Neocarzilin A Is a Potent Inhibitor of Cancer Cell Motility Targeting VAT-1 Controlled Pathways

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Supporting Information

ABSTRACT: The natural product neocarzilin A (NCA) was discovered decades ago, and despite its potent cytotoxic effects, no mode of action studies have been performed up to date. Synthesis of neocarzilins A, B, and C and a stereoisomer of NCA provided insights into structural preferences as well as access to probes for functional studies. NCA turned out to be the most active member and was not only effective against cell proliferation but also migration, a novel and so far overlooked activity. To decipher the molecular mode of action, we applied chemical proteomics for target discovery and revealed that NCA targets cancer cell migration via irreversible binding to the largely uncharacterized synaptic vesicle membrane protein VAT-1. A corresponding knockout of the protein confirmed the phenotype, and pull-down studies showed the interaction with an intricate network of key migration mediators such as Talin-1. Overall, we introduce VAT-1 as a promising novel target for the development of selective migration inhibitors with the perspective to limit toxicity in the absence of antiproliferative effects.

INTRODUCTION

Natural products remain a major resource for modern drug development. In cancer medicine, for example, a large fraction of all approved therapeutics is of natural origin.1 These compounds comprise diverse structural classes such as nonribosomal peptides, alkaloids, isoprenoids, and polyketides.2 A wealth of human targets is addressed by these molecules with hot spots in DNA-affecting proteins, cytoskeletal proteins, and tyrosine kinases. While these targets have been successfully exploited by drugs such as irinotecan and paclitaxel, as well as various kinase inhibitors, toxicity is an inevitable challenge when going after pathways essential for viability.

The exploitation of novel modes of action that do not address cell viability is, therefore, an important task requiring the discovery of suitable drug−target pairs. A closer inspection of previously identified but so far neglected structures could be a viable strategy for the identification of new anticancer leads including detailed mode of action studies. Specifically, with the advent of advanced proteomic methods for target identification such as activity-based protein profiling (ABPP), slight synthetic modifications of the parent natural product are sufficient to unravel the full complement of target proteins in living cells.3−5 ABPP has been widely applied to unravel targets of natural products in cancer cells with a strong focus on compounds inhibiting proliferation.6

While inhibition of cancer cell proliferation remains a primary goal of drug development and natural product screening campaigns, inhibition of migration, the crucial step in metastasis, represents an intriguing additional pathway as the spread of cancer can be controlled and less toxicity associated side effects are to be expected.7 Migration is a complex process initiated by actin polymerization to protrude the cell membrane and facilitate interaction with the surrounding tissue.8,9 Altered integrin signaling, which is mediated by diverse integrin associated proteins such as Talin-1, fosters formation of focal adhesions to the extracellular matrix as a key step for propagating migration.8,9 Until now, clinical development of antimigratory drugs has largely focused on a limited number of targets including integrins and matrix metalloproteinases.10,11 To expand the spectrum of druggable targets and further

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elucidate the molecular mechanisms of migration, new chemical entities are needed. Here, natural products could serve as valuable and evolutionary preselected tools in the discovery of essential hot spots in cellular migration.12

In this study, the antimigratory potential of the polyenone neocarzinilin A (NCA), produced by Streptomyces carzinostaticus, was investigated in vitro and in vivo. Detailed mode of action studies revealed that NCA irreversibly binds to VAT-1, a largely uncharacterized enzyme involved in cell migration. Accordingly, VAT-1 knockout studies matched the NCA phenotype of reduced motility. Whole proteome analysis paired with the identification of cellular VAT-1 interaction partners facilitated insights into an intricate regulation network highlighting that VAT-1 interacts with Talin-1, a key driver of migration.

■ RESULTS

Neocarzinilin A Exhibits Potent Antiproliferative and Antimigratory Effects. Neocarzinilins (NC) A and B, long chain polyenones bearing a characteristic trichloromethylke-
tone group, were isolated in the 1990s (Figure 1A). Despite their ease of synthesis and potent cytotoxicity against K562 chronic myelogenous leukemia cells with an IC$_{50}$ of 185 nM for NCA (the only compound tested so far), this class of molecules attracted only little attention. Studies into the corresponding polyketide biosynthesis gene cluster later...
revealed an additional derivative termed NCC exhibiting a dichloro- instead of a trichloromethyl group (Figure 1A). A total synthesis of NCA was reported and provides structural access to all members of the class for in depth functional studies. Following a shortened procedure of Nozoe et al., NCA was synthesized in 6 steps and an overall yield of 19% (Scheme S1). In brief, the synthesis of NCA and NCC started from commercially available (S)-2-methylbutan-1-ol, which was converted to the corresponding aldehyde via TEMPO oxidation. An HWE olefination, followed by a reduction/oxidation sequence yielded the αβγδ-unsaturated aldehyde 3, which was transformed to trienone 4 via Wittig reaction. The formation of the Li-enolate and subsequent quenching with either trichloroacetic anhydride (NCA) or dichloroacetic anhydride (NCC) yielded the desired natural products. To analyze the relevance of the stereocenter, we also prepared the opposite enantiomer (NCA′) via an analogous reaction sequence starting from (R)-2-methylbutan-1-ol (Figure 1A, Scheme S2). Neocarzilin B was synthesized in a similar manner to NCA, starting from isobutyraldehyde (Figure 1A, Scheme S3). With all NCs in hand, we systematically analyzed their antiproliferative potency against a panel of cancer cell lines. NCA exhibited IC_{50} values ranging from about 300 to 800 nM for different cancer cell lines, corroborating previous literature data (Figure 1B, Figure S1A). In contrast, the effects observed for NCA′, NCB, and NCC were less pronounced, with IC_{50} values ranging from about 1–6 μM for human MDA-MB-231 (Figure 1B) and murine 4T1-luc2 breast cancer cells (Figure S1B). Interestingly, while NCA′, NCB, and NCC exhibited minimal antimigratory effects in breast carcinoma cells, NCA significantly diminished the migration of MDA-MB-231 (Figure 1C and D). Comparable effects were obtained in 4T1-luc2 cells albeit with reduced potency of NCA (Figure S1C). These data suggest that already minor structural alterations in stereochemistry and the trichloromethyl group, respectively, influence the overall bioactivity. The antimigratory phenotype was also confirmed by a significant reduction of the forward migration index, a measure of directed migration, determined in a single-cell chemotaxis assay (Figure 1E). Moreover, NCA treatment reduced the directness of cells migrating toward a chemoattractant (Figure S1D). In addition, invasion of NCA treated breast cancer cells was also severely impaired (Figure S1E). NCA had no apoptotic effects on MDA-MB-231 cells at concentrations which are relevant for inhibition of migration (Figure S1F). Of note, apoptosis assays of NCA treated 4T1-luc2 cells revealed minor induction of cell death at concentrations and time points considerable for migration inhibition (Figure S1G), which could slightly impact the antimigratory readout. Whereas cell cycle progression and microtubule network organization were not affected by NCA (Figure S1H and I), activation of the small Rho GTPase Rac1 was compromised in NCA treated cells as shown by Rac1 pulldown assays (Figure 1F). In addition, confocal microscopy images demonstrated reduced formation of lamellipodia and aberrant localization of Rac1 in migrating cancer cells treated with NCA (Figure 1G). In order to investigate the in vivo efficacy of NCA, luciferase tagged 4T1 murine cells were injected into the tail vein of Balb/c mice. Treatment with 10 mg/kg of NCA was suggested to reduce tumor cell dissemination into the mouse lungs compared to solvent control treated animals after 5 days (Figure S1J). Of note, the body weight of the mice during the experiment increased in both groups, thus suggesting a suitable safety profile of the compound (Figure S1J).

**Chemical Proteomics Identify VAT-1 As Cellular Target of Neocarzinilin A.** To unravel NCA’s mode of action, we applied ABPP to target discovery and designed a corresponding alkynylated probe NC-1 (Figure 2A and B, Scheme 1). Given the importance of the trichloromethyl group for biological activity, we hypothesized that this electrophilic structural element might covalently interact with the dedicated protein target. Thus, probe design commenced with an NCA analog bearing a terminal alkyne at the polyene tail (Scheme 1). Upon in situ protein binding and subsequent cell lysis, the alkyne serves as a unique handle to identify protein targets via click chemistry (CC) to biotin azide, avidin enrichment, and quantitative LC-MS/MS analysis (Figure 2A). The synthetic strategy followed the established natural product assembly and utilized hexyn-1-ol as a starting material (Scheme 1). The resulting Neocarzinilin probe (NC-1) was tested for antiproliferative and antimigratory effects. As depicted in Figure S2A, the antiproliferative activity significantly dropped in MDA-MB-231 and 4T1-luc2 cells (IC_{50}s of 24–34 μM) compared to NCA. In contrast the antimigratory activity was only slightly reduced (Figure S2B). In accordance with the results obtained with NCA, neither cell death nor induction of apoptosis was observed upon treatment of the breast carcinoma cell lines with NC-1 (Figure S2C and D). Overall, these results suggest that the probe is suitable for the labeling of NC antimigratory targets. Prior to MS studies, optimal labeling conditions were adjusted via gel-based analysis of probe treated MDA-MB-231, MCF-7, HepG2, and T24 cells followed by CC to rhodamine azide and subsequent fluorescent SDS-PAGE (Figures 2C, S3A and B). One intense 40 kDa protein band was observed in all four cell lines after 1 h of probe incubation. This band remained visible even at low concentrations of 10–100 nM and was outcompeted by the addition of 1- and 5-fold NCA (Figure S3C). The labeling of this band remained even after extended incubation times of 24 and 48 h suggesting sufficient stability upon binding of the protein target (Figure S3D). To unravel the identity of all targets in MDA-MB-231 and HepG2 cells, two complementary proteomic procedures, i.e., stable isotope labeling of amino acids in cell culture (SILAC) and label-free quantification

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**Scheme 1. Synthesis of Probe NC-1:** (a) 1. DMSO (2.22 equiv), (COCl)_{2} (1.11 equiv), NEt_{3} (4.50 equiv); 2. LiHMDS (1.00 equiv), Ethyl (E)-4-(Diethoxyphosphoryl)-but-2-enoate (4.15) (1.00 equiv), CH_{2}Cl_{2}, THF, −78 °C → rt, 4 h, 38% over Two Steps; (b) DIBAL-H (2.10 equiv), MnO_{2} (20.0 equiv), THF, Hexane, −78 °C → rt, 5 h, 54% over Two Steps; (c) 1-(Triphenyl-phosphoranylidene)-2-propanone (2.00 equiv), Toluene, 100 °C, 16 h, 54%; (d) 1. LiHMDS (2.00 equiv); 2. Trichloroacetic Anhydride (1.00 equiv), THF, −78 °C, 3 h, 45%

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were applied to maximize the overall confidence of obtained hits. \textsuperscript{21,22} Cells were incubated with 100–500 nM NC-1 or DMSO as a background control and processed for LC-MS/MS proteome analysis (Figures 2D and E, S4A and B). Detected proteins are visualized in volcano plots displaying enrichment over the background (log\(_2\) (enrichment) > 2) on the x-axis and significance (\(p < 0.05\)) on the y-axis. Importantly, both SILAC and LFQ revealed comparable...
results with one protein protruding as the most significant and highly enriched target in both cell lines, namely, the synaptic vesicle membrane protein (VAT-1, 41.2 kDa; Figures 2D and E, S4A and B). This direct comparison of SILAC and LFQ analysis in ABPP studies demonstrates the adequate and reproducible readout of both methods. To further enhance the cellular pathways and interaction partner of VAT-1 in the context of cell migration. (A) Volcano plot of co-IP of VAT-1 with 2 mM DSSO in MDA-MB-231 (n = 3). GO enrichment analysis of hits (log2(enrichment) > 1, p-value < 0.05) was performed with the Cytoscape BINGO app (SI Tables 8−10), whereas frequencies of enriched GO terms are compared between the co-IP and the global proteome. (B) Volcano plot of global proteome analysis with LFQ in MDA-MB-231 treated with 500 nM NCA for 24 h (n = 6). Proteins identified in the co-IP in A are shown with the same color code. (A,B) Hits of volcano plots are listed in Tables 6 and 7 in the SI. (C) Co-IP of VAT-1 and Talin-1. VAT-1 was precipitated from MDA-MB-231 cell lysates after 24 h of stimulation with NCA. Amount of Talin-1 determined by Western blot was normalized to VAT-1, and results were normalized to the control. Bars represent the mean ± SEM of two independent experiments, two-tailed unpaired Student’s t test, *P < 0.033. (D) T24 cells treated with NCA were engaged in a Scratch assay and costained for VAT-1 (green), Talin-1 (cyan), and actin (red). Nuclei were stained with Hoechst 33342. (C,D) A representative experiment out of three independent experiments is shown.
confidence of the identified targets, competitive studies with an excess of 5-fold NCA were performed via LFQ in both cell lines (Figures 2F, S4C). Again, extended incubation times with 500 nM NC-1 for 24 h did not change the result (Figure S4D). Overall, VAT-1 was presented by far as the best hit in enrichment as well as competition experiments throughout both cell lines. Most of the hits either represent additional proteins of high abundance frequently targeted by electrophilic compounds (e.g., heme oxygenase HMOX2) or displayed insufficient competition (Figures 2F, S4C). These proteins were thus discarded from further analysis and VAT-1 selected for validation.

**VAT-1 Plays an Essential Role in Cancer Cell Migration.** To confirm VAT-1 as a target of NCA directly, we prepared the recombinant protein via overexpression in *E. coli*. Gel-based labeling with NC-1 and fluorescent tagging revealed a clear protein band for *E. coli* lysate after overexpression as well as the recombinant protein (Figure SSA and B). Heat inactivation of the protein prior to labeling strongly reduced the signal, demonstrating binding of the probe and the natural product to the folded and active protein (Figure S5B). Despite intense efforts including MS/MS fragmentation of the labeled protein (purified and endogenous) as well as by applying a strategy to detect the modified peptide using a cleavable linker or a desthiobiotin modification (Figure 3A), we were unable to decipher the NCA binding site. However, we detected an unmodified tryptic peptide by MS/MS, which was eluted from the beads after desthiobiotin enrichment, suggesting that the probe was detached during the MS preparation procedure. In order to confirm this peptide and further narrow down the binding site, we sequentially mutated all nucleophilic amino acids within its sequence (Figure 3B). The resulting His-TEV-VAT-1 constructs were tested for probe labeling in MDA-MB-231, and solely in case of the E113Q mutant, no fluorescent gel band could be observed (Figures 3C, S5C). Of note, the corresponding peptide is only present in one out of three native VAT-1 isoforms (Figure 3B), which are all expressed in MDA-MB-231. Solely the E113 containing isoform is labeled by the probe further corroborating the correct localization of the NCA binding site (Figure 3D). Several additional targets of NCA represent proteins with highly electrophile-sensitive cysteine residues (e.g., GSTO1 (C32), HMOX2 (C265, C282), FAM213A (C85, C88)), which suggests that the trichloroacetylketone group of NCA has some potential for reacting with cysteine residues as well.23–25

Previous studies showed increased mRNA and protein levels of VAT-1 in glioblastomas and benign prostatic hyperplasia compared to healthy tissue.26,27 A corresponding siRNA based VAT-1 knockdown significantly impaired glioma cell migration but not proliferation.26 This functional link to cancer cell motility matches the observed phenotype of NCA and merits further investigation. MDA-MB-231 cells transfected with siRNA against VAT-1 displayed significantly reduced labeling of the signature 40 kDa protein with NC-1, thus directly confirming its identity as VAT-1 and independently validating the MS results (Figure 3D, successful knockdown of VAT-1 in siRNA transfected cells is shown in Figure S6A).

VAT-1 knockdown in MDA-MB-231 and 4T1-luc2 cells had no effect on apoptosis (Figure S6B) and caused 50–80% inhibition of migration (Figures 3E, S6C). At the same time, proliferation was unaffected (Figure S6D). In addition, CRISPR-Cas9 VAT-1 knockout clones were generated by deleting exon 2 of VAT-1 in HEK293 cells (Figure S6E). In comparison to wildtype HEK293 cells and mock-transfected cells (CRISPR control), the VAT-1 knockout clones displayed a strong impairment of migration (to a comparable extent to that observed with NCA; Figure 3F), whereas the proliferative capacity of these cells was not altered (Figure S6F). While this supports that NCA’s cell motility phenotype is predominantly mediated through VAT-1 inhibition, it suggests that the antiproliferative effect could be contributed via a different target.

**VAT-1/Talin-1 Interaction.** Given the strong impact of NCA on migration, we further focused on elucidating the functional role of VAT-1 in cancer metastasis via proteomic studies. Co-immunoprecipitation (co-IP) with an immobilized anti-VAT-1 antibody was carried out in the presence of the DSSO cross-linker in order to capture also transient interactions as shown previously.28 Subsequent analysis of pulled down proteins via LC-MS/MS revealed several significantly enriched hits in MDA-MB-231 cells (Figure 4A). In comparison, the overall effect of NCA on the total proteome was very limited, and no significant effect of NCA on the expression of co-IP hits could be observed, which was further corroborated by independent Western blot data (Figure 4B, Figure S7). Interestingly, several of the identified VAT-1 interaction partners functionally cluster into the regulation of cell adhesion (e.g., gelsolin, GSN; fibronectin, FN1), integrin activation (e.g., Talin-1, TLN), and lamellipodium organization (e.g., RAC2). Foremost, Talin-1, an important activator of integrins and thus involved in regulating migration, protruded as one of the strongest hits. The interaction with Talin-1 could be independently verified by a Western blot-based co-IP assay, in which VAT-1 was pulled down from whole cell lysates (Figure 4C). Of note, treatment of the cells with NCA seemed to even further enhance the interaction of VAT-1 with Talin-1 (Figure 4C). Moreover, immunostaining revealed colocalization of both proteins in lamellipodia at the leading edge of migrating cells (Figure 4D).

**DISCUSSION**

The detailed investigation of NCA in the context of cancer decades after its discovery demonstrates the power of chemical proteomics. While the molecule was only known for potent cell toxicity,23 its antimigratory effects have been overlooked. By investigating the impact of NCA and its derivatives on the inhibition of migration and elucidating its mode of action via ABPP-based target-fishing approaches, we demonstrate that VAT-1 is a promising novel drug target in cancer research. This largely uncharacterized protein emerges as the by far most prominent hit in both SILAC and LFQ methodologies as well as in competitive labeling approaches, which emphasizes the high specificity of NCA to its target VAT-1. Interestingly, no other proteins presented as prominent hits, suggesting that our simplified probe design lacking the methyl-stereocenter of NCA may have lost affinity for the target(s) responsible for the antiproliferative effects. The importance of this stereocenter was corroborated by synthesis of NCA′ bearing the opposite absolute configuration, resulting in a drop in potency.

Although we were unable to decipher the exact binding mode of NCA to VAT-1, we identified the region of the protein that is modified and unraveled E113 as the likely point of irreversible attachment. Future studies involving cocystalization will have to show how this interaction affects the function of the protein on a molecular level.
Little is known to date about VAT-1: It is proposed to play a role in cancer cell motility and shows putative ATPase activity and calcium dependency.\textsuperscript{26,31,35} In addition, a homology to \textit{E. coli} quinone oxidoreductases as well as eye lens zeta-Crystallin has been described.\textsuperscript{33} The present study further illustrates that inhibiting the expression of VAT-1 either by specific siRNA or CRISPR-Cas9 knockout strongly abrogates migration of the cells, thus paralleling the robust biological antimigratory effects of NCA. Of note, in contrast to the effect of NCA on inhibition of proliferation, genetically impairing VAT-1 expression does not affect cellular growth. Hence, whereas the antimigratory effects of NCA are mediated by its target VAT-1, further studies will focus on the so far unknown binding partners of NCA, which are implicated in inhibition of proliferation.

Proteome analysis to unravel the role of VAT-1 in regulating cellular migration revealed that VAT-1 interacts with a network of proteins associated with cell motility. Intriguingly, Talin-1 could be identified as a direct interaction partner of VAT-1 both by MS-based co-IP experiments as well as by immunoblotting. Talin-1 is one of the key proteins activating integrin signaling, mediating linkage of integrins to the cytoskeleton, formation of focal adhesions, and subsequently promoting migration.\textsuperscript{34} Although treatment of the cells with NCA seemed to enhance the interaction of its target VAT-1 with Talin-1, future studies need to elucidate how this interaction regulates this network on a molecular level.

In summary, VAT-1 could be identified as a so far unknown player in regulation of cancer cell migration that directly interacts with crucial cell migration proteins such as Talin-1. Moreover, with the identification of NCA as a potent migration inhibitor, chemically easily assessable VAT-1 probes are now available for further studies. Hence, VAT-1 with its unique mode of action is set on stage as a novel drug target for anticancer strategies preventing migration and metastasis.

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\section*{ABBREVIATIONS}

VAT-1, synaptic vesicle membrane protein VAT-1; ABPP, activity-based protein profiling; NC, Neocarzinil; CC, click chemistry; SILAC, stable isotope labeling of amino acids in cell culture; LFQ, label-free quantification; WT, wildtype; CRISPR, clustered regularly interspaced short palindromic repeats; co-IP, co-immunoprecipitation; FNI, Fibronectin; GSN, Gelsolin; TNL1, Talin-1; Rac2, Ras-related C3 botulinum toxin substrate 2; siRNA, small interfering RNA

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