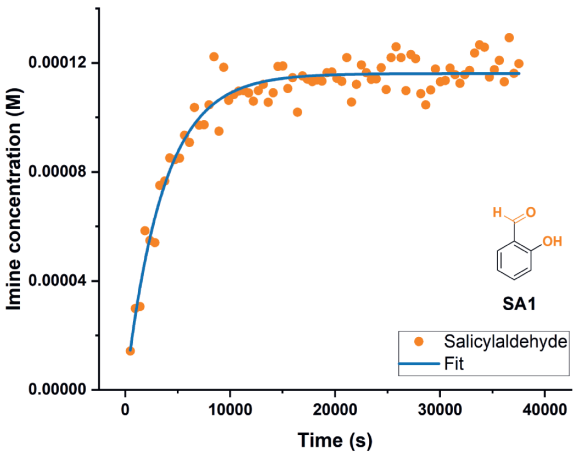


Fig. 11. Kinetic for the corresponding imine in the reaction between salicylaldehyde SA1 and L.

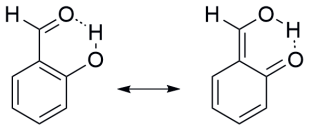


Fig. 10. Tautomers of salicylaldehyde.

The overall reaction rates at neutral pH depend on the dehydration rate (k_2) and the equilibrium constant for the addition of amine to the aldehyde to form the hemiaminal (k_1). Similar rate constants were obtained for salicylaldehydes with electron-withdrawing substituents, except for **SA8**, which had a significantly enhanced rate (Table 1). The pyridine nitrogen might assist in stabilizing the hemiaminal intermediate but likely also eases the elimination of water due to electron effects and hereby leading to an acceleration of the rate-determining dehydration step (Fig. 12). Further in-depth studies would be needed to investigate the structure-rate relationships and detailed mechanistic aspects. The reaction with both OMe substituted aldehydes **SA5** and **SA6** were by far the slowest among the substrates tested and had the poorest curve fits.

3. Discussion

In this study, we used a model lysine-substrate to benchmark aldehydes and salicylaldehydes for their reactivity. We believe that these findings will provide insights into the design and synthesis of reversible covalent compounds targeting lysine. Firstly, careful selection of the warhead is crucial to avoid excessive off-target reactivity and minimize unspecific interactions. Here, the presence of electron-withdrawing substituents may enhance the binding affinity but could potentially compromise selectivity when exposed to more complex settings with multiple lysines present. Given that lysine is highly prevalent in the proteome and often found on protein surfaces, using highly reactive warheads with nearly irreversible binding profiles can pose a challenge. Compounds with highly reactive warheads may be influenced more by their reactivity and less by their non-covalent, specificity-determining binding interaction. Therefore, designing compounds with appropriate substituents will be key to balance potency and specificity.

Moreover, the slow reaction kinetics observed for salicylaldehydes highlight the need to consider the time required for the compound to reach equilibrium, particularly in biochemical assays with short incubation times. As this information becomes critical when designing studies and interpreting the results obtained from experiments involving salicylaldehyde-containing compounds, we recommend monitoring effects over extended time periods to both verify a covalent mechanism and gain an understanding of its time-dependency. Ultimately, a thorough understanding of the kinetics will allow for precise optimization of reaction times and substrate concentrations to accurately assess the desired biological effects.

Table 1. Rate constants for the imine formation with salicylaldehydes. [a]Poor fit due to slow rate

Compound	K_D [mM]	k [1/s] 10^{-4}
SA1	23 ± 1	1.06
SA2	7.3 ± 0.4	3.02
SA3	12 ± 0.5	1.06
SA4	3.7 ± 0.5	1.53
SA5	60 ± 12	0.27[a]
SA6	10 ± 1	0.27[a]
SA7	0.58 ± 0.04	2.41
SA8	7.2 ± 0.2	769

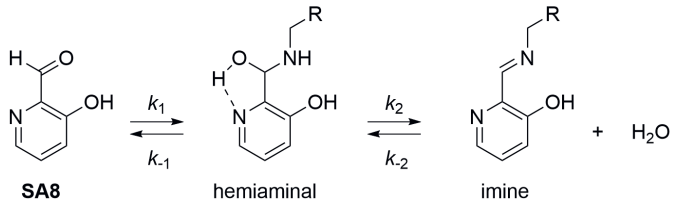


Fig. 12. Intermediates of imine formation for SA8 and L.

4. Conclusion

In summary, our study provides insights into the reactivity profiles and kinetics of aldehydes and salicylaldehydes in imine-based chemistries. The differences in K_D values and reaction rates observed among the compounds emphasize the influence of warhead design for covalency and binding dynamics with lysine residues. This work aims to inspire future studies and applications of reversible covalent compounds to further enable chemically tailored modulation of biological processes and pharmacological interventions.

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Author Contributions

I. K. and P. R. S. conceived the project. I. K. selected compounds, analyzed and interpreted the results, and calculated the K_D values. E. S. developed the NMR reactivity assay. C. D. and C. H. performed the reactivity assay experiments. U. H. helped with K_D calculations and discussion of the data and performed the fits and calculation of the rate constants. I. K. and P. R. S. wrote the manuscript with input from all authors.

Supporting Information

Supporting informaiton is available on https://www.chima.ch/chimia/article/view/2025_152

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