Cell-permeable CaaX-peptides affect K-Ras downstream signaling and promote cell death in cancer cells

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Introduction
Protein prenylation is an irreversible post-translational modification (PTM) found in eukaryotic cells and includes farnesylation (15-carbon body) and geranylgeranylation (20-carbon body) at a C-terminal CaaX box, comprising cysteine (C) and aliphatic (a) and variable (X) amino acids [1–3]. Attachment of these isoprenoids to cysteine occurs by prenyltransferases (farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase)) and results in a stable thioether bond depending on the identity of the variable amino acid X (Fig. 1A) [4–7]. Subsequent steps include proteolysis to release the terminal aaX tripeptide by the endoprotease RAS-converting enzyme 1 (RCE1) [8,9], and methylation of the free carboxylic function of the terminal cysteine residue by isoprenylcysteine carboxyl methyltransferase (ICMT) [10,11]. Cysteine prenylation has been studied extensively owing to its involvement in numerous biological processes, particularly signal

Abbreviations
CF, 5,6-carboxyfluorescein; CLSM, confocal laser scanning microscopy; CPP, cell-penetrating peptides; ER, endoplasmic reticulum; FTase, farnesyltransferase; FTI, farnesyltransferase inhibitor; GGTase, geranylgeranyltransferase; HVR, hypervariable region; ICMT, isoprenylcysteine carboxyl methyltransferase; PDAC, pancreatic ductal adenocarcinoma; PTM, post-translational modification; RCE1, RAS-converting enzyme 1; SPPS, solid-phase peptide synthesis.
transduction pathways that regulate cell growth and proliferation [4,12]. In this respect, the activation of small GTP-binding Ras proteins, which induces the cycling between an active GTP-bound and an inactive GDP-bound state [13], is of high interest. In mammalian cells, the Ras subfamily consists of several isoforms, including HRas, N-Ras, and the splice variants K-Ras-4A and K-Ras-4B [14]. All four family members show high-sequence homology in their N-terminal globular domain but differ in their C-terminal hyper-variable region (HVR) including the secondary signal and the CaaX box (Fig. 1 for HRas and K-Ras-4B) [15]. Ras proteins are important nodes in many cellular processes, such as cell proliferation, growth, survival, and intracellular signaling networks, and their dysregulation is responsible for severe malignancies [4]. In fact, RAS genes are the most commonly mutated oncogenes in cancer and roughly 20–30% of all tumors show enhanced RAS activity due to specific mutations [16,17].

For example, 90% of pancreatic carcinomas show activating K-Ras (point mutations at codon 12, 13, or 61) [20–22]. Earlier studies showed that gain-of-function mutations result in an arrest of Ras proteins in their active GTP-bound state, and oncogenic Ras is considered as a promising target for anticancer therapy. But so far, active Ras mutants are undruggable due to the lack of suitable pockets for binding small molecule inhibitors [15,19]. An alternative targeting strategy implies interference of the post-translational processing of Ras proteins [12,23], which requires membrane association promoted by protein prenylation for initiation of downstream signaling cascades [24,25]. Early studies already highlighted that loss-of-membrane association prevents Ras mutants to induce cell transformation [26]. Initial attempts to develop pharmacological Ras inhibitors focused on FTase inhibitors (FTI) [27], but resulted in disappointing clinical trials [28]. This was partly due to the fact that K-Ras can also be a substrate for GGTase, leading to geranylgeranyl cross-prenylation and rescue of its oncogenic activity [28,29].

However, owing to its involvement in multiple biochemical pathways, many studies are still ongoing to develop tools that help to understand enzymes involved in the prenylation process and to find novel drug targets, particularly in terms of Ras function and

![Fig. 1](image-url)
inhibition. For instance, analogs of isoprenoid diphosphates are utilized to analyze the mechanisms of prenylation, to identify prenylated proteins, and to validate enzymatic activities of prenyltransferases [30,31]. Additionally, libraries of short peptides having free CaaX-termini were screened against substrate specificity of prenyltransferases [32]. To temporally control prenylation, caged substrates (either isoprenoids or peptides) were developed that can be released upon photolysis and were used to characterize the prenylation reaction in more details [33]. While those studies helped to get a clearer picture about structural and mechanistic features, as well as the enzymology of prenyltransferases, still much has to be learned about protein prenylation in living cells. Recently, an approach in this direction was provided by Flynn et al. that created a calibrated sensor for cellular FTase activity [34]. Moreover, also cell-penetrating prenylated peptides were generated as tools to study enzymatic processing and intracellular trafficking related to this PTM [35,36].

Herein, we designed cell-permeable peptides incorporating the C-terminal sequence of Ras proteins and investigated their impact on intracellular prenylation processes. The so-called CaaX-peptides include a cell-penetrating peptide (CPP) sequence, namely sC18* [37,38], and the CaaX boxes CVLS and CVIM, which are derived from HRas and K-Ras-4B, respectively (Fig. 1). Our results highlight that these peptides are specifically recognized by prenyltransferases (most probably FTase; Fig. 1B) based on their PTM motifs. Thus, we present an intriguing example of how the intracellular accumulation of cell-permeable peptides can be controlled. Strikingly, these novel tools induced notable downstream effects on the cellular level, particularly in cancer cells, what makes them a promising, novel concept to address Ras proteins, and their signaling cascades.

### Results and Discussion

#### Design and synthesis of cell-permeable CaaX-peptides

Three different CaaX-peptides were designed comprising each a cell-penetrating peptide sequence at the N-terminus, followed by the so-called secondary signal [25] and the CaaX motif (see Table 1 for sequences). In case of the K-Ras-4B-derived peptide CaaX-1, we replaced parts of the secondary signal, which is responsible for plasma membrane association in acidic regions (Fig. 1) [16,39]. In our opinion, this polybasic feature was perfectly fulfilled by the sequence of sC18*. This truncated CPP version still demonstrates cell-penetrating abilities [38,40] and was used in order to keep the general length of all peptide hybrids as short as possible. Moreover, the penultimate amino acid of the CaaX motif of HRas (CaaX-2), which is in this case a leucine, was exchanged with a glycine (yielding peptide CaaX-3). This amino acid substitution was previously shown to result in loss-of-membrane association and increased cytosolic distribution for an eGFP-CVGS construct [34]. All designed peptides were synthesized by solid-phase peptide synthesis (SPPS) using an optimized synthesis strategy (Fig. 2).

Subsequently, they were labeled with 5(6)-carboxyfluorescein (CF) or biotin, respectively, and after cleavage from the resin and purification, the amino acid sequence was substantiated by mass spectrometry. As controls, peptides having the CaaX box cysteine substituted with serine were generated (SaaX-1 and SaaX-2), since prenylation does not take place at serine [34].

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**Table 1.** Names, sequences, and analytical data of investigated peptides.

| Names | Descriptions | Sequences | MW_{calc} [Da] | MW_{exp} [Da] | Net charge |
|-------|--------------|-----------|----------------|----------------|------------|
| CaaX-1 | sC18*-KRas4B(181-188) | GLRKRLKFRNK-SKTK-CVIM-OH | 2463.1 | 2463.6 | +9 |
| SaaX-1 | sC18*-KRas4B(181-188), C185S | GLRKRLKFRNK-SKTK-SVIM-OH | 2447.0 | 2447.5 | +9 |
| CaaX-2 | sC18*-HRas(181-189) | GLRKRLKFRNK-GCMSCK-CVLS-OH | 2584.2 | 2584.7 | +8 |
| CaaX-3 | sC18*-HRas(181-189), L188G | GLRKRLKFRNK-GCMSCK-CVGS-OH | 2528.2 | 2528.5 | +8 |
| SaaX-2 | sC18*-HRas(181-189), C186S | GLRKRLKFRNK-GCMSCK-SVLS-OH | 2568.2 | 2568.9 | +8 |
| sC18* | – | GLRKRLKFRNK-NH2 | 1571.9 | 1571.4 | +8 |
| Control peptides | | | | | |
| sC18*-KR | sC18*-KRas4B(181-184) | GLRKRLKFRNK-SKTK-NH2 | 2015.5 | 2015.8 | +10 |
| KR-C-1 | KRas4B(181-188) | SKTK-CVIM-OH | 909.2 | 909.1 | +2 |
| KR-S-1 | K-Ras4B(181-188), C185S | SKTK-SVIM-OH | 893.1 | 893.2 | +2 |
| sC18*-HR | sC18*-HRas(181-185) | GLRKRLKFRNK-GCMSCK-NH2 | 2180.7 | 2180.7 | +9 |
| HR-C-2 | HRas(181-189) | GCMSCK-CVLS-OH | 1030.3 | 1030.9 | +1 |
| HR-C-3 | HRas(181-189), L188G | GCMSCK-CVGS-OH | 974.2 | 973.8 | +1 |
| HR-S-2 | HRas(181-189), C186S | GCMSCK-CVLS-OH | 1014.2 | 1014.0 | +1 |
addition, several more control peptides were included, lacking either the CaaX boxes or the CPP (Table 1).

Cell-permeable CaaX-peptides get intracellularly enriched

We first assessed the cellular uptake of CaaX-peptides in the human cancer cell lines HeLa and MCF-7, as well as in noncancerous mouse fibroblasts (MEF), which all express the wild-type Ras protein. Cells were treated with 10 μM of CF-labeled peptide solutions for 0.5 h at 37 °C (Fig. 3A).

An increase in green fluorescence was recognized when cells were incubated with CaaX-peptides, compared to the control peptides containing the SaaX-motifs and compared to the CPP alone. In fact, we noticed a 20- to 100-fold increased uptake dependent on the CaaX-peptide used (Fig. 3A). For other hybrid CPP-peptide conjugates with improved membrane activity, and thus, increased internalization efficiency, this was a result of a change in secondary structure or amphipathicity [37,38,41–43]. However, the herein obtained results rather suggest that enrichment of the CaaX-peptides is triggered and enhanced after selective intracellular recognition by their CaaX motifs. This hypothesis is in accordance with the observed negligible uptake of the SaaX controls, in which only one amino acid was exchanged (C → S), and which displayed similar secondary structures (Fig. 3C). We further verified this assumption by studying in detail the impact of each motif of the peptides. For this purpose, control peptides lacking the CaaX box, or the CPP,
respectively, were synthesized and their cellular uptake was quantified. Notably, we detected for almost all of these control peptides significantly decreased internalization efficiencies (Fig. 4B, C), underlining again the importance of both moieties for their intracellular function.

Comparing the uptake of the different CaaX-peptides in the studied cell lines, we found that cellular translocation was cell line-dependent; however, the CaaX-3 peptide indicated the highest uptake in all cell lines tested (Fig. 3A). Interestingly, the cellular uptake was lowest for MCF-7 cells, whereby in HeLa and MEF cells relatively similar uptake levels were detected. Moreover, we observed a time-dependent cellular accumulation in HeLa cells, which was highest after 2 h and decreased again after 6 h of incubation pointing to degradation or cellular release of the peptides (Fig. 3B).

To get more insights into the intracellular distribution of the CaaX-peptides, we performed confocal laser scanning microscopy (CLSM) using the same experimental conditions as before. Figure 5 shows the results when cells were incubated for 0.5 and 2 h, which confirm the internalization ability of all CaaX-peptides tested. Again, CaaX-peptides were only hardly detectable (shown for SaaX-1 in Fig. 5F). In addition, CaaX-peptides exhibited a mostly vesicular distribution pattern suggesting an endocytic uptake mechanism (Fig. 3A–C). Interestingly, when inspecting CaaX-3 in HeLa cells after 2 h, the compound displayed also a diffuse cytosolic distribution, corroborating recent results, where it was shown that an EGFP-CVGS mutant was diffusely expressed in HEK293T cells [34,44].

Cell-permeable CaaX-peptides have unexpected impacts on cell viability

To analyze the cytotoxic profile of the novel peptides, we incubated them in several concentrations for 24 h with the aforementioned three cell lines. No significant effects on cellular viability were measured when cells were treated with all of the control peptides, particularly SaaX-1, SaaX-2, and the CPP sC18*, respectively.
Fig. 4. Synthesis and analysis of control peptides (Table 1). (A) Design of control peptides missing either the CaaX box or the CPP (B). (C) Cellular uptake of synthesized control peptides into HeLa cells quantified by flow cytometry. Cells were incubated with 10 μM of CF-labeled peptide solution for 0.5 h at 37 °C. Fluorescence intensities were normalized to the internalization of sC18*. (D) Cytotoxicity of peptides when incubated with HeLa cells at 37 °C for 24 h with indicated concentrations. Cellular viability was related to untreated cells (100%). Error bars of (B–D) represent the standard deviations of n = 9 values.

Fig. 5. Microscopic analysis of CaaX-peptides using (A) HeLa, MEF, MCF-7, PANC-1, and Capan-1 cells. (B) Images of control peptides incubated with HeLa and PANC-1 cells. Cells were incubated with 10 μM of CF-labeled peptide solution for 0.5 or 2 h at 37 °C. Green: CF-labeled peptide; blue: nuclear Hoechst 33342 stain; scale bar is 10 μm. (C) K-Ras localization assessed by immunofluorescence staining via the K-Ras antibody and visualized with IgGk light chain binding protein (m-IgGk BP; red) after treating PANC-1 cells for 24 h with 30 μM of unlabeled CaaX-1 peptide. Cells were fixed with 4% paraformaldehyde. Live cell imaging (A, B) was conducted in three replicates, while immunostaining (C) was performed twice.
Fig. 6. (A–E) Cytotoxicity profiles of novel peptides. Cells were incubated for 24 h with various concentrations of indicated peptides. Cellular viability was related to untreated cells (100%); assays were performed in triplicates (n = 3), and therefore, error bars of (A–E) indicate the standard deviations of n = 9 values. (F) Flow cytometric analysis of cell apoptosis/necrosis using the Annexin V-FITC/7-AAD kit (Beckman Coulter). PANC-1 cells were incubated with 30 µM of peptides for 24 h. As a positive control, cells were incubated with 7 µM of staurosporine.
The latter is in agreement with previously published data [37,38]. Moreover, no significant decrease in cellular viability was detected when MEF cells were incubated with CaaX-peptides, although high intracellular enrichment near to the range of HeLa cells was observed (Fig. 3A).

Of more interest was that peptides carrying a CaaX motif displayed cytotoxicity when applied in concentrations ≥ 50 µM for HeLa and MCF-7 cells, while in the latter cell line the strongest effects were observed. This was interesting, since MCF-7 cells, like the majority of breast cancer cells, do not carry mutations in Ras proteins. However, it was already shown that cytokines often lead to increased levels of the GTP-bound form of WT-Ras and are able to turn it into tumor-promoting entities regarding breast cancer growth and development [45]. Moreover, antitumor activity of FTase inhibitors (FTIs) in breast cancer was presented recently. This activity was assumedly independent on the mutation status of Ras, but rather dependent on many other factors accompanied by FTase action [46]. Therefore, we speculated that the relatively low cellular uptake in combination with the cytotoxic effects could point to elevated levels of FTase in combination with activated Ras proteins in the MCF-7 cells and, thus, a higher cellular impact of our peptides on intracellular Ras processing and interaction.

### Evaluating the influence of CaaX-1 on K-Ras-mutated cells

Inspired by the above-mentioned observation that CaaX-peptides may have an impact on cells in which Ras isoforms are dysregulated, we focused in the following on the K-Ras-derived peptide CaaX-1 and its function in K-Ras-mutated cells. Indeed, K-Ras was shown to be the most frequent mutated isoform in Ras-driven cancers, with high frequencies of around 40% in colorectal cancers, and more than 90% in pancreatic ductal adenocarcinomas (PDAC) [47]. For our following studies, we thus chose two pancreatic cancer cell lines (PANC-1 and Capan-1), and the colorectal cancer cell line LS174T, which all carry a K-Ras mutation.
mutation at G12 (Fig. 7A). This oncogenic mutation is present predominantly in human cancers (89%), and impairs intrinsic and GAP-mediated GTP hydrolysis [48]. Interestingly, in terms of toxicity, PANC-1 cells were the most sensitive cell line according to the treatment of the CaaX-1 peptide, followed by LS174T and Capan-1 cells (Fig. 7B). Again, no effects on cellular viability were observed for SaaX-1 or Sc18* (Fig. 7C, D). In addition, an apoptosis assay revealed an increased number of PANC-1 cells undergoing apoptosis and necrosis after treatment with CaaX-1 (Fig. 6F).

Regarding the cellular uptake, the results point to comparable internalization efficiencies of CaaX-1 in these three cell lines and MCF-7 cells (Fig. 7E), although the overall uptake was lower in comparison with HeLa and MEF cells (Fig. 3A). To determine whether there was any relationship between the observed toxicity and recognition of prenylation motifs, we validated the expression levels of farnesyltransferase by performing western blot analysis (Fig. 7F). The intensities of FTase expression in the analyzed cell lines correlated well with the degree of toxicity (PANC1>MCF-7>HeLa>LS174T>MEF). In contrast, Capan-1 cells showed no correlation, while displaying high expression levels of FTase, but only low sensitivity to the CaaX-1 peptide. Although the mutation in the K-Ras amino acid sequence of PANC-1 and Capan-1 cells is at the same position (glycine 12), it differs concerning the substituted amino acid (G12V versus G12D), which might also impact gene expression and other factors resulting in this observed effect [48,49].

We then assessed the intracellular distribution after 0.5 and 2 h performing confocal microscopic analysis, which revealed similar phenotypes of PANC-1 and Capan-1 cells after treatment with CaaX-1, demonstrating predominantly punctuate patterns (Figs 5A,B and 7G).

**FTase activity is responsible for intracellular enrichment of CaaX-1**

Given the high activity of CaaX-1 in PANC-1 cells, we focused in the following on this cell line. First, we further investigated the time-dependent uptake of CaaX-1 (Fig. 8A). We observed a 4.5-fold increase in internalization after 2 h, and in contrast to HeLa cells (Fig. 3B), the signal did not decrease after 6 h, which might be due to the hyperactivated status of K-Ras. In the next step, we explored the cellular uptake of CaaX-1 in a three-dimensional cell model by preparing 3D-PANC-1 spheroids (Fig. 8B,C).

Here, mean fluorescence intensities were lower in comparison with the inspected 2D cell culture models. More interestingly, we recognized a relatively homogeneous internalization into all PANC-1 cell layers. Our data were supported by a nearly Gaussian intensity distribution (Fig. 8C). Generally, for a peptide that internalizes only in the periphery of the spheroid the intensity distribution is expected to be broader, than for a peptide that passes through different layers of the spheroid [50]. In comparison, the intensity distribution of the control peptide SaaX-1 displayed more heterogeneity with two populations pointing to a low peripheral uptake only into the outer layers of the spheroids. Also, in this case, uptake efficiencies were negligible supporting again the importance of the presence of the CaaX box for intracellular enrichment.

In order to analyze whether the CaaX-peptides were indeed identified by farnesyltransferase, we studied the impact of different inhibitors on the internalization efficiencies of CaaX-1. Therefore, we used the farnesyl analog AFCME [51], the FTI tipifarnib [52] and we applied Mevastatin [53], which inhibits conversion of hydroxymethylglutaryl (HMG)-CoA into mevalonate, an upstream precursor of FPP and geranylgeranylprophosphate (GGPP) [54]. We preincubated PANC-1 cells with the different inhibitors, respectively, before adding CaaX-1, followed by flow cytometry analysis of the intracellular uptake (Fig. 8D). Interestingly, uptake of CaaX-1 was not altered after incubation with tipifarnib and even increased after incubation with AFCME, which was supposedly the result of cross-prenylation by GGTase [55], a process recently described [56,57]. Therefore, to completely inhibit prenylation of K-Ras, both pathways need to be disrupted. Accordingly, treatment of PANC-1 cells with Mevastatin resulted in significantly decreased cellular uptake of CaaX-1, proving a prenylation-dependent accumulation of CaaX-1.

To further substantiate CaaX-1 interaction with FTase, we synthesized biotinylated CaaX-1 and performed pull-down assays. Indeed, when magnetic streptavidin beads were preloaded with biotinylated CaaX-1, specific interaction with either recombinant rat FTase or prenyltransferases of PANC-1 cell lysate was detected (Fig. 8E,F). Based on our obtained data thus far, we suggest the following model for CaaX cellular uptake and intracellular fate: After internalization of CaaX-peptides, they are recognized and processed by prenyltransferases (e.g., FTase), and assumedly modified with an isoprenoid lipid. This intracellular modification would then induce a removal of the CaaX-peptides from the internalization equilibrium, triggering intracellular enrichment (Fig. 9).

Based on this model, we wondered whether internalization of CaaX-1 would also be responsible for an
upregulated level of FTase, sort of a consequence of its intracellular modification. We measured the expression level of FTase in CaaX-1- and SaaX-1-treated PANC-1 cells, in comparison with untreated cells. As depicted in Fig. 8G, band intensities did indeed reinforce our hypothesis. We also speculated that CaaX-1 peptides did not act as FTIs, but more as competitors to other proteins serving as substrates for prenyltransferases, such as K-Ras. This is supported by the finding that K-Ras expression levels remained unaffected (Fig. 8H) and we did not observe any alteration in K-Ras localization (Fig. 3C). Therefore, we concluded that off-target effects might have led to the observed strong cytotoxicity in PANC-1 cells.

Proteome profiling of CaaX-1-treated PANC-1 cells reveals changes in K-Ras downstream signaling

We further examined the proteomic changes caused by CaaX-1 incubation in comparison with SaaX-1-treated and untreated (control) PANC-1 cells. A label-free quantitative proteome analysis was performed after incubating cells for 24 h with 30 µM peptide solutions. We quantified in total 4361 proteins, of which 186 were significantly altered between the three conditions (based on ANOVA, *P < 0.05; **P < 0.005; ***P < 0.0005). Hierarchical clustering revealed four different protein clusters (n = 146), from which cluster 1 (n = 73) and cluster 4 (n = 36) contained proteins that were exclusively upregulated or downregulated after treatment with CaaX-1, respectively (Fig. 10A). Among the upregulated proteins in cluster 1, we found candidates involved in actin binding and actin filament organization including Thymosin beta-4, Thymosin beta-10, and LIM and SH3 domain protein 1. The organization of the actin cytoskeletal is mediated by Ras-regulated signaling pathways, and its dysregulation assumedly is related to a dysfunction of Ras proteins after CaaX-1 treatment [57]. Furthermore, within both clusters (1 and 4), we identified proteins associated with the ER, an important organelle for protein folding, post-translational...
modifications, and the delivery of proteins to their correct destination [58]. In particular, we detected upregulated proteins implicated in protein folding as well as the unfolded protein response, which is usually triggered by ER stress, for example, heat-shock 70 kDa protein 5, Yip1 interacting factor homolog A, and SEC61 translocon beta subunit. As downregulated proteins, we identified subunit of the eukaryotic translation initiation factor 2, which is known to relieve the ER workload during ER stress by attenuating protein translation [59,60], and the calcium sensor programmed cell death protein 6 playing a key role in ER to Golgi vesicular transport [61].

Of more interest was that we could allocate several proteins in both clusters (1 and 4), which were directly related to Ras signaling cascades. Cluster 1 contained STAM-binding protein, which is involved in the negative regulation of the PI3K-AKT pathway and Ras-related signal transduction. Moreover, we found inositol-3-phosphate synthase 1 in cluster 4, which is a rate-limiting enzyme in the synthesis of all inositol-containing compounds, including PIP3, which is an important mediator in AKT signaling pathways. Also, we found cAMP-dependent protein kinase type I-alpha in cluster 4, which is a regulatory subunit of the protein kinase A, and, in fact, is a negative regulator of the RAF/MEK/ERK (MAPK) pathway [62,63]. Indeed, it was shown that PRKAR1A inactivation is associated with dysregulated PKA resulting in increased expression and activation of the MAPK pathway [64,65]. In line with this, we further identified IRF-2-binding protein 2 in cluster 4, a negative regulator of the NFAT1 transcription factor [66] being one of the many downstream effectors of the RAF/MEK/ERK pathway. We also performed a 1D annotation enrichment analysis to identify global changes in pathways between CaaX-1-treated and untreated control cells (Fig. 10B). We detected again effects in actin polymerization (Arp2/3 complex formation) and ER trafficking (SRP-dependent proteins). However, more of note was that we found changes in pathways related to the PI3K/AKT pathway, affecting PTEN and PIP3, matching our previous observations. Notably, these reside downstream the insulin receptor/insulin-like growth factor receptor cascades, which were also significantly influenced by the CaaX-peptides.

Since it was recently shown that pancreatic cancer initiation, progression, and maintenance depend on K-Ras-PI3K-Pdk1 signaling [67,68], we furthermore assessed the activation state of direct downstream effectors of K-Ras by western blot analysis, including members of the RAF/MEK/ERK and PI3K/AKT pathway (Fig. 10C). While protein levels of MEK1/2, ERK1/2, and AKT were unaffected after treatment, the phosphorylation state of all three proteins was altered compared with untreated samples. Surprisingly, the treatment of PANC-1 cells with CaaX-1 increased phosphorylation of MEK and ERK kinases. In contrast, a decrease in phosphorylation of AKT indicated an inhibition of the PI3K/AKT pathway, which is in accordance with the data obtained in our proteome profiling (Fig. 10A,B). The reason for the relatively low levels of phosphorylated ERK1/2 and MEK1/2 in the control cells may be correlated with the fact that constant activation of the ERK pathway may lead to growth arrest [68].

Fig. 9. Model of internalization and processing of the synthesized CaaX-peptides and their possible intracellular effects. Assumedly, CaaX-peptides are taken up mainly by endocytic mechanisms, whereas also direct translocation might be possible. After internalization, they are recognized and processed by prenyltransferases, which potentially modify them with an isoprenoid lipid, possibly leading to localization to membranous structures. This intracellular modification would then induce a removal of the CaaX-peptides from the internalization equilibrium, triggering intracellular enrichment, and possibly also a positive feedback regulation in terms of FTase expression levels.
It was already shown that transient activation of ERK was followed by sustained lower levels of ERK activity inducing cell proliferation in many cell systems [69–72]. This finding would also explain the differences of control and SaaX-1-treated samples. In summary, our results indicated once again that CaaX-1 peptides might interfere with FTase and thus, compete with the post-translational modification of farnesylated K-Ras, or with other processes important for the dysregulated K-Ras status in PANC-1 cells. For instance, it was recently demonstrated that Ras dimerization is important to activate effector pathways. Probably, Ras–Ras dimer formation and following protein binding processes were impaired by the intracellular accumulation

Fig. 10. (A) Hierarchical clustering and line-plot of Z-score-normalized protein intensities. Clusters were identified by hierarchical clustering of significantly changed proteins (ANOVA, \( P \) value < 0.05). Representative proteins included in the clusters are listed, those related to Ras signaling pathways are highlighted in light gray. (B) 1D annotation enrichment identified significantly changed terms (FDR < 0.02). Box plots show relative fold change between CaaX-1 and untreated cells for proteins annotated with the indicated terms, whereby significant changes related to Ras signaling pathways are highlighted in light gray. (C) Western blot analysis showing the activation state of the RAF/MEK/ERK and PI3K/AKT pathways in PANC-1 cells after treatment with CaaX-1 (\( n = 3; \) loading control: \( \beta \)-actin).
of CaaX-1 peptides [73]. Moreover, it might be assumed that downstream signaling pathways of K-Ras were directly altered by CaaX-1, leading to a switch from the PI3K/AKT pathway observed in K-Ras-mutated PANC-1 cells to the physiological RAF/MEK/ERK pathway [74].

**Conclusion**

Within this work, we designed and synthesized so-called CaaX-peptides bearing the cell-penetrating peptide sC18* and C-terminal sequences of Ras proteins, which are required for protein prenylation. The purpose of this study was to investigate this novel combination of such PTM motifs with a CPP and to analyze whether these chimeras would potentially be recognized and processed by the protein PTM machinery. Our first finding was that CaaX-peptides are intracellularly highly enriched dependent on their CaaX box. As illustrated in Fig. 3, by using the C-terminal CVGS motif, it was even possible to increase cytotoxic peptide distribution. Since one bottleneck in the use of cell-penetrating peptides is their often only poor endosomal release, our newly created peptides might offer a really advantage in this direction. Moreover, we showed that a cysteine to serine mutation led to significantly lower internalization efficiencies of such peptides, suggesting an uptake pathway certainly dependent on correct intracellular recognition of CaaX-peptides. This was additionally supported by decreased intracellular accumulation after the treatment of PANC-1 cells with different control peptides and inhibitors, for example, Mevastatin. Furthermore, we highlighted cytotoxic effects of CaaX-peptides in several cancerous cells, which approximately corresponds with the distinct cellular expression levels of prenyltransferases, for example, FTase. Focusing then on PANC-1 cells, we also proofed the direct interaction of CaaX-1 with farnesyltransferase by pull-down assays and verified an upregulation in expression levels of CaaX-1 peptides with farnesyltransferase by pull-down assays and verified an upregulation in expression levels.

In future, we will study how these peptides interfere and probably compete with Ras processing, since, within this work, we highlighted significant changes in Ras-related signaling pathways. Particularly, K-Ras would be an intriguing target in terms of PDACs, one of the most severe and K-Ras addicted cancers. Taken all these results into account, our approach might also open up a novel field for the development and study of therapeutics targeting other prenylated proteins, for instance such that are substrates of FTase and occur in the genomes of bacteria and viruses.

**Materials and methods**

**Peptide synthesis and purification**

All peptides were synthesized according to the Fmoc/tBu strategy using an automated multiple solid-phase peptide synthesizer Syro I (multiSynTech), as previously described [41]. As solid support, preloaded Wang resins were used and peptides were labeled N-terminally with 5(6)-carboxyfluorescein (CF) to enable their intracellular detection. Labeling of the peptides was performed on resin with fully protected side chains by using 5 equivalents (eq.) of fluorophore, Oxyma, and D,N,N'-disopropylcarbodiimide (DIC) (overnight (o/n), RT). For synthesis of biotinylated conjugates, biotin coupling was performed on resin using 3 eq. of biotin, Oxyma, and DIC and (o/n, RT, N-methyl-2-pyrrolidone (NMP)). Peptides were cleaved from the resins using trifluoroacetic acid (TFA)/thioanisole/1, 2-ethanediethiol (90:7:3, v/v/v) within 3 h at RT, then precipitated in ice-cold diethyl ether and lyophilized. Analytical data were obtained from RP-HPLC (Agilent with Nucleodur column: 100-5; c18ec; 4.6 × 125 mm) using a gradient from 10% to 60% of acetonitrile (ACN) in H2O with 0.1% formic acid for sample separation, followed by electrospray-ionization mass spectrometry (ESI/MS, Thermo Scientific LTQ-XL, Darmstadt, Germany) measurements. Purification of peptides was achieved by preparative RP-HPLC (Hitachi Elite LaChrom, San Jose, CA, USA) on a 15 × 250 mm Jupiter 4 μm Proteo 90 A column (Phenomenex, Torrance, CA, USA) using linear gradients from 15% to 45% B in A (A = 0.1% TFA in water; B = 0.1% TFA in ACN) over 45 min and a flow rate of 6 mL-min⁻¹.

**Peptide sequencing by tandem mass spectrometry**

For determining the position of not properly incorporated amino acids, fractions containing side-products in high purity were analyzed by LC-MS/MS. Samples were dissolved in H2O/MeOH/FA (50:50:0.1, v/v/v) and diluted to a final concentration of 100 pmol-μL⁻¹. Full scans were conducted, and side product ions isolated for fragmentation with a collision energy of 35–40%. Samples were injected by a syringe-pump at a flow rate of 3 μL-min⁻¹.

**Secondary structure analysis**

Circular dichroism (CD) spectroscopy of the CaaX-peptides (20 μm in 10 mM phosphate buffer, pH 7.0, or 10 mM phosphate buffer/2,2,2-trifluoroethanol (TFE) (1:1 v/v)) was recorded in triplicate using a Jasco Corp J-715 spectropolarimeter in a range from 190 to 260 nm using instrument settings as described in Ref. [75]. The resulting signal was converted from ellipticity (mdg) to molar ellipticity [Q] in
Annexin V-FITC/7-AAD staining solutions. Cells were incubated for 150 min in the dark with the binding buffer from the kit (Beckmann Coulter, Brea, CA, USA) and incubated for 10 min with 70% EtOH. Cells were resuspended in ice-cold binding buffer and washed with PBS. In the negative controls, the cell pellet was resuspended in phenol red-free medium. Cells were centrifugated (7.5 min, 106 g) and the cell pellet was resuspended in phenol red-free trypsin, and resuspended in phenol red-free medium. For cell culture experiments, cells were always grown to a confluency of up to 80%. All experiments were performed minimum twice in triplicates.

Cell culture

All cell lines used during this work were cultured as subconfluent monolayers in 10-cm petri dishes at 37 °C, in humidified atmosphere containing 5% CO2. HeLa, MCF-7, LS174T, and PANC-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 2-4 mM glutamine. MEF and Capan-1 cells were cultured in DMEM medium with 10% FBS and 4 mM glutamine for MEF and Capan-1. When reaching a confluency of ~80–90%, cells were split by using 0.5 mg-mL−1 trypsin-EDTA for cell detachment. For cell culture experiments, cells were always grown to a confluency of up to 80%. All experiments were performed minimum twice in triplicates.

Cellular viability

The cellular viability was determined with a resazurin-based cytotoxicity assay (Sigma-Aldrich), as previously reported [41,78]. Therefore, cells were seeded (HeLa: 1.7 × 104; MCF: 2 × 104; LS174T: 2.4 × 104; PANC-1: 1.9 × 104; MEF: 1.7 × 104; Capan-1: 2.0 × 105 cells) in 96-well plates and grown to subconfluence (~70–80%) o/n. On the next day, cells were incubated with different peptide concentrations in the appropriate serum-free medium. After 24 h, cells were washed twice with PBS, while the positive controls were treated for 10 min with 70% EtOH. Cells were incubated with a 1:10 dilution of the reagent (v/v, in serum-free medium) for 1–2 h at 37 °C. The resazurin product was monitored at 595 nm (λex = 550 nm) on a Tecan infinite M200 plate reader.

Annexin V-FITC/7-ADD assay

PANC-1 cells were seeded (1.2 × 105) in 24-well plates and grown to subconfluence o/n. Medium was removed, and cells were incubated with 30 µM of unlabeled peptides for 24 h at 37 °C. Positive controls were treated with 7 µM of staurosporine (Sigma-Aldrich), while untreated cells served as negative controls. After incubation, cells were washed twice with PBS, detached with phenol red-free trypsin, and resuspended in phenol red-free medium. Cells were centrifugated (7.5 min, 106 g, 4 °C), the supernatant was discarded, and the cell pellet was washed with PBS. In the following, the cell pellet was resuspended in ice-cold binding buffer from the kit (Beckmann Coulter, Brea, CA, USA) and incubated for 150 min in the dark with the Annexin V-FITC/7-AAD staining solutions. Cells were analyzed within 30 min with the guava easyCyteTM System (Merck, Darmstadt, Germany) using the GRN-B (525/30) and the RED-B (695/50) channel.

Cellular uptake and intracellular distribution

To study the intracellular fate of the novel peptides, flow cytometry experiments and confocal scanning microscopy were performed, as already described [41]. For the quantification of the cellular uptake, cells were seeded (HeLa: 1.0 × 105; MCF: 1.2 × 105; LS174T: 1.6 × 105; PANC-1: 1.4 × 105; MEF: 1.0 × 105; Capan-1: 1.5 × 105 cells) in 24-well plates and grown to subconfluence (~70–80%) o/n. On the next day, fluorophore-labeled peptides were incubated for 30 min in the appropriate serum-free medium. After incubation, cells were washed twice with PBS, detached with phenol red-free trypsin, and resuspended in phenol red-free medium. Cellular uptake was determined with the guava easyCyteTM System (Merck) using the GRN-B (525/30), counting 10 000 cells per well. For the assays with inhibitors, we incubated 10 µM AFCME for 30 min, 5 µM tipifarnib for 24 h, and 10 µM Mevastatin for 24 h with the cells. After preincubation, cells were washed with PBS and peptide incubation was started.

For the microscopic analysis, cells were seeded (HeLa: 4.0 × 105; MCF: 4.5 × 105; PANC-1: 5.0 × 105; MEF: 3.0 × 105, Capan-1: 5.0 × 105 cells) in a μ-slide eight-well plate (Ibidi) and grown to subconfluence (~70–80%). On the next day, cells were incubated with fluorophore-labeled peptides in the appropriate serum-free medium. Cell nuclei were stained for the last 10 min of the incubation time with Hoechst 33342 nuclear dye. Medium was removed, and external fluorescence was quenched with trypan blue (150 µM in 0.1 M acetate buffer, pH 4.1) for 30 s. After washing the cells once and adding fresh medium, microscopic analyses were performed on a Leica TCS SP8 confocal scanning microscope using a 63× immersion oil objective and images were processed with Fiji.

For the immunostaining, PANC-1 cells were seeded as described above. On the next day, cells were incubated for 6 h with 30 µM of CaaX-1 peptide in serum-free medium. Afterward, cells were washed twice with PBS and fixed using 2% paraformaldehyde (PFA) in PBS (30 min, RT). Cells were washed 3 times with 100 mM glycine in PBS (10 min, gentle shaking) and blocked (0.3% Triton X-100, 5% bovine serum albumin (BSA) in PBS) for 2 h at RT under shaking. The anti-K-Ras (Santa Cruz, Dallas, TX, USA; sc-30; 1:500) antibody was incubated overnight (RT, gentle shaking). Cells were washed again 3 times with 100 mM glycine in PBS (10 min, gentle shaking), and the secondary antibody was incubated for 2 h (anti mouse igG2a; 1:500), and afterward, cell nuclei were stained for 15 min with Hoechst 33342 nuclear dye. Cells were washed twice with PBS, once with ddH2O, mounted with Mowiol, and analyzed on the same microscope as described above.
Preparation of spheroids

3D spheroids of PANC-1 cells were generated with the hanging drop method to analyze the penetration ability of the peptides into more complex tissue. Therefore, a confluent petri dish of PANC-1 cells was washed, trypsinized, and harvested by centrifugation (300 g, 4 °C, 5 min). Cells were seeded in droplets containing each 1.5 × 10^5 cells onto an inverted lid of a petri dish supplemented with 1.2 mg·mL⁻¹ methylcellulose in the appropriate serum-free medium. After 2 days, spheroids were formed and incubation of compounds was performed by addition of CF-labeled peptides directly into the droplets (final concentration: 10 µM). Incubation was carried out for 2 h at 37 °C, and afterward, spheroids were harvested by soaking them carefully with 1 mL into a tube. For quantification of the uptake, cells were centrifuged (1000 r.p.m., 5 min, 4 °C), the supernatant was discarded, and the pellet was washed twice with PBS. PANC-1 spheroids were treated with phenol red-free trypsin under gentle shaking at 37 °C and after falling apart, resuspended in phenol red-free medium. Cellular uptake was determined as described above.

Cell lysates and western blotting

Cells were grown to confluency in 6-well plates, washed twice with PBS, and trypsinized using 500 µL trypsin. After detachment, cells were resuspended with 6 mL of the appropriate full medium. Cells were centrifuged (5 min, 1000 r.p.m. and 4 °C), the supernatant was discarded, and the pellet was washed twice with PBS. PANC-1 spheroids were harvested by centrifugation (1000 r.p.m., 5 min, 4 °C), the supernatant was discarded, and the cell pellet washed with 1 mL PBS and centrifuged using the same conditions. The pellet was resuspended in ~ 200 µL lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM TCEP, 2 mM EDTA, 1% Triton X-100, 1 : 100 HaltTM protease inhibitor cocktail) and rotated for 40 min at 4 °C. Cell debris was removed by centrifugation (20817 g, 30 min, and 4 °C). Protein concentration was measured with Roti®-Nanoquant (Promega), and the appropriate amount of lystate was denatured in Laemmli buffer (10 min, 95 °C). Samples were electrophoresed on a 10% polyacrylamide gel via Tricine-SDS/PAGE, transferred onto a PVDF membrane (1.5 h, 100 mV), and blocked with 5% milk in PBS T for 1.5 h. Incubation with primary antibodies was carried out overnight followed by an incubation with HRP-conjugated rabbit or mouse antibody for 1.5 h (Cell Signaling Technology (CST), Danvers, MA, USA; 1 : 3000). The following primary antibodies were used as follows: anti-FNTA (Abnova, Taipei, Taiwan Cat/#4326; 1 : 250), anti-ERK1/2 (CST Cat/#4695; 1 : 1000), anti-phospho-ERK1/2 (CST Cat/#4370; 1 : 1000), anti-MEK1/2 (CST Cat/#8727; 1 : 1000), anti-phospho-MEK1/2 (CST Cat/#9154; 1 : 1000), anti-AKT (CST Cat/#9272; 1 : 1000), anti-phospho-AKT (CST Cat/#4060; 1 : 1000), and anti-K-Ras (Santa Cruz; sc-30; 1 : 1000). Samples were visualized by using the SignalFire™ ECL Reagent (CST). To obtain a loading control, the membrane was washed 2 × 10 min with stripping buffer (0.2 M glycine, 0.1% SDS, 1% Tween 20, pH 2.2), blocked again, and incubated with an anti-β-actin antibody (Santa Cruz; sc-47778 HRP; 1 : 1000) for 1.5 h. Samples were again visualized using the ECL Reagent.

Pull-down analysis

The interaction between CaaX-1 and the prenyltransferases was studied with crude lysates (a) and recombinant rat FTase (b) (Jena Bioscience, Jena, Germany) via a pull-down assay. Since high amounts of crude lysates were required, PANC-1 cells were seeded in 100 × 200 mm petri dishes and lysed as described above when reaching a confluency of around 80%. Magnetic streptavidin beads (Dynabeads™ MyOne™ Streptavidin C1 (Thermo Scientific)) were washed three times with (a) lysis buffer or (b) PBS and preloaded for 30 min at RT with 40 µg of biotinylated CaaX-1 or SaaX-1 in (a) lysis buffer or (b) PBS. Beads were washed once with (a) lysis buffer or (b) PBS, and unbound streptavidin was blocked with 1 mM biotin in PBS. After washing once with lysis buffer, beads were rotated for 2 h at 4 °C with (a) 500 µg crude PANC-1 lysate or (b) 400 ng recombinant rat FTase which was again followed by three times washing with lysis buffer. Elution was carried out, by rotating the beads for 15 min at RT in hexafluoroisopropanol (HFIP). HFIP was evaporated for 20 min at 40 °C under continuous N2 flow (Xcel-Vap, Biotage, Uppsala, Sweden). Western blotting was performed as described above.

Quantitative proteome analysis

8.0 × 10^5 PANC-1 cells were seeded in 6-well plates and grown to confluency. Cells were incubated for 24 h with 30 µM of the peptide solutions. On the next day, cells were lysed with modified RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1% Triton, 1 : 100 HaltTM Protease Inhibitor Cocktail) as described above. DNA was shared by sonification for 10 min at 4 °C with a Bioruptor. In the following, proteins were precipitated with cold acetone o/n. On the next day, sample was centrifugated (15 000 g, 10 min, 4 °C), the pellet was washed twice with acetone and then air-dried. For the in-solution digest, the pellet was dissolved in 6 M urea/2 M thiourea and incubated for 30 min at RT with 5 mM DTT to reduce disulfide bonds. To alkylate cysteines, 40 mM iodoacetamide was added to the samples and the mixture was incubated for 20 min in the dark (RT). Endoproteinase Lys-C was added (enzyme: substrate ratio of 1 : 100) for further 3 h at RT. Afterward, samples were first diluted with 50 mM ABC to an urea concentration of 2 M and trypsin (enzyme: substrate ratio of 1 : 100) was added.
added o/n. To stop the digestion, formic acid (FA) was supplemented to a final concentration of 1%. Before MS analysis, samples were desalted on Stage tips. Proteomic analysis was performed as already described [79], using an Easy nLC 1000 UHPLC coupled to a QExact Plus mass spectrometer (Thermo Fisher). Peptides were resuspended in solvent A (0.1% FA), picked up with an autosampler, and loaded onto in-house made 50-cm fused silica columns (internal diameter (I.D.) 75 μm, C18 2.7 μm, Poroshell beads, Agilent) at a flow rate of 0.75 μL·min⁻¹. A 240-min segmented gradient of 5–34% solvent B (80% ACN in 0.1% FA) over 215 min, 34–55% solvent B over 5 min, and 55–90% solvent B over 5 min at a flow rate of 250 nL·min⁻¹ was used to elute peptides. Eluted peptides were sprayed into the heated transfer capillary of the mass spectrometer using a nanoelectrospray ion source (Thermo Fisher Scientific). The mass spectrometer was operated in a spectrometer using a nanoelectrospray ion source (Thermo Fisher Scientific). The Orbitrap acquired full MS scans (300–1750 m/z) at a resolution (R) of 70 000 with an automated gain control (AGC) target of 3 × 10⁶ ions collected within 20 ms. The dynamic exclusion time was set to 20 s. From the full MS scan, the 10 most intense peaks (z ≥ 2) were fragmented in the high-energy collision-induced dissociation (HCD) cell. The HCD normalized collision energy was set to 25%. MS/MS scans with an ion target of 5 × 10⁵ ions were acquired with R = 17 500, with a maximal injection time of 60 ms and an isolation width of 2.1 m/z. The raw files were processed using MaxQuant software and its implemented Andromeda search engine [80].
Parameters were set to default values. ANOVA and 1D annotation enrichment analysis were performed using Perseus [81]. Hierarchical clustering was performed using Instant Clue [82].

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

AK, KS, and JLW performed the experiments. AK, KS, JLW, MK, and IN analyzed the data. AK, JLW, MK, and IN wrote the manuscript.

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