

Covalent Activation of the C-type Lectin DC-SIGN

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Abstract: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a C-type lectin receptor expressed on antigen-presenting cells, crucial for pathogen recognition and immune modulation. The shallow and polar carbohydrate binding site of DC-SIGN presents challenges for ligand design. Here, we explored covalent modification targeting specific lysine residues as a novel strategy to modulate DC-SIGN function. Screening a lysine-targeted electrophilic fragment library using orthogonal functional assays identified two potent activators. Structural analyses via NMR spectroscopy, mass spectrometry and computational modeling confirmed structural perturbations of the carbohydrate recognition domain (CRD) and revealed distinct mechanisms of activation. While both activators significantly enhanced DC-SIGN's affinity for monosaccharide ligands, one compound induced oligomerization via covalent coupling and non-covalent secondary site interactions, whereas the other selectively modified lysine K373 directly within the primary carbohydrate binding site. These findings demonstrate the potential of lysine-targeted covalent compounds as a novel therapeutic strategy for modulating DC-SIGN function and potentially C-type lectins in general.

Introduction

DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) is a Ca^{2+} -dependent lectin expressed on antigen-presenting cells, playing a key role in innate and adaptive immunity.^[1–4] Upon recognition of mannosylated and fucosylated glycan patterns on various pathogens and self-antigens, DC-SIGN facilitates antigen uptake, cellular adhesion, modulation of immune response, and in some cases, immune evasion by pathogens, such as HIV-1 and SARS-CoV-2.^[5–9] This has made DC-SIGN an attractive target for targeted delivery of vaccines, immune modulation, and anti-infective strategies.^[10,11] However, targeting DC-SIGN's carbohydrate binding site with small molecules remains notoriously difficult due to its

inherently low druggability and the typically weak affinity of monosaccharide-based ligands.^[12,13] On the one hand, efforts to develop ligands have predominantly focused on carbohydrate-based ligands, often depending on multivalent presentation on various supports to increase potency.^[10,12,14] On the other hand, identification of several secondary sites and moderate hit rates in fragment screening campaigns have suggested DC-SIGN to be amenable to fragment-based drug discovery (FBDD) approaches.^[15–20]

As an alternative strategy, covalent ligand approaches have emerged as a promising method to efficiently modulate challenging targets and to enhance both potency and selectivity.^[21,22] By forming irreversible or prolonged interactions with target proteins, covalent ligands can overcome the limitations of conventional affinity-driven ligand design. In

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addition, covalent fragment approaches combine the advantages and effectiveness of FBDD and target identification by covalent labeling.^[23] Covalent modulation can be achieved by ligand-first or electrophile-first approaches. In the former case, a high-affinity ligand is equipped with a well-positioned electrophilic functional group, referred to as a warhead. In the latter approach, a path commonly followed for challenging targets without ligands, small electrophilic fragments are screened to find starting points for FBDD approaches.^[24] Although covalent drugs have seen a resurgence in medicinal chemistry, examples of covalent modulation of lectins remain scarce and largely limited to bacterial systems.^[25–28] In contrast, covalent approaches targeting mammalian lectins have primarily served as chemical-biology tools for mapping and stabilizing transient interactions, enabling, for example, mapping of glycan-lectin contacts on cell surfaces.^[29] Yet, covalent modulators designed for functional perturbation of human or mammalian C-type lectins, specifically of DC-SIGN, have not been explored.

In this study, we pursued an electrophile-first approach by designing and screening a Lys-targeted covalent fragment library against DC-SIGN. We identified and characterized the first covalent activators of DC-SIGN, providing novel insights into mechanisms of glycan-binding enhancement through covalent modification. Detailed biophysical and structural investigations elucidate distinct modes of action, demonstrating how covalent targeting of lysine residues can effectively modulate glycan recognition by DC-SIGN through induction of oligomerization or by directly affecting monosaccharide recognition. These findings open new avenues for exploiting covalent strategies in the development of DC-SIGN-targeted but also C-type lectin-targeted small molecules in general.

Results and Discussion

Library Design and Chemistry

We have compiled a 34-membered library of reactive fragments based on known covalent warheads reported to target lysine residues in proteomic studies or in targeted covalent inhibitor (TCI) development (Table S1).^[23,30–32] The library was designed to identify suitable warheads for lysine targeting by emphasizing the covalent labeling step rather than non-covalent recognition. Therefore, the fragments were kept small, chemically diverse, and largely apolar, representing a focused set of aminophilic electrophiles with broad reactivity and geometry coverage.

Seventeen fragments were commercially available, while the other half of the fragments were synthesized (for the details of the syntheses, see Supporting Information). First, we evaluated the reactivity of the library members with *N*- α -acetyl lysine in an HPLC-MS-based kinetic assay, measuring pseudo-first-order rate constants and the formation of the corresponding adducts at pH 10.2. At this pH, the ϵ -amine of lysine would be deprotonated, which mimics the intrinsic reactivity of Lys residues at protein binding sites, where its pK_a can range from 5 to 11 depending on the microenvironment^[23,33–35] (Figure 1a; Table S2). The results

confirmed that the library covers a wide range of reactivities (Figure 1b). We found 14 fragments reacting immediately ($t_{1/2} < 5$ min), while five and four showed reactivity under 20 or 90 min. The half-life of eleven fragments was over 24 h, including three mildly reactive compounds (>48 h). The aqueous stability of the fragments was then evaluated under physiological conditions (pH 7.4). Among the highly reactive compounds, six showed $>80\%$ stability after 1 h, while for five fragments the stability was $<50\%$. Thirteen of the 20 less reactive fragments showed acceptable stability ($>80\%$), and only three were found to be unstable ($<50\%$). Based on the wide range of reactivity, we assumed that lysines in proteins showing diverse nucleophilicity can be targeted (Figure 1b; Table S2). Having many highly reactive warheads supports the diversity in targeting lysines with low reactivity, while low-reactivity warheads might provide specific labeling.

Functional Screening Identifies DC-SIGN Activating Electrophiles

The first screening assay selected is based on the multivalent interaction of the glycoprotein horseradish peroxidase (HRP) with the carbohydrate binding site of the tetrameric DC-SIGN ectodomain (ECD). The assay has been previously utilized to screen non-covalent inhibitors as well as to evaluate the inhibitory or activating mutations on the DC-SIGN carbohydrate recognition domain (CRD).^[36,37] To test the covalent compounds, the purified protein was immobilized on plates and incubated with 0.5 mM of compound overnight at 4 °C or room temperature. Activity of the modified protein was then assessed by addition of HRP followed by detection of its peroxidase activity (Figure 2a).

Using a cutoff of $\sim 50\%$ activity change compared to the DMSO control, we identified only **30**, a naphthalene-2,3-dicarbaldehyde *ortho*-phthalaldehyde (OPA)-type fragment, as a potential inhibitor, whereas eight compounds (**11**, **17**, **19**, **22**, **25**, **26**, **31**, **33**) showed $\geq 50\%$ activation of DC-SIGN (Figure 2b).^[38] Moreover, the temperature-dependent increase in activity of all selected compounds suggested a covalent mechanism underlying the compound activities. We further confirmed **30** as an inhibitor and seven of the eight activators (salicylaldehydes **25** and **26**, squarate **33**, difluorostyrene **19**, dichlorotriazine **17**, formylacetylene **31**, and *N*-hydroxysuccinimide (NHS)-ester **11**) in dose-response experiments, revealing micromolar activities (Figures 2c, S1, and S2). The fragments identified as hits react through various mechanisms, i.e., through acylation (**11**), conjugate addition-elimination (**33**), imine formation (**25**, **26**, **31**), and substitution (**17**, **19**, **33**).

To orthogonally validate activity of these compounds in solution, we established a grating-coupled interferometry (GCI) assay, in which altered affinity of DC-SIGN for its monosaccharide ligand can be evaluated based on binding to mannose-PEG³-amine coupled to the sensor chip (Figure 2d). Upon injection, DC-SIGN ECD showed sufficient binding to the chip with affinities similar to previous surface plasmon resonance setups utilizing mannosylated BSA on the sensor surface (Figure S3a).^[39] In control experiments with mannose,

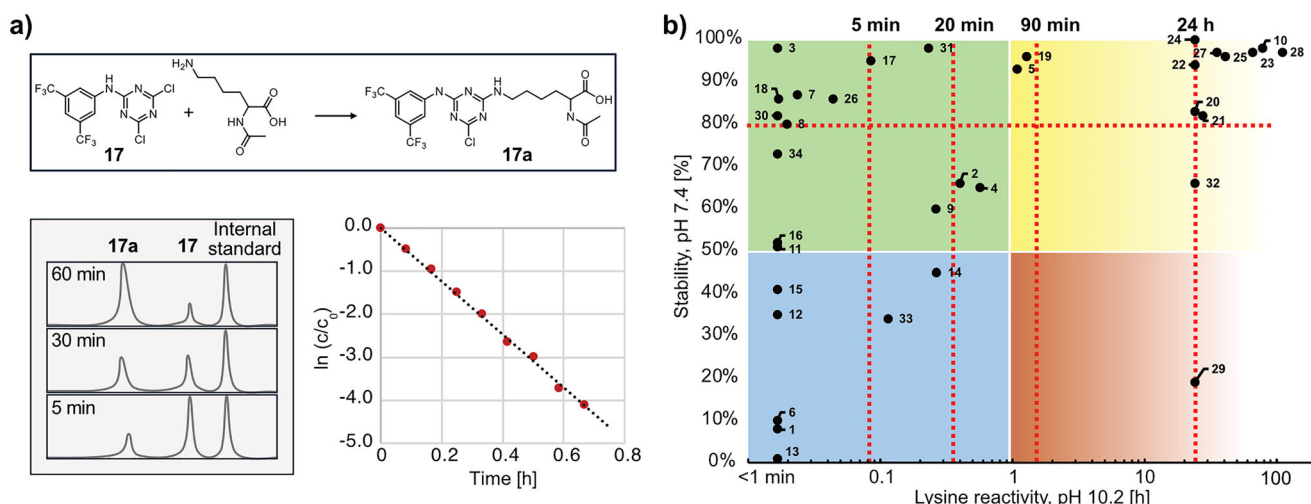


Figure 1. The electrophilic fragment library displays a wide range of reactivities. a) Schematic representation of the reaction of **17** with *N*-α-acetyl lysine. Time-resolved HPLC chromatograms for the reactivity assay and plotting the decrease of the fragment for the $\ln(k)$ and $t_{1/2}$ determination by linear regression. b) Correlation between buffer stability and *N*-α-acetyl lysine reactivity. The y-axis represents the percentage of compound remaining after 1 h, indicating stability under physiological conditions. The x-axis shows the reactivity of each compound, expressed as the half-life ($t_{1/2}$) derived from pseudo-first-order kinetic measurements of *N*-α-acetyl lysine conjugation. The plot illustrates the relationship between compound stability and reactivity, with shorter half-lives indicating faster reaction rates. Red dashed lines correspond with thresholds mentioned in the text (Table S2).

the assay yielded IC_{50} s comparable to published data and results from titrations in the HRP assay, demonstrating dependency of the interaction on the carbohydrate binding site (Figure S4a).^[40]

For the covalent hits, DC-SIGN ECD was preincubated with the compounds overnight and quenched by the addition of *tris*(hydroxymethyl)aminomethane (Tris) in five-fold excess relative to the ligand prior to injection (Figure 2d). While the inhibitor **30**, as well as activators **26**, **25**, **19**, and **31** precipitated the protein or showed no activity compared to the DMSO control, compounds **11**, **33**, and **17** activated binding to the chip surface (Figures 2e,f, S5, and S6). Notably, although no precipitation upon incubation with **11**, **33**, or **17** was observed, compound concentrations exceeding 0.25 mM led to a reduction in binding response, indicating covalent modification at additional less reactive sites. Yet, saturation of the binding response by all three compounds allowed for fitting of binding parameters and yielded EC_{50} s comparable to those obtained in the plate-based assay. Hill slopes >1 of the binding isotherms indicated a positive cooperative effect induced by the compounds. We also observed a drastic decrease in off-rates, indicating that the compounds alter the kinetics of the DC-SIGN ECD-glycan interaction, likely by increasing affinity, avidity, or both (Figure S7).

Selective glycan recognition by DC-SIGN is not limited to simple monosaccharides at its canonical carbohydrate binding site but additionally depends on an extended site, establishing contacts with carbohydrate units of di- and trisaccharides and more complex oligosaccharides, such as those present on HRP.^[41–43] To determine whether the covalent activators could modulate glycan binding via this extended site, we also tested DC-SIGN modified with covalent adducts (**11**, **17**, **33**) on a sensor chip coupled to the disaccharide Man(α1,2)Man-

PEG3-amine (Figures S3b and S4b). We obtained similar results as for the mannose-PEG3-amine sensor, suggesting that the activity of the covalent compounds does not originate from modulating additional contacts to larger glycans outside of the canonical carbohydrate binding site (Figures S8 and S9).

Finally, to confirm the covalent activity of the three hits, we quenched the reaction at different time points in our plate-based assay and detected activity by binding to HRP. This revealed a clear time dependency of the EC_{50} of **33** and indicated irreversible modification of the protein. **11** showed fast reaction followed by reduced activity at incubation times exceeding 10 min. For **17**, a time-dependent increase in response was observed that was, however, increasingly linear, suggesting an unspecific reaction at longer incubation times (Figure 2g). Consequently, we excluded **17** from further investigation.

Taken together, our functional screening and validation of the reactive fragment library against DC-SIGN ECD identified and validated two covalent activators and no true inhibitors. While the exclusive identification of activators is surprising, control experiments with mannan, mannose, and EDTA confirmed that the observed responses depend on Ca^{2+} -mediated carbohydrate recognition rather than non-specific interactions, excluding methodological artifacts as a source of apparent activation. Moreover, activation by the covalent hits was consistently observed for immobilized DC-SIGN and in solution and showed clear time dependence, further supporting a covalent mechanism underlying the observed effects. Considering the different warhead chemistries as well as products and selective reactivity of hits **11** and **33** for specific Lys sidechains, we speculated a general tendency of covalent modification of Lys residues to activate DC-SIGN (Figure 2h).

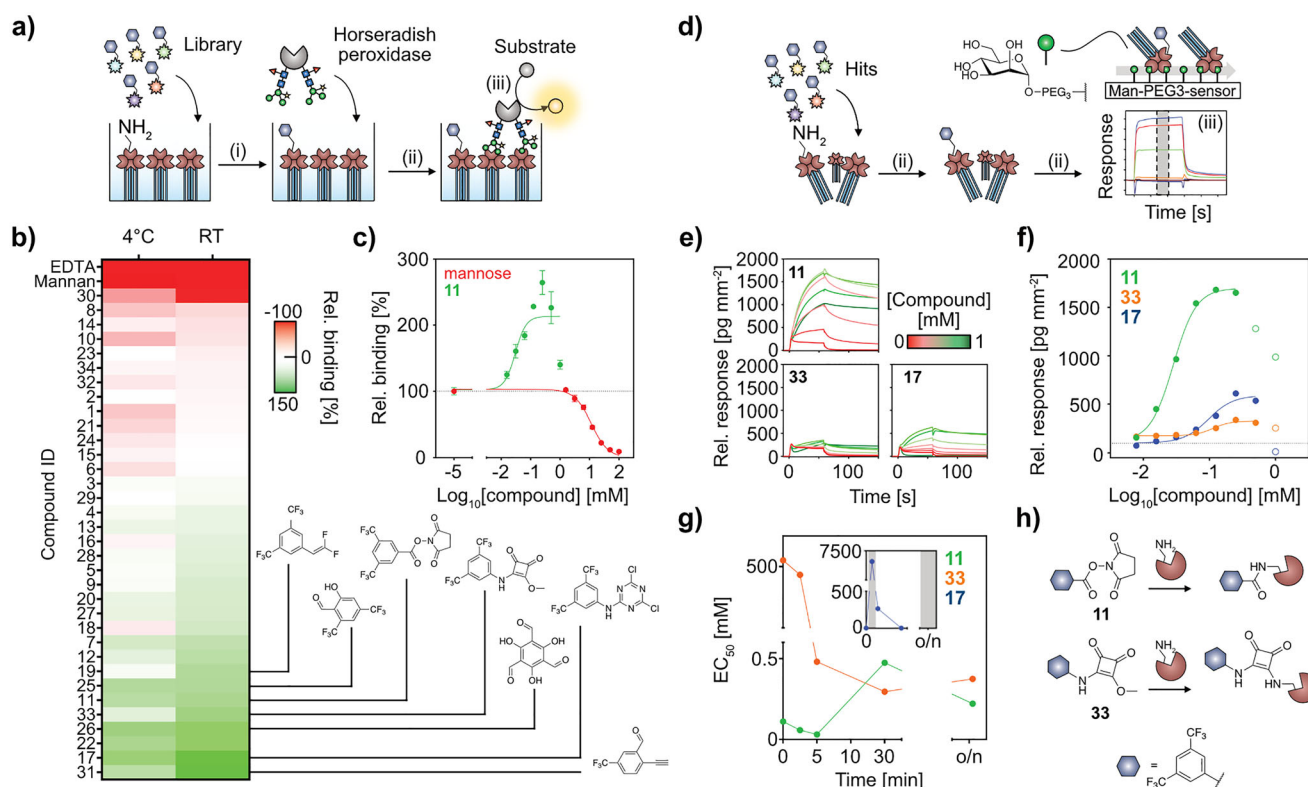


Figure 2. Functional screening and validation of the covalent warhead library against DC-SIGN ECD. a) The HRP assay was used to screen the electrophilic fragments at single concentrations in dose-response experiments. Plate-immobilized DC-SIGN ECD is incubated with 0.5 mM of each compound overnight (i). After washing, HRP is added to the wells (ii). HRP interacts with DC-SIGN ECD via its endogenous glycosylation. The amount of HRP bound is evaluated via its peroxidase activity (iii). b) Heatmap displaying screening results for incubation at 4 °C or at RT. EDTA and mannan were used as controls for the DC-SIGN-glycan interaction. Overall, more electrophilic fragments activating DC-SIGN binding to HRP were found. Structures of activators displaying an increase in DC-SIGN binding activity of $\geq 50\%$ relative to the DMSO control are shown. Results represent the mean of three biological replicates. c) Example dose-response curves after overnight incubation at RT, showing mannose as a control inhibitor ($IC_{50} = 11.4$ mM) and **11** as an activator ($EC_{50} = 0.03$ mM). Dose-response curves of other hit compounds are shown in Figures S1 and S2. d) A GCI assay was established to orthogonally validate hit compounds in dose-response experiments. DC-SIGN ECD in solution is incubated with each compound (i). Modified DC-SIGN is injected over a quasi-planar GCI sensor modified with mannose-PEG3-amine, allowing for multivalent protein-glycan interactions (ii). Binding is evaluated from resulting sensorgrams using response at equilibrium (iii). e) Sensorgrams of DC-SIGN ECD modified with validated activators **11**, **33**, and **17** showing drastic change in relative response and reduced off rates of the interaction. f) EC_{50} fit of binding response from sensorgrams in e) yielded micromolar activities for the hit compounds **11** ($EC_{50} = 0.03$ mM), **33** ($EC_{50} = 0.1$ mM), and **17** ($EC_{50} = 0.1$ mM). Data points without filling were not used for fitting. g) Dose-response curves of the validated hit compounds after varying incubation times using the HRP assay indicated clear time dependency for **11** and **33**, but not for **17**. Grey areas indicate timepoints where the EC_{50} could not be fitted reliably. h) The two validated hit compounds, **11** and **17**, share the same non-covalent 3,5-bis(trifluoromethyl)phenyl moiety but yield different reaction products.

Covalent Activators Modulate Glycan Binding Kinetics of the Carbohydrate Recognition Domain via Different Mode-of-Actions

The neck domain of DC-SIGN ECD contains several Lys residues involved in coiled-coil interactions important for tetramerization and therefore multivalent interactions of the receptor with glycans (Figure 3a).^[44] To exclude that the activity of the hit compounds relies on an unspecific reaction with the neck domain instead of the CRD, we transferred our GCI assay to the isolated DC-SIGN CRD.

Similar to experiments with the ECD, mannose titration revealed monovalent IC_{50} s in the expected range and an increase in affinity for the Man($\alpha 1,2$)Man surface compared to the mannosylated one, both confirming the carbohydrate specificity of the interaction (Figures S10 and S11). Using the same setup as for the ECD, dose-response experiments

with the covalent hits **11** and **33** confirmed the activating potential of the compounds (Figure 3b). No reduction in binding response was observed at higher concentrations, suggesting that either additional reaction sites at the neck domain or changes in the relative orientation of the CRDs in the ECD tetramer upon reaction could lead to inhibition of carbohydrate binding. Interestingly, **11** showed a significantly higher EC_{50} and a similar Hill slope of >1 when compared to experiments with the ECD (Figure 3c). Apart from indicating the activity of **11** to be partially dependent on the presence of the neck domain, this suggested cooperativity of the **11**-modified CRD interacting with the chip. In contrast, the EC_{50} of **33** was similar to experiments with the ECD, and a Hill slope ≈ 1 suggested **33** to specifically modulate the CRD in both the tetrameric ECD and the isolated CRD. When tested on the Man($\alpha 1,2$)Man surface, activity of **11** and **33** was

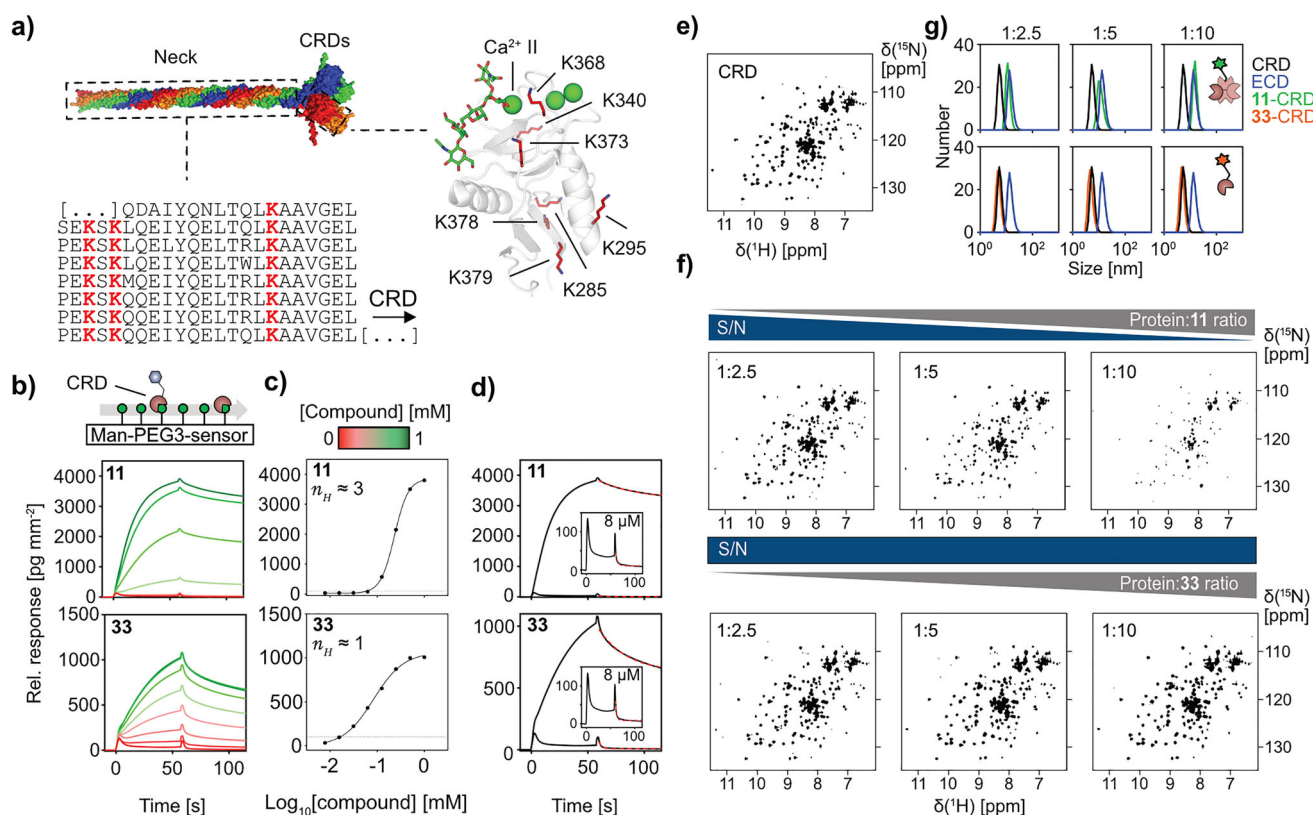


Figure 3. Electrophilic fragment hits activate DC-SIGN CRD via different mechanisms. a) Model of the DC-SIGN ECD tetramer, colored by monomers. The sequence of the neck domain with lysines highlighted in red and the CRD in complex with an oligosaccharide (PDB: 1K9I) with lysines as red sticks is shown. b) The GCI assay implemented for DC-SIGN ECD was transferred to the CRD to test the effect of activators **11** and **33** on the monovalent DC-SIGN-glycan interactions. c) The activity of **11** was reduced compared to experiments with the ECD ($EC_{50} = 0.2$ mM), and the Hill slope suggested cooperativity effects. **33** showed comparable activity ($EC_{50} = 0.1$ mM) on the CRD as on the ECD and a Hill slope of ~ 1 . d) Off-rates (k_{off}) of the CRD-mannose-PEG3 interaction are drastically reduced for both **11**- and **33**-modified DC-SIGN CRD when comparing low (0.008 mM; $k_{off,11} \approx 0.25$ s $^{-1}$, $k_{off,33} \approx 0.41$ s $^{-1}$) and high (1 mM; $k_{off,11} \approx 0.03$ s $^{-1}$, $k_{off,33} \approx 0.04$ s $^{-1}$) concentrations of the compounds. e) 1H ^{15}N HSQC NMR spectrum of ^{15}N -labeled wild-type DC-SIGN CRD in the absence of compound. f) 1H ^{15}N HSQC NMR spectra of DC-SIGN CRD modified with **11** at increasing ratios show reduced S/N, while **33** does not change spectral quality. g) Comparison of the protein hydrodynamic radius in DLS upon modification indicates an increase in size of **11**-modified DC-SIGN CRD. At a protein-**11** ratio of 1:10, DC-SIGN CRD has a similar size as the unmodified DC-SIGN ECD, suggesting **11** to induce oligomerization. In accordance with 1H ^{15}N HSQC NMR data, **33**-modified DC-SIGN CRD displays no increase in size.

only slightly increased, further supporting a model in which changes in monosaccharide binding instead of oligosaccharide binding dominate the mode of action of the compounds (Figure S12).

In line with observations from experiments with the ECD, we observed reduced off-rates upon modification of the CRD with the compounds (Figure 3d). As the isolated CRD cannot oligomerize in the absence of the neck domain, we reasoned that reduced off-rates could point toward modulation of the CRD-mannose-PEG3 interaction.^[45] This was further supported by a decreased dissociation constant (K_D) for the sensor surface for both **11**- and **33**-modified DC-SIGN CRD (Figures S13 and S14). Finally, to confirm the dependency of the observed activation by **11** and **33** on covalent bond formation of the DC-SIGN CRD, we conducted titration experiments with compounds inactivated with Tris prior to reacting it with the protein. In accordance with the expected low affinity of the non-covalent ligand, no activation of the response was observed (Figure S15).

Next, we aimed to elucidate binding sites and structural perturbations induced by **11** and **33** interacting with DC-SIGN CRD. We incubated ^{15}N -labeled DC-SIGN CRD with 0.25 mM of each compound, followed by dialysis to remove unreacted ligand. We recorded 1H ^{15}N HSQC NMR spectra of ^{15}N -labeled wild-type DC-SIGN CRD in the absence of ligand to establish a reference for subsequent comparison (Figure 3e). This baseline allowed us to attribute later spectral changes specifically to the non-covalent moieties of **11** or **33**, anchored through covalent attachment at the modified lysine residues.^[46]

Strikingly, 1H ^{15}N HSQC NMR spectra of **11**- and **33**-modified DC-SIGN CRD wild type revealed drastic differences. Spectra of **11**-modified DC-SIGN CRD showed significantly reduced signal-to-noise (S/N) compared to the unmodified and **33**-modified protein (Figure 3f). As no precipitation was observed visually, we reasoned that an increase in size and therefore an increase in relaxation rates of the protein upon reaction with **11** is the origin of the reduction

in S/N. This was confirmed by dynamic light scattering (DLS), showing a clear shift toward a population of larger particles (Figure 3g). Since the reduction in S/N and the population of larger particles increased with the ligand-to-protein ratio, we concluded that compound **11** induces oligomerization of DC-SIGN in a concentration-dependent manner. The size of the **11**-CRD particles was close to those of the DC-SIGN ECD tetramers, and no soluble aggregates were observed, suggesting a distinct oligomerization pathway.

Taken together, our GCI and HSQC NMR assays suggested distinct mechanisms underlying DC-SIGN activation by **11** and **33**. While **11** triggered the formation of CRD oligomers, **33** showed selective covalent modification without altering the protein monomeric state.

Activation by **33** Relies on Covalent Coupling to K373 at the Carbohydrate Binding Site

To study the covalent and non-covalent interaction of **33** with the DC-SIGN CRD, we calculated chemical shift perturbations (CSPs) relative to the wild-type protein from ^1H ^{15}N HSQC NMR spectra, informing about changes in the chemical environment of the ^{15}N -labeled backbone amide of each residue.^[47]

We observed only one new cross-peak upon modification with **33**, supporting the formation of a new amide bond by reaction with a single Lys sidechain (Figure 4a). In line with this, CSP mapping suggested a localized effect of covalent modification by **33**. Perturbations clustered around residues forming the Ca^{2+} -dependent carbohydrate binding site, including the long loop (E349, N350, V351), β -strands 3 and 4 (E358, D366), and adjacent loops (F313, A372) (Figure 4b,c). These residues either directly participate in Ca^{2+} coordination or are involved in mono- and oligosaccharide binding, indicating that **33** perturbs the carbohydrate binding site.^[42,48] Within this region, two lysines, K368 and K373, lie in spatial proximity, suggesting that covalent modification of their sidechains could sterically position the non-covalent moiety toward the affected residues (Figure 4d). Computational analysis of Lys solvent-accessible surface areas (SASA) and predicted pK_a values revealed comparable nucleophilicity, with K368 being more exposed and K373 more buried (Table S3).

To unambiguously determine the covalent binding site of compound **33** at the carbohydrate binding site, we mapped labeling sites in chymotrypsin and ProAlanase-digested peptides by LC-MS/MS. This identified K373 within the peptide NDDKCNLAK³⁷³F as the site of modification for **33** (Figures S16 and S17; Table S4). Furthermore, kinetic analysis of time-dependent intact-MS data yielded $k_{\text{inact}}/K_{\text{I}} = 54.96 \text{ M}^{-1} \text{ min}^{-1}$, consistent with a slow, saturation-limited reaction characteristic of site-specific covalent engagement at K373 rather than a non-specific reaction at the DC-SIGN CRD (Figure S18 and Table S5).

Having previously established that activation by **33** depends on covalent bond formation in our GCI experiments, we reasoned that mutation of K373 to a non-nucleophilic amino acid should deplete activity of the compound, therefore

confirming K373 as a specific reaction site (Figure S15). Since a K373N mutation has been previously shown to alter binding of DC-SIGN to glycans, we inserted a K373R mutation into the ECD construct, consequently removing nucleophilicity while maintaining positive charge in this site.^[41,49] We also generated negative control mutants K295R and K340R that were expected to have no effect on the activity of **33** (Figure 3a). Strikingly, when tested in our HRP assay, **33** only showed drastically reduced activity after incubation with K373R, while K295R and K340R displayed EC_{50} s similar to that of the wild type (Figure 4e, top). Notably, compared to the wild type, K373R showed reduced binding to HRP. To exclude that depletion of activity is resulting from this change in glycan binding, we also measured activity in **11** modified mutants. No change in EC_{50} was observed, further confirming selectivity of **33** for K373 (Figure 4e, bottom).

To rationalize the binding mode of **33** at the carbohydrate binding site of DC-SIGN, we applied an induced fit docking protocol guided by CSPs observed in our ^1H ^{15}N HSQC NMR experiments. This indicated that the warhead of **33** is located close enough to K373 (3.5 Å $\text{N}(\text{H}_2)\text{-C}(\text{OMe})$) for covalent bond formation, which was further confirmed by independent covalent docking (Figure 4f). In the best-scored pose, the 3,5-bis(trifluoromethyl)phenyl moiety and squarate linker orient toward a poorly conserved region containing residues of the canonical and extended carbohydrate binding sites (Figures 4g and S19). The covalently modified lysine K373 itself is also weakly conserved and absent in other mannose-binding C-type lectins such as langerin, DC-SIGNR, dectin-2, and the mannose receptor, suggesting that covalent engagement at this position is DC-SIGN-specific and accounts for the selective enhancement of glycan affinity by **33** (Figure S20).

Taken together, the combination of HSQC NMR, LC MS/MS, mutational analysis, and docking suggested **33** to covalently modify K373, where its non-covalent moiety could either stabilize the Ca^{2+} -bound conformation of the binding site or pre-organize the carbohydrate binding site, thereby facilitating glycan engagement by the DC-SIGN CRD (Figure 4h).

Compound **11** Induces Oligomerization via Non-covalent Binding to a Secondary Site

As the NHS-ester warhead of **11** cannot covalently cross-link the protein, we speculated whether a non-covalent interaction at a neighboring CRD could support the formation of oligomers.

We have previously identified a druggable secondary site in DC-SIGN, which was specifically targeted by phenol and fragments resembling the non-covalent moiety of our hits (Figure 5a).^[15,18] Because binding to this site was shown to be blocked by an M270F mutation, we reasoned that if oligomerization by **11** depends on this interaction, the mutation should abolish oligomer formation (Figure 5a).^[18,37] Strikingly, ^1H ^{15}N HSQC NMR experiments with the ^{15}N -labeled CRD M270F mutant showed no decrease in S/N, and DLS further confirmed lack of oligomerization upon reaction

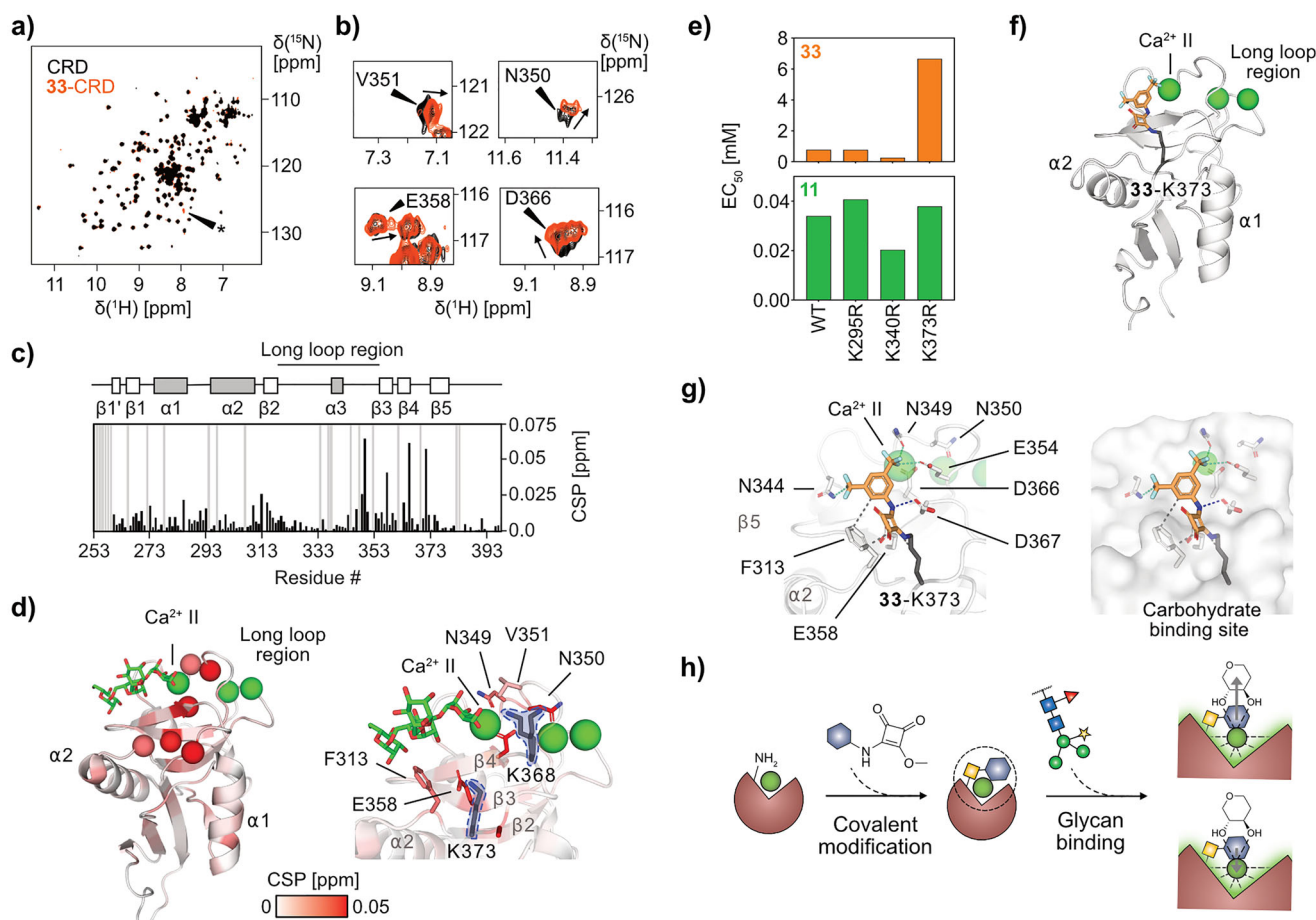


Figure 4. Compound **33** activates DC-SIGN by modifying residue K373 at the carbohydrate binding site. a) Superposition of ^1H ^{15}N HSQC NMR spectra of unmodified ^{15}N -labeled and **33**-modified DC-SIGN CRD wild type. b) Examples of residues showing CSPs compared to the unmodified protein. c) and d) CSP mapping onto the X-ray crystallographic structure of DC-SIGN CRD (PDB: 1SL4) indicates **33** to perturb the long loop region and β -strands 3 and 4 forming the Ca^{2+} -dependent carbohydrate binding site of DC-SIGN. α atoms of residues showing CSP > 0.025 ppm are shown as spheres. Magnification of the region around the carbohydrate binding site suggests K368 or K373 as the major modification sites. e) Comparison of EC_{50} values obtained for the Lys mutants in HRP binding assays. The K373R mutant shows decreased activity upon reaction with **33**. For compound **11** no differences in EC_{50} values were observed. f) Covalent docking of **33** to K373 predicts the binding mode of **33** at the carbohydrate binding site of DC-SIGN. g) Close-up of the predicted binding mode of **33** docked to DC-SIGN. Both the 3,5-bis(trifluoromethyl)phenyl moiety and the squarate linker interact with residues of the extended (F313, E358) and the canonical carbohydrate binding site (N349, N350, E354). h) Schematic representation of the mode-of-action of **33** activating DC-SIGN. Upon covalent modification of K373, **33** either stabilizes the Ca^{2+} -bound conformation of the binding site (bottom) or pre-organizes the carbohydrate binding site (top), thereby facilitating glycan engagement by the DC-SIGN CRD. The 3,5-bis(trifluoromethyl)phenyl moiety and the squarate linker are colored blue and yellow, respectively.

with **11** (Figure 5b,c). On the one hand, this allowed us to exclude non-specific aggregation through, for example, charge neutralization, as a driving factor of the activity of **11**. On the other hand, this highlighted that covalent modification alone is not sufficient to induce oligomerization but equally depends on non-covalent interactions at the secondary site. In the absence of oligomer formation, we were able to calculate CSPs induced at the site of covalent modification by **11** (Figure 5d). Mapping of CSPs on the X-ray structure of DC-SIGN revealed clustering of larger perturbations in α -helix 1 and the loop connecting α -helix 1 and α -helix 2 (Figure 5e). As blocking of the secondary site restricts specific non-covalent interactions, we argued that these CSPs result from covalent modification of one or several of the four Lys residues located in this region (K285, K295, K378, and K379) (Figure 5e). This was supported by additional peaks appearing in the spectra,

resulting from the formation of new amide bonds at the side chains of modified Lys residues (Figures 5b and S21).

To define the reaction site in more detail, we digested the labeled protein by chymotrypsin and ProAlaase, revealing **11** to label K379 within the peptide KFWICKK³⁷⁹S (Figures S22 and S23; Table S6). This is in agreement with our nucleophilicity prediction, suggesting K379 as one of the most reactive lysines available for covalent targeting in the DC-SIGN CRD (Table S3). Kinetic analysis of intact-MS data further confirmed rapid covalent engagement (k_{inact} 0.3442 min^{-1}), yielding a $k_{\text{inact}}/K_{\text{I}}$ ratio of $5.5 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$ (Figure S24 and Table S7).

Notably, K379 lies opposite to a secondary site we identified as critical for the oligomerizing activity of **11** and as a secondary binding site. Because these two sites are located on opposing faces of the CRD, we hypothesized that

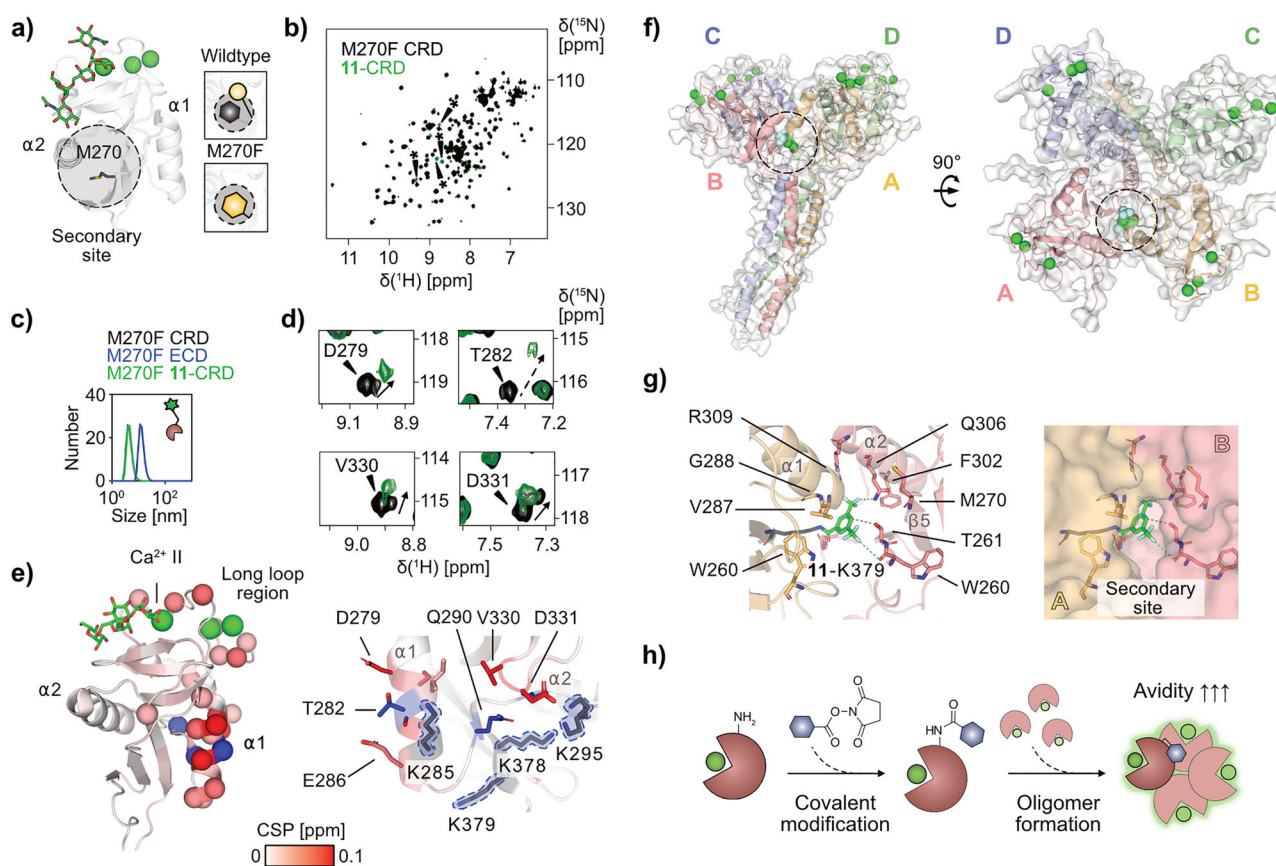


Figure 5. Compound **11** induces oligomerization by modifying K379 and interacting with a secondary site. a) Model of the secondary site below α -helix 2, centered at residue M270, previously found to interact with phenol and drug-like fragments. The secondary site can be blocked by inserting an M270F mutation.^[18,37] b) Superposition of ^1H ^{15}N HSQC NMR spectra of unmodified ^{15}N -labeled and **11**-modified DC-SIGN CRD M270F. No reduction in S/N was observed upon reaction with **11** at protein:**11** ratios of 1:10. New cross-peaks marked by stars appear upon modification with **11**, indicative of the formation of several new amide bonds (Figure S21). c) Size comparison of unmodified and **11**-modified DC-SIGN CRD M270F and DC-SIGN ECD M270F confirms absence of oligomerization due to blocking of the secondary site. d) The ^1H ^{15}N HSQC NMR spectra of **11**-modified DC-SIGN CRD M270F enable analysis of CSPs due to improved S/N compared to wild-type **11**-modified DC-SIGN CRD. Examples of residues showing large CSPs compared to the unmodified protein. e) CSPs mapped onto the X-ray crystallographic structure of DC-SIGN CRD (PDB: 1SL4) show clustering of larger CSPs at α -helix 1, located opposite to the secondary site. α atoms of residues showing CSP > 0.025 are shown as spheres. Blue spheres indicate vanishing or severely broadened resonances upon modification with **11**. Magnification of the region around α -helix 1 reveals several lysine residues in the vicinity of the most perturbed residues. f) Modeling of the complex of tetrameric DC-SIGN including two C-terminal neck repeats with compound **11**.^[50] Covalent coupling at CRD A positions the ligand distal from the carbohydrate binding site at the interface to CRD B, supporting oligomerization. **11** is shown as spheres. The interaction site in CRD B is highlighted with a dotted circle. Ca^{2+} ions are shown as green spheres. g) Close-up of the interface between CRD A and B. Covalent modification of K379 directs the 3,5-bis(trifluoromethyl)phenyl moiety of **11** to interact with a previously described secondary site in CRD B.^[18] The ligand directly interacts with T261 and Q306 of the secondary site, facilitating protein–protein interactions supporting oligomer formation. h) Schematic representation of the mode-of-action of **11** activating DC-SIGN. Upon covalent modification of K379, the non-covalent moiety of **11** supports oligomerization, allowing the protein to interact with glycans with higher avidity. The 3,5-bis(trifluoromethyl)phenyl moiety is colored in blue.

covalent modification at K379 in one CRD (CRD A) and non-covalent binding at the secondary site in a neighboring CRD (CRD B) could promote inter-CRD interactions. To explore this, we analyzed molecular dynamics simulations of the DC-SIGN tetramer modified with **11**, which revealed that such a dual interaction is geometrically feasible at the CRD-CRD interface (Figure 5f). In this complex, the 3,5-bis(trifluoromethyl)phenyl moiety is directly interacting with residues of the secondary site, consequently explaining the lack of oligomerization upon modification of the M270F mutant (Figure 5g).

Collectively, our data support a model in which the covalent coupling of **11** to K379 in CRD A and non-

covalent interaction with the secondary site at another CRD B stabilizes a defined oligomeric assembly. As illustrated by the increase in binding to the mannose-modified sensor with drastically decreased off-rates and a Hill slope of >1, the CRDs in this assembly are arranged to simultaneously engage multiple glycans on the sensor, thereby enhancing avidity through chelation and statistical rebinding effects. The observed oligomerization thus reflects a productive, DC-SIGN-specific process requiring precise spatial complementarity between the covalent and non-covalent binding sites. The structural context of DC-SIGN further supports this specificity. Unlike related C-type lectins such as langerin or DC-SIGNR, which exhibit rigid CRD orientations,

DC-SIGN possesses a flexible CRD-neck junction that allows dynamic rearrangement of individual CRDs to accommodate diverse glycan geometries.^[44,50,51] This conformational adaptability likely determines accessibility of the secondary site and enables the inter-CRD cross-linking geometry required for **11**-induced oligomerization. Most probably, the same mechanism is also present in the ECD, for which we have previously shown that the secondary site is accessible in the flexible relative arrangement of CRDs in the tetramer.^[37]

Conclusion

We identified and characterized the first covalent activators of the human C-type lectin DC-SIGN through systematic functional screening of an electrophilic fragment library, biophysical validation, and structural analyses. Our results reveal distinct mechanisms by which these activators modulate glycan recognition and emphasize the benefits of considering a variety of warheads in covalent screening libraries. In addition to their different reactivity that enables effective mapping of the tractable sites of the target, different pre-reaction, transition state, and post-reaction geometries due to their different labeling chemistries might help explore different functional mechanisms.

Here we identified two distinct mechanisms driving covalent activation of glycan binding of DC-SIGN. The squarate-containing **33** represents a selective, non-oligomerizing activator that covalently modifies K373 within the carbohydrate binding site. This site-specific engagement enhances carbohydrate affinity, likely by stabilizing the glycan-binding-competent conformation. In contrast, the NHS-ester-containing **11** activates DC-SIGN through a distinct, oligomerization-dependent mechanism, combining covalent modification at K379 with specific non-covalent engagement of a secondary site. This dual interaction promotes the formation of defined oligomers that increase avidity through controlled multivalency.

By stabilizing distinct conformational or oligomeric states, these two compounds provide mechanistic starting points for covalently modulating DC-SIGN-dependent cellular processes such as endocytosis, antigen routing as well as signaling response.^[1–11] In particular, the well-defined, single-site mechanism of **33** provides a tractable framework for designing probes or ligands with refined selectivity and reactivity. Likewise, although the complex oligomerization mechanism of **11** may present challenges for structure-based optimization, it offers a conceptually valuable tool to tune and dissect multivalent interactions and their downstream response. These findings therefore establish a structural and mechanistic foundation for the rational design of covalent modulators capable of selectively probing or controlling DC-SIGN-mediated immune functions.

Author Contributions

J.L. expressed proteins, conducted functional screening and validation, analyzed NMR data and wrote the manuscript.

M.B. expressed proteins, conducted NMR experiments and participated in writing the manuscript. N.Cs. synthesized, collected and characterized the library members and participated in protein MS and MS/MS measurements. K.G. participated in NMR and biochemical measurements. Z.O. performed computational modelling. G.S. performed MS and MS/MS measurements. I.A.B. performed the glycan synthesis, characterized the compounds and participated in GCI experiments. P.Á.B. supervised the research work, curated data and participated in writing the manuscript. G.M.K. conceived and supervised the research, acquired funding and participated in writing the manuscript. C.R. designed and directed the research, acquired the funding and supported writing of the manuscript.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: DC-SIGN • Covalent inhibition • Glycobiology • Protein activation • Fragment-based drug design

- [1] A. Engering, T. B. H. Geijtenbeek, S. J. v. Vliet, M. Wijers, E. v. Liempt, N. Demaurex, A. Lanzavecchia, J. Franssen, C. G. Figdor, V. Pigué, Y. v. Kooyk, *J. Immunol.* **2002**, *168*, 2118–2126, <https://doi.org/10.4049/jimmunol.168.5.2118>.

- [2] Y. V. Kooyk, T. B. H. Geijtenbeek, *Immunol. Rev.* **2002**, *186*, 47–56, <https://doi.org/10.1034/j.1600-065X.2002.18605.x>.
- [3] T. B. H. Geijtenbeek, D. S. Kwon, R. Torensma, S. J. v. Vliet, G. C. F. v. Duijnhoven, J. Middel, I. L. M. H. A. Cornelissen, H. S. L. M. Nottet, V. N. KewalRamani, D. R. Littman, C. G. Figdor, Y. v. Kooyk, *Cell* **2000**, *100*, 587–597, [https://doi.org/10.1016/S0092-8674\(00\)80694-7](https://doi.org/10.1016/S0092-8674(00)80694-7).
- [4] T. B. H. Geijtenbeek, R. Torensma, S. J. v. Vliet, G. C. F. v. Duijnhoven, G. J. Adema, Y. v. Kooyk, C. G. Figdor, *Cell* **2000**, *100*, 575–585, [https://doi.org/10.1016/S0092-8674\(00\)80693-5](https://doi.org/10.1016/S0092-8674(00)80693-5).
- [5] E. Navarro-Sanchez, R. Altmeyer, A. Amara, O. Schwartz, F. Fieschi, J. L. Virelizier, F. Arenzana-Seisdedos, P. Desprès, *EMBO Rep.* **2003**, *4*, 723–728, <https://doi.org/10.1038/sj.embor.embor866>.
- [6] T. B. H. Geijtenbeek, D. J. E. B. Krooshoop, D. A. Bleijs, S. J. v. Vliet, G. C. F. v. Duijnhoven, V. Grabovsky, R. Alon, C. G. Figdor, Y. v. Kooyk, *Nat. Immunol.* **2000**, *1*, 353–357, <https://doi.org/10.1038/79815>.
- [7] Q. Lu, J. Liu, S. Zhao, M. F. G. Castro, M. Laurent-Rolle, J. Dong, X. Ran, P. Damani-Yokota, H. Tang, T. Karakousi, J. Son, M. E. Kaczmarek, Z. Zhang, S. T. Yeung, B. T. McCune, R. E. Chen, F. Tang, X. Ren, X. Chen, J. C. C. Hsu, M. Teplova, B. Huang, H. Deng, Z. Long, T. Mudianto, S. Jin, P. Lin, J. Du, R. Zang, T. T. Su, et al., *Immunity* **2021**, *54*, 1304–1319.e1309.
- [8] M. Thépaut, J. Luczkowiak, C. Vivès, N. Labiod, I. Bally, F. Lasala, Y. Grimoire, D. Fenel, S. Sattin, N. Thielens, G. Schoehn, A. Bernardi, R. Delgado, F. Fieschi, *PLoS Pathog.* **2021**, *17*, e1009576.
- [9] S. I. Gringhuis, J. d. Dunnen, M. Litjens, M. v. d. Vlist, T. B. H. Geijtenbeek, *Nat. Immunol.* **2009**, *10*, 1081–1088, <https://doi.org/10.1038/ni.1778>.
- [10] J. Cramer, A. Lakkaichi, B. Aliu, R. P. Jakob, S. Klein, I. Cattaneo, X. Jiang, S. Rabbani, O. Schwaradt, G. Zimmer, M. Ciancaglini, T. A. Mota, T. Maier, B. Ernst, *J. Am. Chem. Soc.* **2021**, *143*, 17465–17478, <https://doi.org/10.1021/jacs.1c06778>.
- [11] M. M. Alam, C. M. Jarvis, R. Hincapie, C. S. McKay, J. Schimer, C. A. Sanhueza, K. Xu, R. C. Diehl, M. G. Finn, L. L. Kiessling, *ACS Nano* **2021**, *15*, 309–321, <https://doi.org/10.1021/acsnano.0c03023>.
- [12] B. Ernst, J. L. Magnani, *Nat. Rev. Drug Discovery* **2009**, *8*, 661–677, <https://doi.org/10.1038/nrd2852>.
- [13] J. Cramer, *RSC Med. Chem.* **2021**, *12*, 1985–2000, <https://doi.org/10.1039/d1md00238d>.
- [14] L. Medve, S. Achilli, J. Guzman-Caldentey, M. Thépaut, L. Senaldi, A. L. Roy, S. Sattin, C. Ebel, C. Vivès, S. Martin-Santamaria, A. Bernardi, F. Fieschi, *Chem. - Eur. J.* **2019**, *25*, 14659–14668, <https://doi.org/10.1002/chem.201903391>.
- [15] J. Aretz, H. Baukman, E. Shanina, J. Hanske, R. Wawrzinek, V. A. Zapol'skii, P. H. Seeberger, D. E. Kaufmann, C. Rademacher, *Angew. Chem. Int. Ed.* **2017**, *56*, 7292–7296, <https://doi.org/10.1002/anie.201701943>.
- [16] J. Aretz, E.-C. Wamhoff, J. Hanske, D. Heymann, C. Rademacher, *Front. Immunol.* **2014**, *5*, 323, <https://doi.org/10.3389/fimmu.2014.00323>.
- [17] H. Zhang, O. Daněk, D. Makarov, S. Rádl, D. Kim, J. Ledvinka, K. Vychodilová, J. Hlaváč, J. Lefebvre, M. Denis, C. Rademacher, P. Ménová, *ACS Med. Chem. Lett.* **2022**, *13*, 935–942, <https://doi.org/10.1021/acsmchemlett.2c00067>.
- [18] R. Wawrzinek, E.-C. Wamhoff, J. Lefebvre, M. Rentzsch, G. Bachem, G. Domeniconi, J. Schulze, F. F. Fuchsberger, H. Zhang, C. Modenutti, L. Schnirch, M. A. Marti, O. Schwaradt, M. Bräutigam, M. Guberman, D. Hauck, P. H. Seeberger, O. Seitz, A. Titz, B. Ernst, C. Rademacher, *J. Am. Chem. Soc.* **2021**, *143*, 18977–18988, <https://doi.org/10.1021/jacs.1c07235>.
- [19] M. J. Borrok, L. L. Kiessling, *J. Am. Chem. Soc.* **2007**, *129*, 12780–12785, <https://doi.org/10.1021/ja072944v>.
- [20] J. Schulze, H. Baukman, R. Wawrzinek, F. F. Fuchsberger, E. Specker, J. Aretz, M. Nazaré, C. Rademacher, *ACS Chem. Biol.* **2018**, *13*, 3229–3235, <https://doi.org/10.1021/acscmbio.8b00875>.
- [21] X. Xie, T. Yu, X. Li, N. Zhang, L. J. Foster, C. Peng, W. Huang, G. He, *Signal Transduction Targeted Ther.* **2023**, *8*, 335, <https://doi.org/10.1038/s41392-023-01589-z>.
- [22] L. Boike, N. J. Henning, D. K. Nomura, *Nat. Rev. Drug Discov.* **2022**, *21*, 881–898, <https://doi.org/10.1038/s41573-022-00542-z>.
- [23] N. Csorba, P. Abranyi-Balogh, G. M. Keseru, *Trends Pharmacol. Sci.* **2023**, *44*, 802–816, <https://doi.org/10.1016/j.tips.2023.08.014>.
- [24] T. Tang, J. Luo, D. Zhang, Y. Lu, W. Liao, J. Zhang, *Eur. J. Med. Chem.* **2025**, *284*, 117202, <https://doi.org/10.1016/j.ejmech.2024.117202>.
- [25] J. Singh, R. C. Petter, T. A. Baillie, A. Whitty, *Nat. Rev. Drug Discovery* **2011**, *10*, 307–317, <https://doi.org/10.1038/nrd3410>.
- [26] S. Wagner, D. Hauck, M. Hoffmann, R. Sommer, I. Joachim, R. Muller, A. Imberty, A. Varrot, A. Titz, *Angew. Chem. Int. Ed. Engl.* **2017**, *56*, 16559–16564, <https://doi.org/10.1002/anie.201709368>.
- [27] G. Antonini, A. Bernardi, E. Gillon, A. Dal Corso, M. Civera, L. Belvisi, A. Varrot, S. Mazzotta, *J. Med. Chem.* **2024**, *67*, 19546–19560, <https://doi.org/10.1021/acs.jmedchem.4c01876>.
- [28] L. Fischer, R. C. Steffens, T. J. Paul, L. Hartmann, *Polym. Chem.* **2020**, *11*, 6091–6096, <https://doi.org/10.1039/D0PY00975J>.
- [29] S. Han, B. E. Collins, P. Bengtson, J. C. Paulson, *Nat. Chem. Biol.* **2005**, *1*, 93–97, <https://doi.org/10.1038/nchembio713>.
- [30] M. E. Abbasov, M. E. Kavanagh, T. A. Ichu, M. R. Lazear, Y. Tao, V. M. Crowley, C. W. Am Ende, S. M. Hacker, J. Ho, M. M. Dix, R. Suci, M. M. Hayward, L. L. Kiessling, B. F. Cravatt, *Nat. Chem.* **2021**, *13*, 1081–1092, <https://doi.org/10.1038/s41557-021-00765-4>.
- [31] S. M. Hacker, K. M. Backus, M. R. Lazear, S. Forli, B. E. Correia, B. F. Cravatt, *Nat. Chem.* **2017**, *9*, 1181–1190, <https://doi.org/10.1038/nchem.2826>.
- [32] N. Peczk, Z. Orgovan, P. Abranyi-Balogh, G. M. Keseru, *Expert Opin. Drug Discovery* **2022**, *17*, 413–422, <https://doi.org/10.1080/17460441.2022.2034783>.
- [33] U. P. Dahal, A. M. Gilbert, R. S. Obach, M. E. Flanagan, J. M. Chen, C. Garcia-Irizarry, J. T. Starr, B. Schuff, D. P. Uccello, J. A. Young, *MedChemComm* **2016**, *7*, 864–872, <https://doi.org/10.1039/C6MD00017G>.
- [34] P. Abranyi-Balogh, L. Petri, T. Imre, P. Szij, A. Scarpino, M. Hrast, A. Mitrovic, U. P. Fonovic, K. Nemeth, H. Barreateau, D. I. Roper, K. Horvati, G. G. Ferenczy, J. Kos, J. Ilas, S. Gobec, G. M. Keseru, *Eur. J. Med. Chem.* **2018**, *160*, 94–107, <https://doi.org/10.1016/j.ejmech.2018.10.010>.
- [35] K. E. Gilbert, A. Vuorinen, A. Aatkar, P. Pogany, J. Pettinger, E. K. Grant, J. M. Kirkpatrick, K. Rittinger, D. House, G. A. Burley, J. T. Bush, *ACS Chem. Biol.* **2023**, *18*, 285–295, <https://doi.org/10.1021/acscmbio.2c00633>.
- [36] S. Ng, N. J. Bennett, J. Schulze, N. Gao, C. Rademacher, R. Derda, *Bioorg. Med. Chem.* **2018**, *26*, 5368–5377, <https://doi.org/10.1016/j.bmc.2018.08.036>.
- [37] J. Lefebvre, M. Besch, M. D. Gamarra, J.-O. Kapp-Joswig, A. Balke, H. Flatau, G. Suchy, E. Georgieva, P. Scheerer, B. G. Keller, C. P. Modenutti, C. Rademacher, *Biorxiv* **2025**, 2025.08.19.671048, <https://doi.org/10.1101/2025.08.19.671048>.
- [38] Q. Zhang, Y. Zhang, H. Liu, H. Y. Chow, R. Tian, Y. M. Eva Fung, X. Li, *Biochemistry* **2020**, *59*, 175–178, <https://doi.org/10.1021/acs.biochem.9b00787>.
- [39] V. Porkolab, C. Pifferi, I. Sutkeviciute, S. Ordanini, M. Taouai, M. Thépaut, C. Vives, M. Benazza, A. Bernardi, O. Renaudet, F. Fieschi, *Org. Biomol. Chem.* **2020**, *18*, 4763–4772, <https://doi.org/10.1039/D0OB00781A>.
- [40] A. Holla, A. Skerra, *Protein Eng., Des. Sel.* **2011**, *24*, 659–669, <https://doi.org/10.1093/protein/gzr016>.

- [41] Y. Guo, H. Feinberg, E. Conroy, D. A. Mitchell, R. Alvarez, O. Blixt, M. E. Taylor, W. I. Weis, K. Drickamer, *Nat. Struct. Mol. Biol.* **2004**, *11*, 591–598, <https://doi.org/10.1038/nsmb784>.
- [42] H. Feinberg, R. Castelli, K. Drickamer, P. H. Seeberger, W. I. Weis, *J. Biol. Chem.* **2007**, *282*, 4202–4209, <https://doi.org/10.1074/jbc.M609689200>.
- [43] M. Wuhler, C. I. Balog, C. A. Koeleman, A. M. Deelder, C. H. Hokke, *Biochim. Biophys. Acta* **2005**, *1723*, 229–239, <https://doi.org/10.1016/j.bbagen.2005.02.013>.
- [44] G. Tabarani, M. Thépaut, D. Stroebel, C. Ebel, C. Vivès, P. Vachette, D. Durand, F. Fieschi, *J. Biol. Chem.* **2009**, *284*, 21229–21240, <https://doi.org/10.1074/jbc.M109.021204>.
- [45] H. Feinberg, Y. Guo, D. A. Mitchell, K. Drickamer, W. I. Weis, *J. Biol. Chem.* **2005**, *280*, 1327–1335, <https://doi.org/10.1074/jbc.M409925200>.
- [46] E. Weber, P. Abranyi-Balogh, B. Nagymihaly, D. K. Menyhard, N. Peczká, M. Gadanecz, G. Schlosser, Z. Orgovan, F. Bogar, D. Bajusz, G. Kecskemeti, Z. Szabo, E. Bartus, A. Tokoli, G. K. Toth, T. V. Szalai, T. Takacs, E. de Araujo, L. Buday, A. Perczel, T. A. Martinek, G. M. Keseru, *Angew. Chem. Int. Ed. Engl.* **2025**, *64*, e202410435, <https://doi.org/10.1002/anie.202410435>.
- [47] M. P. Williamson, *Prog. Nucl. Magn. Reson. Spectrosc.* **2013**, *73*, 1–16, <https://doi.org/10.1016/j.pnmrs.2013.02.001>.
- [48] H. Feinberg, D. A. Mitchell, K. Drickamer, W. I. Weis, *Science* **2001**, *294*, 2163–2166, <https://doi.org/10.1126/science.1066371>.
- [49] C. Delaunay, S. Pollastri, M. Thepaut, G. Cavazzoli, L. Belvisi, C. Bouchikri, N. Labiod, F. Lasala, A. Gimeno, A. Franconetti, J. Jimenez-Barbero, A. Arda, R. Delgado, A. Bernardi, F. Fieschi, *Chem. Sci.* **2024**, *15*, 15352–15366, <https://doi.org/10.1039/D4SC02980A>.
- [50] V. Porkolab, M. Lepšák, S. Ordanini, A. S. John, A. L. Roy, M. Thépaut, E. Paci, C. Ebel, A. Bernardi, F. Fieschi, *ACS Cent. Sci.* **2023**, *9*, 709–718, <https://doi.org/10.1021/acscentsci.2c01136>.
- [51] H. Feinberg, C. K. W. Tso, M. E. Taylor, K. Drickamer, W. I. Weis, *J. Mol. Biol.* **2009**, *394*, 613–620, <https://doi.org/10.1016/j.jmb.2009.10.006>.
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