VIP-derived sequences modified by N-terminal stearyl moiety induce cell death: the human keratinocyte as a model

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Abstract Vasoactive intestinal peptide (VIP) is a recognized growth factor affecting many cell types. We have previously developed a series of lipophilic VIP analogues containing an N-terminal covalently attached stearyl moiety. The current studies identified stearyl-Nle^{17}-VIP and stearyl-Nle^{17}-neurotensin$_{6-11}$VIP$_{7-28}$, acting at μM concentrations, as cytotoxic to human keratinocytes. The core C-terminal active VIP-derived peptide, stearyl-Lys-Lys-Tyr-Leu-NH$_2$ (St-KKYL-NH$_2$), was identified as being responsible for the observed cytotoxicity. Cytotoxicity coincided with marked reduction in intracellular cyclic GMP and was abolished by co-treatment with the endonuclease inhibitor, aurine-tricarboxylic acid, suggesting apoptotic mechanisms. Stearyl-VIP derivatives thus offer lead compounds for future drug development against hyperproliferative skin conditions.

Key words: Vasoactive intestinal peptide; Keratinocyte cytotoxicity; cGMP; Lipophilic peptide; Aurine-tricarboxylic acid

1. Introduction

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) are members of a family of regulatory peptides that include secretin and glucagon [1]. VIP, a basic 28 amino acid peptide, originally isolated from the gastrointestinal system [2], was identified as a modulator of growth, survival and differentiation in many cell systems, including the brain, the gastrointestinal tract, lung and immune cells, of primary origin and cancerous one [1].

VIP effects have also been documented for skin cells [3]. The epidermis, the outermost skin layer, provides the first line of defense against the external environment. The major cell type in the epidermis is the keratinocyte [4]. Normal human skin contains a variety of neuropeptides that are either derived from sensory neurons or from skin cells such as keratinocytes, microvascular endothelial cells or fibroblasts. In addition, immune cells, that either constitutively reside in the skin or infiltrate under inflammatory conditions, have been reported to produce neuropeptides [5]. VIP was directly implicated in keratinocyte proliferation [6–8].

Apoptosis is an evolutionary conserved, gene-directed, active cell death that follows an orderly pattern of morphologic and biochemical changes, functioning in developmental remodeling, regulation of cell numbers, and defense against damaged, virus-infected, auto-reactive and transformed cells [9]. Agents and events that activate the apoptotic pathway include growth factors, hormones, cytokines, UV and γ irradiation, disruption of oxidative pathways and cell matrix interactions [10]. The pathway is conceptually divided into three mechanistically distinct phases: induction (or initiation), effector and degradation [11]. The epidermis is a continually renewing tissue where homeostasis is maintained by factors that influence the interrelated processes of the keratinocytic life cycle. Premature, excessive or deficient apoptosis have all been linked to the homeostatic dysregulation characteristic of skin disorders, such as psoriasis [12].

A lipophilic super-agonist of VIP, stearyl-Nle^{17}-VIP (SNV), was previously described [13], which contains modifications intended to confer stability, membranal penetration and increased bioavailability. This analog exhibited a 100-fold potency as compared to VIP [14,15] in promoting neuronal survival, through a cyclic GMP (cGMP)-associated mechanism [16]. Another previously described VIP analog is the hybrid antagonist, neurotensin$_{6-11}$VIP$_{7-28}$ (VHA, [17]). VHA proved to be a potent antagonist of VIP, inhibiting VIP-mediated cAMP formation and inducing neuronal killing effects in vitro [18]. VHA also antagonized VIP growth promoting actions on mouse embryos [19] and inhibited proliferation of a variety of cancer cell lines [20]. Further modification of the VHA, on the basis of enhanced activity of SNV, yielded the stearyl-VIP hybrid antagonist, stearyl-Nle^{17}-neurotensin$_{6-11}$VIP$_{7-28}$ (SNH) [14]. This antagonist proved to be 100-fold more potent in producing neuronal cell death than VHA [14,18], and more potent in lung cancer growth inhibition [21].

It is desirable to have smaller molecules mimicking the parent peptide activity, while offering applicable benefit in better penetration through biological barriers and reduced possible enzymatic degradation sites. Structure–function analyses, utilizing VIP fragments, have indicated that for most activities, the entire sequence of the peptide is required for full biological function. VIP$_{2-28}$ retained a similar biological activity to the parent peptide [22], while shorter fragments (amino acids 7–28, 15–28, 14–28) exhibited $<$10–1000-fold potency as

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Abbreviations: VIP, vasoactive intestinal peptide; SNV, stearyl-Nle^{17}-VIP; SNH, stearyl-Nle^{17}-neurotensin$_{6-11}$VIP$_{7-28}$; St-KKYL-NH$_2$, stearyl-Lys-Lys-Tyr-Leu-NH$_2$; ATA, aurine-tricarboxylic acid; PACAP, pituitary adenylate cyclase activating peptide; VHA, neurotensin$_{6-11}$VIP$_{7-28}$; LDH, lactate dehydrogenase; GnRH, gonadotropin releasing hormone; CHI, cycloheximide; Pr, propionyl; K-SFM, keratinocyte serum free medium; EGF, epidermal growth factor; MEM, minimal Eagle's essential medium; FCS, fetal calf serum

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measured for smooth muscle relaxation [23], and lower potencies for other activities [22]. While VIP_{10-28} antagonized VIP activities [18,24], a smaller fragment derived from the C-terminal of the VIP peptide (amino acids 20–23), stearyl-Lys-Lys-Tyr-Leu-NH_2 (St-KKYL-NH_2), was identified as the core active site of VIP, responsible for neuroprotective effects of VIP/SNV [25].

The focus of the present study was the development and evaluation of VIP analogs as potential inhibitors of keratinocytes' growth, with emphasis on relevance to possible therapeutic applications. More specifically, these include the identification of VIP-derived minimal sequences, modified by N-terminal addition of a stearyl moiety and an amidated C-terminus, capable of capturing the activity of the full sequence, as well as characterization of the mechanism involved in cell death induction.

2. Materials and methods

2.1. Solid phase peptide synthesis

Peptides were prepared by solid phase synthesis using the Fmoc chemistry as described [13,26]. VIP, PACAP27 [27] and VHA were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a semipreparative RP-18 Lichrospher, 10 μm 250×10 mm column (Merck, Darmstadt, Germany), eluted by a linear gradient of aqueous acetonitrile (ACN) in 0.1% TFA: $t=0$ min, 0% ACN; $t=0$–5 min, 15% ACN; $t=5$–60 min, 52.5% ACN at a flow rate of 5 ml/min. SNV, SNH and St-KKYL-NH_2 were purified by a preparative RP-8 Lichrosorb, 7 μm 250×25 mm column as above: $t=0$ min, 0% ACN; $t=0$–10 min, 37.5% ACN; $t=10$–50 min, 75% ACN at a flow rate of 10 ml/min. Shorter, more hydrophobic peptides required a longer ACN = 75% run. The isolated peptides were subjected to analytical RP-HPLC under similar conditions as above (flow rate 1 ml/min) to confirm their purity and to amino acid analysis to verify their composition. Mass spectrometry was employed for molecular weight determinations.

2.2. Cell cultures

2.2.1. Neonatal keratinocytes. Primary human neonatal keratinocytes were purchased from Clonetics (NHEK #CC-2503, CA, USA) in their cryopreserved form, and cultivated in keratinocyte serum free medium (K-SFM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 25 μg/ml bovine pituitary extract (BPE) and 0.1 ng/ml human recombinant epidermal growth factor (EGF). This medium is based on MCDB 153 medium, enriched by various hormones (insulin 5 μg/ml, tri-iodothyronine, transferrin, hydrocortisone 0.5 μg/ml, cholera toxin, adenine). Subculturing was achieved under serum free conditions, using trypsin:EDTA solution (0.025:0.01%), and neutralizing by soybean trypsin inhibitor (0.05 mg/ml, Biological Industries, Beit Haemek, Israel).

2.2.2. HaCaT. Human HaCaT keratinocytes were a kind gift from Prof. Fusenig [28]. The cells were routinely propagated in minimal Eagle's essential medium, MEM, supplemented with 1% Pen-Strep-nystatin, 2 mM L-Gln and 10% fetal calf serum (FCS, Biological Industries, Beit Haemek, Israel) under a humidified atmosphere with 10% CO_2 at 37°C. Subculturing was achieved by trypsinization with Versene solution (trypsin:EDTA 0.25:0.02%), and neutralizing by serum containing medium.

2.2.3. HT29. The human colon carcinoma cell line was routinely propagated in RPMI 1640 supplemented with 1% Pen-Strep-nystatin, 2 mM L-Gln and 10% FCS under a humidified atmosphere with 10% CO_2 at 37°C. Subculturing was achieved by trypsinization with Versene solution and neutralizing by serum containing medium.

2.3. Cell viability assays

2.3.1. Neonatal keratinocytes. For cell proliferation assays, cells were seeded at 10 000/well in 96 well microtiter plates (Nunc, Roskilde, Denmark), in K-SFM supplemented with BPE and EGF. The following day, medium was changed to K-SFM without EGF and BPE (basal medium), in order to achieve a quiescent state. Following 48 h, the medium was replaced by fresh basal medium supplemented with peptides at the indicated concentrations for an additional 22–24 h. Cells were used in their third or fourth passage. Cell viability was monitored by 1.5–3 h incubation with MTS reagent (CellTiter 96 AQ_{ueous} cell proliferation kit, #G5430, Promega, Madison, WI, USA), oxidation of which by active mitochondria results in color development, at 490 nm.

2.3.2. HaCaT. For cell proliferation assays, cells were seeded at 4000/well in 96 well microtiter plates, in 5% FCS-supplemented MEM. The following day, cells were starved by changing the medium to MEM supplemented with 0.1% bovine serum albumin (BSA) for a 48 h period, after which the peptides were added, in fresh medium, at the specified concentrations and incubation proceeded for a 22–24 h period. Cell viability was monitored by 1.5–3 h incubation with the MTS reagent. Alternatively, for moderate starvation or a non-starvation protocol, cells were seeded in 10% FCS-supplemented MEM, and peptides were added the following day in fresh 2% or 10% FCS-supplemented medium for a 72 h or a 48 h period, respectively. Cell viability was monitored by 1.5–3 h incubation with the MTS reagent.

2.3.3. HT29. For cell proliferation assays, cells were seeded at 4000/well into 96 well plates in 10% supplemented RPMI 1640 (Biological Industries, Beit Haemek, Israel). Medium was changed to 0.1% BSA-supplemented RPMI the following day, for a 48 h period. Stearyl peptides were added, in fresh 0.1% BSA medium for an additional 22–24 h. Cell viability was monitored by addition of the MTS reagent during the last 3 h of incubation.

2.3.4. Lactate dehydrogenase (LDH) determination. Supernatants from HaCaT cells exposed to stearyl peptides, with or without 25 μM aurine-tricarboxylic acid (ATA), the endonuclease inhibitor (Sigma, St. Louis, MO, USA), were collected, from the same wells used for MTS determination, and LDH activity evaluated by a LDH cytotoxicity detection kit (Boehringer-Mannheim, Mannheim, Germany), according to the manufacturer's instructions. The combined LDH activity from burst cells treated with 5% Triton X-100 in 50 mM Tris–HCl, and the LDH values obtained from their respective medium (prior to Triton treatment), were used to define 100% LDH activity (per well). Values obtained from samples were normalized according to their ratio relative to this 100% value.

2.4. Measurements of intracellular cGMP accumulation

HaCaT cells were seeded, 4×10^5 per 35 mm dish (Corning, MA, USA), in 5% FCS-supplemented MEM, as above. On the following day, cells were starved by changing the medium to MEM supplemented with 0.1% BSA for a 48 h period. On the fourth day, the medium was changed to fresh MEM (without supplements). SNV was added for a stimulation period of 15 min. Control cultures received saline. Following stimulation, the cultures were washed three times with cold phosphate-buffered saline, and the cyclic nucleotides extracted by 30 min incubation at 4°C in 80% ethanol and mechanical removal of the cells. The resulting suspension was pelleted by centrifugation at 2000×*g* for 15 min at 4°C. The supernatants were transferred to fresh tubes and dried by an Eppendorf concentrator 5301 (Eppendorf, Hamburg, Germany). cGMP content in the samples was assayed by a cGMP enzyme immunoassay system kit (Amersham, International Plc., Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions.

2.5. Statistical analyses

Statistical tests employed ANOVA one way analysis of variance with all pairwise multiple comparison procedures (Student–Newman–Keuls method).

3. Results

3.1. Anti-proliferative effects of stearyl modified VIP-related peptides on HaCaT cells

The lipophilic VIP antagonist SNH was tested for its anti-proliferative activity on HaCaT cells following a 24 h incubation period (Fig. 1). Results indicated that at 10 μM, SNH produced essentially complete cell killing. In order to estimate the time dependence of cytotoxic effect manifestation, SNH was applied for a shorter exposure period. Thus, activity of SNH was already evident after a 2 h exposure, when only 32%

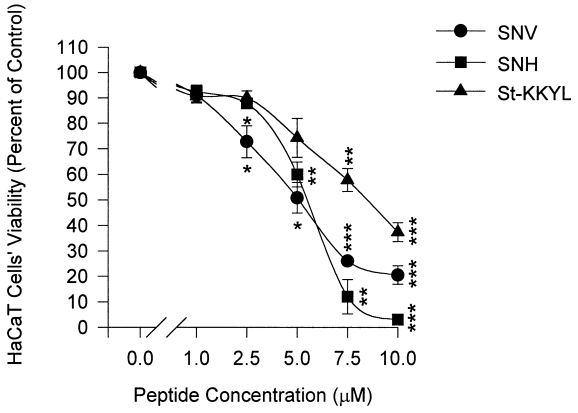

Fig. 1. Starved HaCaT cells′ viability, as assessed by the metabolic dye, MTS, in the presence of the indicated concentrations of stearyl peptides: SNV, SNH and St-KKYL-NH_2. Cells (4000/well) were seeded into 96 well plates in 5% FCS-supplemented MEM. The following day, the medium was switched to 0.1% BSA-supplemented MEM for a 48 h period. Peptides were then added in fresh 0.1% BSA-supplemented medium for an additional 24 h. The MTS reagent was added during the last 3 h of incubation. Means ± S.E.M. of four independent experiments are indicated. Control cultures yielded ~0.7 optical density (OD) at 490 nm. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

of the cells maintained their viability, as compared to control cultures (not shown).

To test whether the stearyl moiety at the micromolar concentrations utilized contributed to the cell killing effect, we further tested SNV. Additional data (see adjoining manuscript) indicated that VIP and SNV induced keratinocyte cell proliferation at picomolar to nanomolar concentrations. However, SNV at a high concentration (10 μM) proved to be a cytotoxic agent for the HaCaT line, inflicting not only inhibition of cell proliferation, but reduced cell survival (Fig. 1).

Based on these observations, aiming to pinpoint the exact part of the VIP peptide, accountable for the observed cytotoxic activity, several stearyl-modified peptides, encompassing fragments derived from the entire VIP peptide, were synthesized and evaluated. Similar to VIP, all stearyl peptides employed were in their C-terminal amidated form. Some of these peptides also served as controls, examining the possibility of non-specific, stearyl-dependent, cytotoxic effect. The various peptides, with the relevant controls, are summarized in Table 1, according to their location in the VIP peptide. Data in Table 1 indicate the following: first, stearic acid by itself was not cytotoxic to HaCaT cells, nor was its mere addition to a peptide (such as represented by gonadotropin releasing hormone, GnRH). Second, the N-terminus of the VIP peptide was less relevant to effect manifestation. Sequences of the N-terminal and middle part of the VIP peptide were either inactive or exhibited markedly reduced activity, while the C-terminus part of the peptide was its active site, specifically, the KKYL sequence (Fig. 1, Table 1). Third, non-covalent mixtures of stearic acid with VIP, Nle^{17}-VIP or VHA were not cytotoxic, nor was the covalently bound propionyl (Pr)-KKYL-NH_2, i.e. the cytotoxic effect was dependent on covalent linkage of both peptide and stearyl moieties. The results in Table 1 were obtained with cells incubated in the absence of serum (in the presence of 0.1% BSA). To assess the effects of increasing serum concentrations on HaCaT survival, cells (seeded in 10% serum for 24 h) were further incubated with the stearyl peptides in the presence of serum (2%, 72 h or 10%, 48 h). Results indicated a gradual diminution of the cytotoxic effect associated with increasing serum concentrations (25% or 60% survival with SNV, 49% or 100% survival with SNH and 29% or 100% with St-KKYL, respectively).

3.2. *Cytotoxic effects on neonatal human keratinocytes and human colon carcinoma HT29 cell line*

Since marked cytotoxic effects of SNV, SNH and St-KKYL-NH_2 were observed with the HaCaT cell line, we examined whether the effect was maintained with human neonatal keratinocytes. As presented in Fig. 2, susceptibility to

Table 1
Effects of stearyl peptides on HaCaT cell viability

	St-peptide sequence	Cell survival (percent of control)		
		5×10^{-6} M	10^{-5} M	5×10^{-5} M
	SNV	54 ± 6	22 ± 4	ND
	SNH	60 ± 6	4 ± 1	ND
N-Terminus:	St-VIP(1–15)	inactive	inactive	80 ± 4
	St-VIP(1–14)	88 ± 3	86 ± 4	ND
Middle part:	St-VIP(8–13) [DNYTRL]	inactive	inactive	ND
	St-VIP(8–14)	inactive	inactive	70 ± 3
	St-VIP(15–21)	inactive	inactive	0
C-Terminus:	St-VIP(15–28)	inactive	87 ± 3	82 ± 4
	St-VIP(15–23)PACAP(24–27)	78 ± 8	22 ± 11	ND
	St-VIP(21–25) [KYLNS]	89 ± 3	86 ± 2	ND
	St-VIP(20–23) [KKYL]	74 ± 9	36 ± 4	ND
Controls:	VIP	inactive	inactive	inactive
	PACAP27	inactive	inactive	ND
	VIP hybrid antagonist (VHA)	inactive	inactive	ND
	Stearic acid	inactive	inactive	inactive
	Stearic acid+VIP	inactive	inactive	inactive
	Stearic acid+VIP(Nle^{17})	inactive	inactive	inactive
	Stearic acid+VHA	inactive	inactive	inactive
	St-GnRH	inactive	82 ± 2	76 ± 3
	Pr-KKYL	inactive	inactive	inactive

The effects of stearic acid and stearyl containing VIP-derived or control peptides (all in their C-terminal amidated form) on HaCaT cell viability was assessed by MTS. Results are presented as mean ± S.E.M. of 3–6 independent experiments. For experimental details, see Fig. 1. ND, not determined. Inactive indicates ± 10% of control values (assigned to 100%). St = stearyl; Pr = propionyl.

stearyl-modified VIP-related peptides was maintained in the neonatal keratinocytes. Similar results were also obtained with the colon carcinoma cell line, HT29, cultured under similar conditions as HaCaT (Fig. 3). All three peptides maintained their impact, demonstrating similar potency and efficacy in the two cell lines, HaCaT and HT29. A somewhat different efficacy was evident in the neonatal keratinocytes. In either case, SNH was the most efficacious analog.

3.3. Mode of cytotoxic effect

Microscopic evaluation revealed a change from the cobble stone appearance under serum-enriched culture conditions (Fig. 4A) to cellular elongation and a higher degree of intracellular spacing under serum-deprived conditions (Fig. 4B). The massive cellular death that has occurred following stearyl peptides treatment (Fig. 1, Table 1) was evaluated microscopically (Fig. 4C, SNH, 10 μM and similar results were obtained for SNV and St-KKYL-NH_2). A characterization of the possible mechanism involved, namely, necrotic, apoptotic or a combination of the two, was performed. To that end, the endonuclease inhibitor, ATA, an apoptosis marker commonly utilized to distinguish between these two types of cell death [29], was applied, at 25 μM concentration, together with the cytotoxic peptides. ATA interferes with the final degradation phase of the apoptotic process (see Section 1). While ATA itself did not dramatically change cellular morphology (Fig. 4D), a remarkable restoration in cell viability was observed in cultures co-treated with SNH and ATA (Fig. 4E).

A quantitative evaluation of the results (MTS) showed that ATA had some intrinsic cytotoxic effect (32 ± 1.5% cell death). Thus, the ATA-restored viability value (about 70 ± 5% survival) in the presence of the stearyl peptides (SNH, SNV and St-KKYL, 5×10^{-6} M and 10^{-5} M, Table 1), as demonstrated by MTS, was limited to the level of viability observed in wells containing ATA only. The LDH activity, an indicator of membranal integrity, exhibited the exact mirror image of the MTS measurements (Fig. 5).

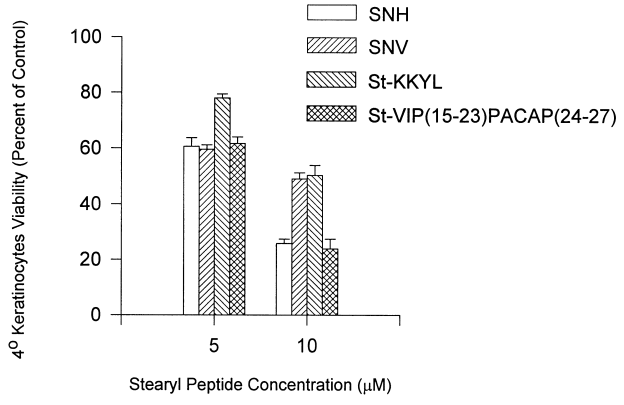

Fig. 2. Starved fourth passage (4°) human keratinocytes viability, as assessed by the metabolic dye, MTS, in the presence of the indicated concentrations of stearyl peptides: SNV, SNH, St-KKYL-NH_2 and St-VIP(15–23)PACAP(24–27). Primary neonatal human keratinocytes that have undergone three trypsinization cycles (i.e. fourth passage (4°)) were seeded into 96 well plates (10 000 cells/well) in complete K-SFM. A day following seeding, the medium was replaced by basal K-SFM for a 48 h period. Peptides were then added in fresh basal K-SFM for an additional 24 h. Means ± S.E.M. of two independent experiments are indicated. Control cultures yielded ∼0.5 OD at 490 nm. $P<0.00001$ for all data presented (peptide-treated versus untreated control = 100% viability).

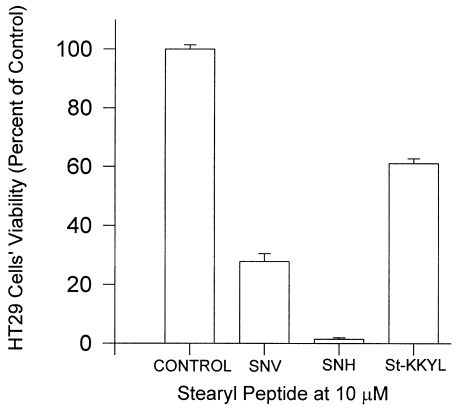

Fig. 3. Starved HT29 cell viability, as assessed by the metabolic dye, MTS, in the presence of 10 μM stearyl peptides: SNV, SNH and St-KKYL-NH_2. Cells (4000/well) were seeded into 96 well plates in 10% FCS-supplemented RPMI. The following day, the medium was switched to 0.1% BSA-supplemented RPMI for a 48 h period. Peptides were then added in fresh 0.1% BSA-supplemented medium for an additional 24 h. The MTS reagent was added during the last 3 h of incubation. Means ± S.E.M. of two independent experiments are indicated. Control cultures yielded ∼0.7 OD at 490 nm. $P<0.000001$ for the three peptides.

The dependency of the process upon new protein synthesis was further explored. To this end, the inhibitor of protein synthesis, cycloheximide (CHI, 10 μg/ml), was added to HaCaT cultures together with the peptide. CHI has a cytotoxic effect by itself, similar in its magnitude to that observed by 5 μM SNH (Table 2). It is not surprising then that co-treatment of CHI and SNH at this concentration did not result in improved survival of the cells. Although the percent of surviving cells is almost five times larger at the 10 μM SNH+CHI, as compared to 10 μM SNH alone ($P<0.005$), the improvement was only marginal compared to a 100% survival.

3.4. cGMP

Control cultures yielded ∼150 fmol/dish (100%). At the cytotoxic doses reported here (e.g. 10^{-5} M), SNV decreased basal cGMP levels in the cells to 38 ± 1% ($P<0.01$).

4. Discussion

Many pathological skin conditions are characterized by hyperproliferation of keratinocytes, such as psoriasis, dermatoses and skin cancers (squamous cell carcinoma and basal cell carcinoma). Thus, inhibition of keratinocytes growth would be highly advantageous as a mean for controlling the symptoms of hyperproliferative skin diseases. The present study demonstrates that several peptides, modified at their N-terminal by a fatty acid moiety, stearyl, acted as potent cytotoxic agents. The lack of activity by their non-modified counterparts emphasizes the important role of the stearyl moiety, and the necessity for the covalently attached fatty and peptide components. We speculate that while the peptide interacts with its specific receptor on the keratinocytic membrane, it assures close proximity of the stearyl moiety, leading to membrane perturbation. Specificity of the phenomenon was demonstrated as follows. (1) Stearic acid, by itself, was inactive in inducing cell death, even at concentrations higher than the ones required for maximal cytotoxic manifestation. (2) Some VIP sequences were inactive despite the stearyl attachment.

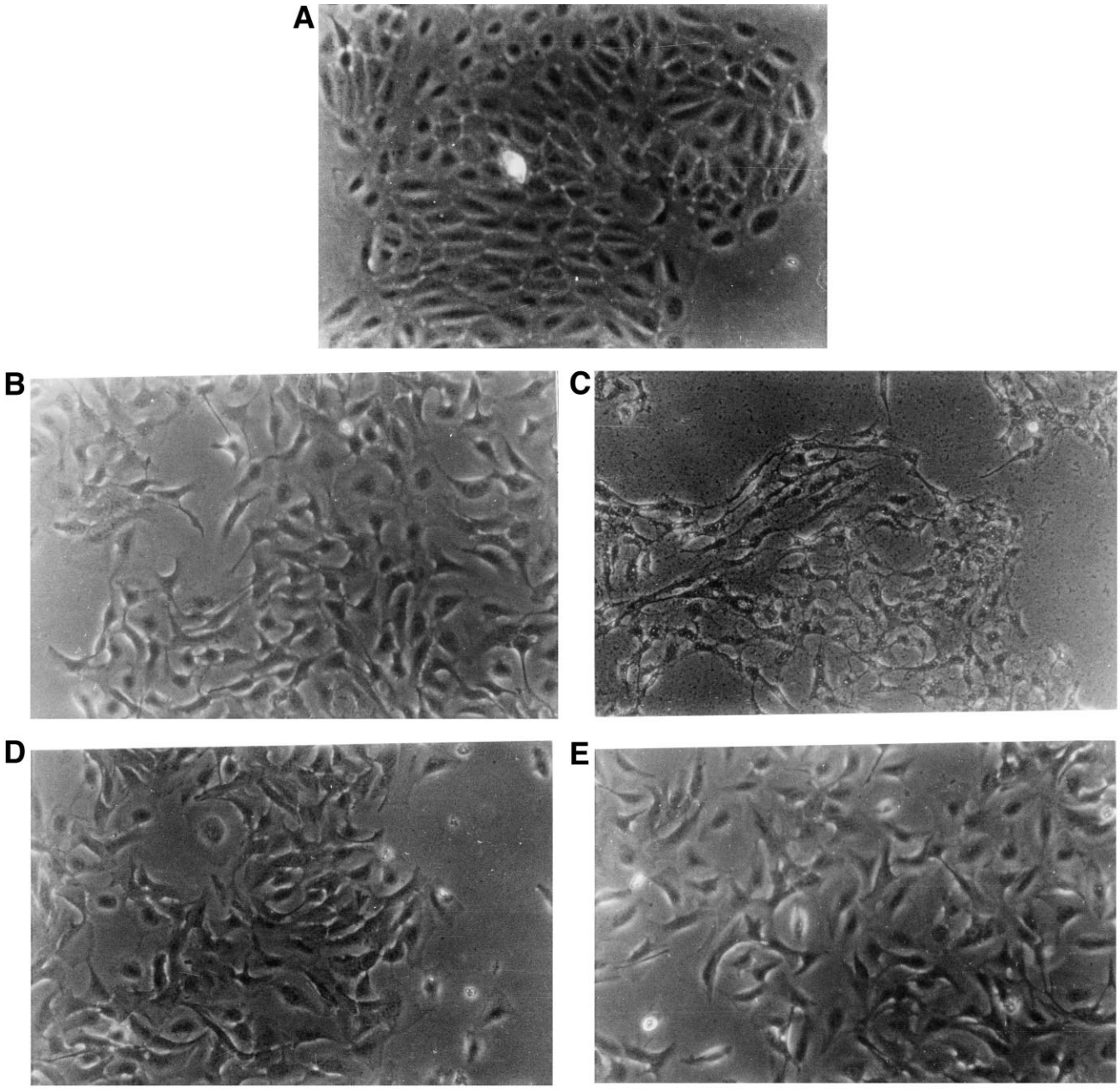

Fig. 4. Phase contrast microscopy of HaCaT cultures. Pictures were taken using an Olympus CK2 microscope equipped with an Olympus SC35 camera. A: Control cultures (10% FCS-supplemented MEM). B: HaCaT cells following a 48 h growth in 0.1% BSA-supplemented MEM (starved HaCaT). C: Starved HaCaT cells following a 24 h treatment with 10 μM SNH. D: Starved HaCaT cells following a 24 h treatment with ATA (25 μM). E: Starved HaCaT cells following a 24 h co-treatment with 10 μM SNH and 25 μM ATA. All photographs were taken under the same conditions.

(3) Short-time exposure to SNH (2 h) resulted in the manifestation of the majority of the cytotoxic effect, a characteristic of receptor-mediated interaction. Lastly, (4) in the process of elucidating the minimal VIP sequence, which maintains the cytotoxic effect, the KKYL sequence was identified, a sequence shared by all the stearyl peptides that turned out to be active. This finding, together with other observations regarding active VIP sequences, further implies that as far as keratinocytes are concerned, the N-terminus of the peptide is less relevant to effect manifestation, as evident by the replacement of the first six amino acids in SNH vs. SNV. Furthermore, while sequences of the N-terminal and middle part of the VIP peptide were less or not active compared to other sequences, activity seemed to reside in the C-terminus part of the VIP peptide, specifically KKYL. Certain amino acids in combination with this core peptide may enhance/improve the KKYL sequence cytotoxic activity. A recent publication from our laboratory [25] has identified the St-KKYL-NH_2 as the core active site of VIP, with picomolar concentrations capturing the neurotrophic effects offered by the entire 28 amino acid parent, SNV, in model systems for Alzheimer's disease.

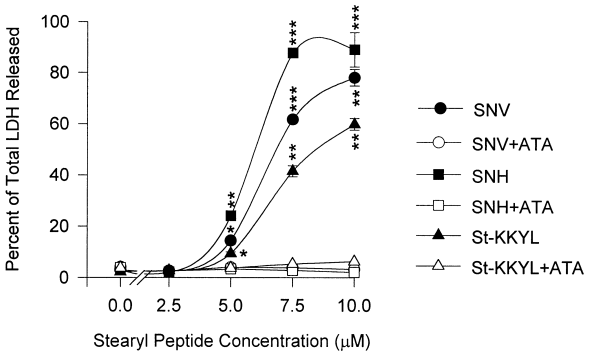

Fig. 5. Restoration of cell viability by simultaneous incubation of starved HaCaT with 25 μM ATA and SNV, SNH or St-KKYL-NH_2. Cell viability was evaluated by LDH release. For experimental details, see legend to Fig. 1. 100% LDH was obtained by combining intracellular LDH values (5% Triton X-100 extract) and the corresponding conditioned medium values. Means ± S.E.M. of three independent experiments are indicated. Significant differences were observed between cultures+peptide+ATA as compared to cultures+peptide only: $*P < 0.002$; $**P < 0.0001$; $***P < 0.00001$.

Previously, Fermor et al. [30] have reported the cytotoxic effect of several fatty acids. All human lines, that were examined, responded in a dose-dependent cell death to the presence of 10–50 μM stearic acid, with varying sensitivities. While no clear answer exists to explain the observed cytotoxic effect, non-specific toxicity as a result of lipid droplets or lipid inclusions in the cytoplasm of the cells (steatosis), leading to irreversible cell degeneration [31], as well as specific effects on membrane function [32] were speculated. Due to the fact that previous studies have associated cytotoxicity with stearic acid, we specifically assessed the possible cytotoxic effects of the free acid. Although no cytotoxic effect of free stearic acid was evident in HaCaT cells, a possible contribution of the fatty moiety to the observed effect should be considered. However, specificity was observed in that St-VIP(1–15) did not show any cytotoxic effect at 10^{-5} M, while under those conditions, SNH treatment resulted in 96% cell death and SNV treatment resulted in 78% death, with their non-lipophilic derivatives being totally inactive (Table 1). These results suggest that a covalent linkage between a certain peptidic structure and stearic acid is required for the cytotoxic effect. As this publication [30] was originally based on the assumption that cancerous cells are more susceptible to fatty acids than normal cells, and the HaCaT line, although not cancerous, may have some characteristics of transformed cells, the effect of the active stearyl peptides on neonatal keratinocytes was examined. Whereas the cytotoxic effect was less pronounced, it was maintained in these cells. The effect was also evident in the human colon carcinoma, HT29. SNV, SNH and St-KKYL-NH_2 all maintained their impact, as well as similar potency ranking in the three cell types, SNH being the most efficacious one in starved cells. The less pronounced effect in the case of the neonatal keratinocytes is attributed to the fact that they are not starved at such harsh conditions as the HaCaT (as K-SFM contains various growth factor additives, even when lacking BPE and EGF). It thus seems that in order to obtain maximal effect manifestation, the cells must be cultured under extreme conditions. This observation is further supported by the finding that incubation of the HaCaT line with the stearyl peptides, but with serum, resulted in diminution of the effect (especially for SNH), until its total abolishment. The fact that SNV maintains its cytotoxicity even in the presence of serum may indicate a higher binding affinity to the receptor, or a higher selectivity to a specific receptor, relative to SNH and St-KKYL-NH_2.

Culturing HaCaT cells in serum-deprived conditions resulted in an apparent increase in VIP receptor 1 ($VPAC_1R$) expression, with no parallel change in VIP receptor 2 ($VPAC_2R$, see adjoining manuscript). Thus, modification in receptors' expression, following starvation, may provide an additional explanation to the observed variation in the degree of cytotoxicity (within HaCaT under different culture conditions and between HaCaT and the neonatal keratinocytes). Viewed this way, the observation of cytotoxic effect being dependent on culture conditions may further support the specificity of the cytotoxic peptide phenomenon, as a higher level of receptors expressed at the cell surface implies a higher level of stearyl in proximity to interfere at the cell membrane. In addition, previous publications from our laboratory have demonstrated that the stearyl-modified peptides differentiate between different VIP receptors [14]. The VIP hybrid antagonist (VHA) inhibited VIP-stimulated cAMP formation in astroglial cells, while SNH was essentially inactive. In contrast, SNH was at least 100-fold more potent than VHA in producing neuronal cell death [14]. Thus, superior activity of SNV and SNH in the present keratinocyte model may also involve a parameter of preference to specific VIP receptors on these cells, and may bear significance in targeting specific VIP-related drugs toward therapeutics.

ATA, a triphenylmethane dye, has previously been used to establish the causal relationship between DNA laddering and cell death. As such, it was shown to inhibit nuclease activity, resulting in reduced DNA fragmentation, associated with increased cell survival [33]. However, ATA may have additional effects, among them are influences on receptors to other growth factors, indicative of a multiple mechanisms of action [33]. Remarkable restoration in cell viability, obtained by co-incubation of stearyl peptides and ATA, thus suggests that the cell death induced by SNV, SNH and St-KKYL-NH_2 was associated with pathways affected by ATA, such as the apoptotic pathway, or involved in growth regulation by other factors.

Furthermore, it seems that new protein synthesis is responsible only in part for the suggested apoptotic cell death in this system. Data in the literature report of many examples where inhibitors of RNA or protein synthesis fail to interfere with the apoptotic pathway. On the contrary, some of these inhibitors were reported to enhance the susceptibility of cells to apoptosis induction or even induced apoptosis by themselves,

Table 2
Effects of CHI co-treatment on SNH cytotoxicity in HaCaT cells

Treatment	Surviving cells (%)
Control	100 ± 2.6
CHI (10 μg/ml)	69 ± 1.4
SNH (5 μM)	66 ± 3.2
SNH (10 μM)	1.8 ± 0.8
SNH (5 μM)+CHI	64 ± 2.8
SNH (10 μM)+CHI	8.8 ± 1.8

CHI was added simultaneously with SNH on the fourth day of culturing. Data represent percent of surviving cells relative to control. Results are presented as mean ± S.E.M. of two independent experiments. For experimental details, see Fig. 1.

thus indicating that macromolecular synthesis is not a general requirement for apoptosis [34]. For instance, DNA degrading enzymes were found to be constitutively present in the nucleus and only require activation.

While cAMP was not implicated in the SNV-induced effects [14], SNV (femtomolar–nanomolar concentrations)-mediated increases in cGMP were implicated in neuronal survival [16]. Here, cytotoxic SNV concentrations significantly reduced cGMP levels. Hence, reduced cGMP levels may, in combination with other signals, initiate apoptosis.

Dysregulation of the apoptotic pathway has been implicated in the pathogenesis and promotion of skin diseases, such as skin cancer and psoriasis. Haake and Polakowska [11] suggested that terminal differentiation of the keratinocyte is a specialized form of apoptosis. Apoptosis was suggested as the default pathway taken in the absence of continued proliferation or normal differentiation. The time between commitment to death and execution of the apoptotic effector phase was suggested to be extended in normally differentiating keratinocytes, relative to rapid apoptosis of abnormal or unnecessary keratinocytes, to allow expression of proteins subserving the differentiated function. For normal function, the apoptotic pathway, as the foundation of keratinocyte terminal differentiation, must be completed. In psoriasis, failure to complete the apoptotic pathway may result in abnormal retention of nuclei in the stratum corneum (parakeratosis [11]). Thus, the cytotoxic effect of the stearyl peptides may be a result of their induction of excessive differentiation in the HaCaT keratinocytes, resulting in unavoidable death. Regardless of the mechanism underlying cell death induction, the development of potent skin permeable agents having anti-proliferative effect is highly favorable. The stearyl peptides were specifically so designed to increase/enable their penetration through biological barriers, such as the skin. Thus, the observed cytotoxic effect of SNH, SNV and even more so the maintenance of effect by St-KKYL-NH_2 seem to have great promise in developing therapeutic agents for the topical treatment of hyperproliferative skin disorders.

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