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The peculiar N- and C-termini of trichogin GA IV are needed for membrane interaction and human cell death induction at doses lacking antibiotic activity

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ABSTRACT

Peptaibiotics, non-ribosomally synthetized peptides from various ascomycetes, are uniquely characterized by di-alkylated α-amino acids, a rigid helical conformation, and membrane permeation properties. Although generally considered as antimicrobial peptides, peptaibiotics may display other toxicological properties, and their function is in many cases unknown. With the goal to define the biological activity and selectivity of the peptaibiotic trichogin GA IV from the human opportunist *Trichoderma longibrachiatum* we analyzed its membrane interaction, cytotoxic activity and antibacterial effect. Trichogin GA IV effectively killed several types of healthy and neoplastic human cells at doses (EC 50% = 4–6 μM) lacking antibiotic effects on both Gram$^-$ and Gram$^+$ bacteria (MIC > 64 μM). The peptaibiotic distinctive C-terminal primary alcohol was found to cooperate with the N-terminal n-octanoyl group to permeate the membrane phospholipid bilayer and to mediate effective binding and active endocytosis of trichogin GA IV in eukaryotic cells, two steps essential for cell death induction. Replacement of one Gly with Lys plus the simultaneous esterification of the C-terminus, strongly increased trichogin GA IV anti-Gram$^+$ activity (MIC 1–4 μM), but further mitigated its cytotoxicity on human cells.

1. Introduction

Peptaibiotics [1–4], a family of fungal peptides, share a high content of α-aminoisobutyric acid (Aib) and other non-coded residues conferring them a stable helical structure, which is responsible for their ability to interact with phospholipid membranes. Their sequences are also characterized by an acyl group at the N-terminus and a 1,2-aminoalcohol at the C-terminus. For this reason these peptides are also named peptaibols. In the past, much effort was devoted to their isolation, structure elucidation, synthesis and antimicrobial activity determination, aiming at finding new antibiotic drugs [1]. However, antiviral, antiparasitic, immunosuppressive, anticoagulant and neuroleptic effects of various peptaibiotics have also been described [4,5]. A few studies deal with neurotoxicity and eukaryotic cells interactions as well [6–10].

A thorough investigation of the numerous biological effects of a peptaibiotic has not yet been reported, partly because of the difficult chemical synthesis that hampered for years the use of the solid-phase technique [11,12]. Therefore, we decided to perform a wide-range, membrane/cell interaction study on the peptaibiotic trichogin GA IV (Tric) [13,14] because of its accessible chemical synthesis [15], strong proteolytic resistance [16] and well-known conformation [14] facilitating the acquisition of structure/activity relationships. Moreover, the producing mold, *Trichoderma longibrachiatum*, widely present as a plant saprophytic agent and in marine sediments, is an emerging human pathogen. It is responsible for invasive mycosis, accompanied with tissue necrosis, in immune-compromised individuals [17–20], but also for local sinusitis in immune-competent patients [20,21]. Therefore, ascertaining the trichogin mechanism of action and its possible human cytotoxicity may have medical relevance.

Tric, whose primary structure is *n*Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol (*n*Oct, *n*-octanoyl; Lol, 1,2-amino alcohol leucinol), is able to permeate/modify model phospholipid membranes [14,22] by a not yet fully understood mechanism. Its mixed α-/3_{10}-helix [13,23], with the four Glys all lying on the same side, is slightly amphiphilic. Considering its short length (spanning about half of a membrane bilayer),

Abbreviations: ATP, adenosine-5′-triphosphate; BafA1, bafilomycin A1; CF, 5(6)-carboxyfluorescein; CFU, colony-forming unit; DMEM, Dulbecco's modified Eagle's medium; DOG, deoxy-D-glucose; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; LB, Luria Bertani; LDH, lactate dehydrogenase; Lol, leucinol; MIC, minimum inhibitory concentration; MFI, mean fluorescence intensity; OD, optical density; PC, phosphatidylcholine; PI, propidium iodide; PM, plasma membrane; PMN, polymorphonuclear leukocytes; PS, phosphatidylserine; SD, standard deviation; SPPS, solid-phase peptide synthesis; U, Aib; Z, benzyloxycarbonyl

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Tric was believed to act mainly through the so-called carpet-mechanism [24], namely accumulating parallel to the membrane surface and, after a threshold concentration, causing the membrane leakage. The *n*Oct group was found to play a crucial role in the interaction with model lipid bilayer and erythrocytes as well [25]. Recently, the possible occurrence of a transmembrane orientation was also suggested [26,27]. No information is available on the role of the other distinctive feature of Tric and other peptaibiotics, that is the C-terminal alcohol moiety. Although Tric was reported to display some antimicrobial action [15,16,28] and to decrease the viability of keratinocytes at high doses [15], no systematic studies are so far available on its cytotoxic efficacy and cellular selectivity so the definition of its real biological function is still elusive. Thus, we investigated in detail the membrane permeabilization ability and the antibacterial, hemolytic, and cytotoxic power of Tric using liposomal models, bacterial strains (two $Gram^-$ and two $Gram^+$), human cells (6 non-tumor and 4 tumor), and erythrocytes. Moreover, several Tric analogs variably modified at the N- and/or C-termini and in the peptide backbone were synthesized to assess the main structural features required for membrane/cell interactions and to test the possibility of modulating cell-efficacy and selectivity.

2. Materials and methods

2.1. Peptide synthesis

The chemical structures of the trichogin analogs investigated are reported in Table 1. The newly synthesized peptides were prepared by SPPS or solution procedures according to published protocols [16,29]. All peptides, characterized by means of NMR and mass spectra, have a purity greater than 96% (HPLC).

2.2. Interactions with model membranes

2.2.1. Circular dichroism

CD spectra were acquired on a Jasco (Tokyo, Japan) J-715 spectropolarimeter. Fused quartz cells (Hellma) of 1 mm path length were used. The values are expressed in terms of $[\Theta]_T$ and total molar ellipticity ($deg \cdot cm^2\ dmol^{-1}$). A 100 mM solution of sodium dodecyl sulfate (SDS) was used as solvent.

2.2.2. Small unilamellar vesicle (SUV) preparation

DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine) and DOPG (1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), while cholesterol (Ch) was a Sigma-Aldrich (St. Louis, MO) product. The lipid mixture, DOPC/Ch (7:3) or DOPG/DOPE (7:3), was dissolved in $CHCl_3$ in a test tube, dried under N_2, and lyophilized overnight. The lipid film was then reconstituted with a solution of carboxyfluorescein (CF) dye in 30 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) buffer (pH 7.4) at room temperature for 1 h. The resulting multilamellar vesicle suspension was sonicated (GEX 400 Ultrasonic Processor, Sigma) on ice until the initially cloudy lipid dispersion became translucent. The excess of fluorescent dye was eliminated by gel filtration on Sephadex G-75 (Sigma). SUVs (Ø = 50–60 nm) were diluted to a concentration of 0.06 mM with Hepes buffer (5 mM Hepes, 100 mM NaCl, pH 7.4). The SUVs were stored at 4 °C and used within 24 h.

2.2.3. Leakage from lipid vesicles

The peptide-induced leakage from SUVs of lipid composition DOPC/Ch (7:3) or DOPG/DOPE (7:3) was measured at 293 K using the CF-entrapped vesicle technique and a Perkin Elmer model MPF-66 spectrofluorimeter. The phospholipid concentration was kept constant (0.06 mM), and increasing [peptide]/[lipid] molar ratios were obtained by adding aliquots of methanol solutions of peptide at different concentrations. The final methanol concentration was kept below 5% by volume. After rapid and vigorous stirring, the time course of fluorescence change corresponding to CF escape was recorded at 520 nm (10-nm band pass) with $\lambda_{exc}$of 488 nm (10-nm band pass). A 1% T emission filter was applied. The percentage of released CF at time *t* was determined as $(F_t - F_0) / (F_T - F_0) \times 100$, with F_0 = fluorescence intensity of vesicles in the absence of peptide, F_t = fluorescence intensity of vesicles at time *t* in the presence of peptide, and F_T = total fluorescence intensity determined by disrupting the vesicles by addition of 50 μl of a Triton X-100 aqueous solution (10%). The kinetics experiments were stopped at 20 min.

2.3. Antibacterial activity

MICs of the peptides were determined using the broth microdilution method. Two-fold serial dilutions (from 1 to 64 μM) of each peptide,

Table 1
Peptide sequences of Tric and its analogs investigated. The molecular weight as determined by ESI–MS is given only for the newly synthesized compounds.

n.	Sequences[a]	Abbreviation	Synthesis[b]	MW_{calcd}	MW_{found}
1	Oct-UGLUGGLUGILol	Tric	[28]	–	–
C-terminal modifications					
2	Oct-UGLUGGLUGIL-**OMe**	Tric-OMe	[25]	–	–
3	Oct-UGLUGGLUGIL-**OH**	Tric-COOH	Auto-SPPS	1080.70	1080.65
N-terminal modifications					
4	**Ac**-UGLUGGLUGILol	Ac-Tric	Auto-SPPS	892.62	892.63
5	**Fitc**-UGLUGGLUGILol	FITC0-tric	Auto-SPPS	1329.66	1329.67
N- and C-terminal modification					
6	**Ac**-UGLUGGLUGIL-**OMe**	Ac-Tric-OMe	[25]	–	–
Replacement of Gly residue					
7	Oct-U**K**LUGGLUGILol	K2	Auto-SPPS	1137.79	1137.84
8	Oct-UGLUG**K**LUGILol	K6	[28]	–	–
9	Oct-UGLUGGLU**K**ILol	K9	Auto-SPPS	1137.79	1137.84
10	Oct-UGLUG**K**(**Fitc**)LUGILol	K6FITC	Auto-SPPS[c]	1527.83	1527.87
Replacement of a Gly residue and C-terminal modifications					
11	Oct-U**K**LUGGLUGIL-**OMe**	K2-OMe	[28]	–	–
12	Oct-U**K**LUGGLUGIL-**COOH**	K2-COOH	Auto-SPPS	1151.77	1151.78
13	Oct-UGLUG**K**LUGIL-**OMe**	K6-OMe	Sol. phase	1165.78	1165.83
14	Oct-UGLUGGLU**K**IL-**OMe**	K9-OMe	[28]	–	–
15	Oct-UGLUG**K**(**Fitc**)LUGIL-**OMe**	K6FITC-OMe	Sol. phase	1554.83	1554.85

Bold indicates the modification at the N- or C-terminal of trichogin.
[a] U, Aib; Fitc, fluorescein isothiocyanate.
[b] Auto-SPPS, SPPS through an automatic synthesizer; man-SPPS, manual SPPS; sol. phase, peptide synthesis in solution.
[c] The final step (Fitc incorporation) was carried out in solution.

previously dissolved in ethanol at the final concentration of 750 μM, were prepared in LB medium and 50 μL per well was arranged in sterile 96-well plates (Falcon). Then, an aliquot (50 μl) of bacterial cell suspension was added to each well, at a final concentration of 5×10^5 CFU/ml. After incubation for 8 h or 24 h at 37 °C, the inhibition of bacterial growth was assessed and the MIC endpoint was defined as the lowest concentration of the antimicrobial peptide that completely inhibited bacterial growth. The effect of the peptides on the growth of four bacterial strains was examined as follows: a bacterial cell suspension with an OD at 625 nm of 0.05 was prepared in LB medium, diluted 1:100 in LB and then allocated in 96-well plates and after adding different concentrations (1–64 μM) of the peptides, the bacterial cultures were grown for up to 24 h, recording the absorbance at 620 nm at 1 h intervals with a Microplate ELISA reader (Amersham Biosciences) to assess the cell growth. Vehicle solvent (EtOH) was used as control at the same concentrations reached in peptide containing media.

2.4. Cell isolation and culture

HeLa, A431 and CCD34-Lu cells were maintained in DMEM (Gibco), A549 in F12 medium (Gibco) and HL60 in RPMI medium (Gibco), supplemented with 10% FCS (Euroclone) and antibiotics (penicillin and streptomycin, Invitrogen) at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2; cells were split every 2–3 days.

Human monocytes, PMNs and lymphocytes were purified from buffy coats of healthy donors, kindly provided by the Centro Immunotrasfusionale, Hospital of Padova. Briefly, for monocyte purification, buffy coats were subjected to two sequential centrifugations on Ficoll and Percoll gradients (GE Healthcare); residual lymphocytes were removed by incubation in 2% FCS RPMI at 37 °C and subsequently removed by washing. Unless otherwise specified, cells were kept at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2 in RPMI-1640 supplemented with 10% FCS. For PMN purification, the pellet of cells obtained after the centrifugation on the Ficoll gradient was subjected to dextran erythrocyte precipitation; residual erythrocytes were removed by hypotonic lysis in 155 mM NH_4Cl, 10 mM $KHCO_3$, and 100 mM Na_2EDTA at pH 7.4 and cells were cultured in RPMI medium, supplemented with 10% FCS. For lymphocyte preparation, buffy coats were incubated with 50 μl/ml of Rosette Sep® Human T Cell Enrichment Cocktail (StemCell Technologies). Blood was then centrifuged over a Ficoll gradient and cells were cultured in RPMI medium, supplemented with 10% FCS. To obtain macrophages, purified monocytes were cultured for seven days in 20% FCS RPMI supplemented with 100 ng/ml of M-CSF (Immunological Sciences). Dendritic cells were obtained by culturing purified monocytes in 10% RPMI containing 50 ng/ml GM-CSF and 20 ng/ml IL-4 (Peprotech).

2.5. MTS and LDH assays

The day before the experiment, A431, HeLa, A549 and CC34-Lu cells were detached by trypsin treatment (Gibco), counted and seeded onto 96 wells/plate (8×10^3 cells/well, Falcon). After purification, monocytes were seeded onto 96 wells/plate (2×10^6 cells/well), left to adhere to the plastic and immediately treated with stimuli. PMNs, lymphocytes (2×10^6 cells/well) and HL60 cells (40×10^3) were seeded onto cell plates and subsequently treated. Different peptides were diluted in culture medium (from 20 μM to 1 μM) and added to the cells. As a positive control, cells were treated with medium without stimuli. After 24 h-treatment, the cell supernatant was collected for subsequent assay (LDH assay) and cells were tested using a CellTiter 96® AQueous One Solution Reagent (Promega). Plates were read with an ELISA reader (Amersham Biosciences) at 492 nm. EC_{50} (the concentration of drug where 50% of cells died) was calculated for each peptide and each kind of tested cell, after a non-linear logistic fit of data means. For LDH assays, 50 μl aliquots of the cell supernatant were tested with a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). EC_{50} (the concentration of drug that induced the lysis of 50% of the total cell number) was calculated for each peptide and each kind of tested cell, after a non-linear logistic fit of data means.

2.6. Hemolysis assay

Fresh citrated blood was mixed at 1:1 (v/v) ratio with different concentrations of Tric and its analogs (0–16 μM) in 150 mM NaCl. Blood diluted with distilled water or with PBS was used as negative or positive control, respectively. Samples were incubated at 37 °C for 2 h and then centrifuged at 2600 rpm for 7 min. Supernatants were collected and incubated at room temperature for 30 min to allow hemoglobin oxidation. Then oxyhemoglobin concentration was measured spectrophotometrically at 620 nm.

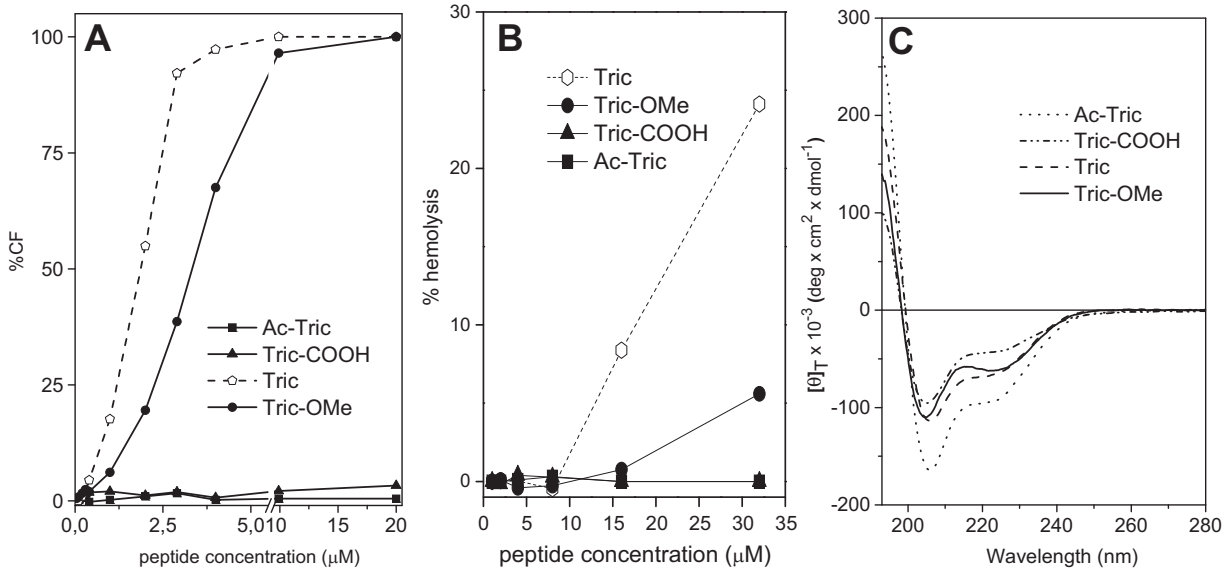

Fig. 1. Interaction of Tric analogs with model and cell membranes. A) CF leakage from SUVs (DOPC/Ch 7:3 — lipid concentration: 60 μM) induced by increasing peptide concentrations. B) Induction of hemolysis by different Tric analogs after 2 h incubation with fresh red blood cells; results were expressed as percentage with respect to total hemolysis induced by water. C) Far-UV CD spectra of trichogin GA IV and its analogs in 100 mM SDS aqueous solution (peptide concentration: 0.1 mM).

2.7. Propidium iodide and Annexin V assay

HeLa cells were seeded on a plastic plate the day before the experiment, while HL60 cells were directly used. Cells were treated for 18 h with different trichogin analogs (up to 20 μM), then they were washed with PBS, detached with trypsin (in the case of HeLa cells), centrifuged at 200 *g* for 5 min, re-suspended in 50 μl of incubation buffer (Roche) and incubated for 15 min at 37 °C with 2 μl of Annexin-V-Fluos (Roche). Cells were finally diluted in 250 μl of the incubation buffer and 15 μl of propidium iodide (PI) (Sigma) was added to each sample just before the acquisition with FACSCanto® (Beckton Dickinson). Percentage of live (Annexin V^-/PI^-), apoptotic (Annexin V^+/PI^-), oncotic (Annexin V^-/PI^+) and late apoptotic/abortive oncotic (Annexin V^+/PI^+) cells of the total (10^6 cells) was calculated using FACSDiva Software.

2.8. Binding and uptake studies

The day before the experiment, HeLa and A431 cells were detached by trypsin treatment and seeded on a 24-well plate (1×10^5 cells/well). On the day of the experiment the different cells (HeLa, A431, monocytes, PMNs, macrophages and dendritic cells) were incubated for 2 h with different FITC-labeled trichogin analogs (from 0.075 to 5 μM), both at 37 °C (binding and endocytosis) and 0 °C (binding) in

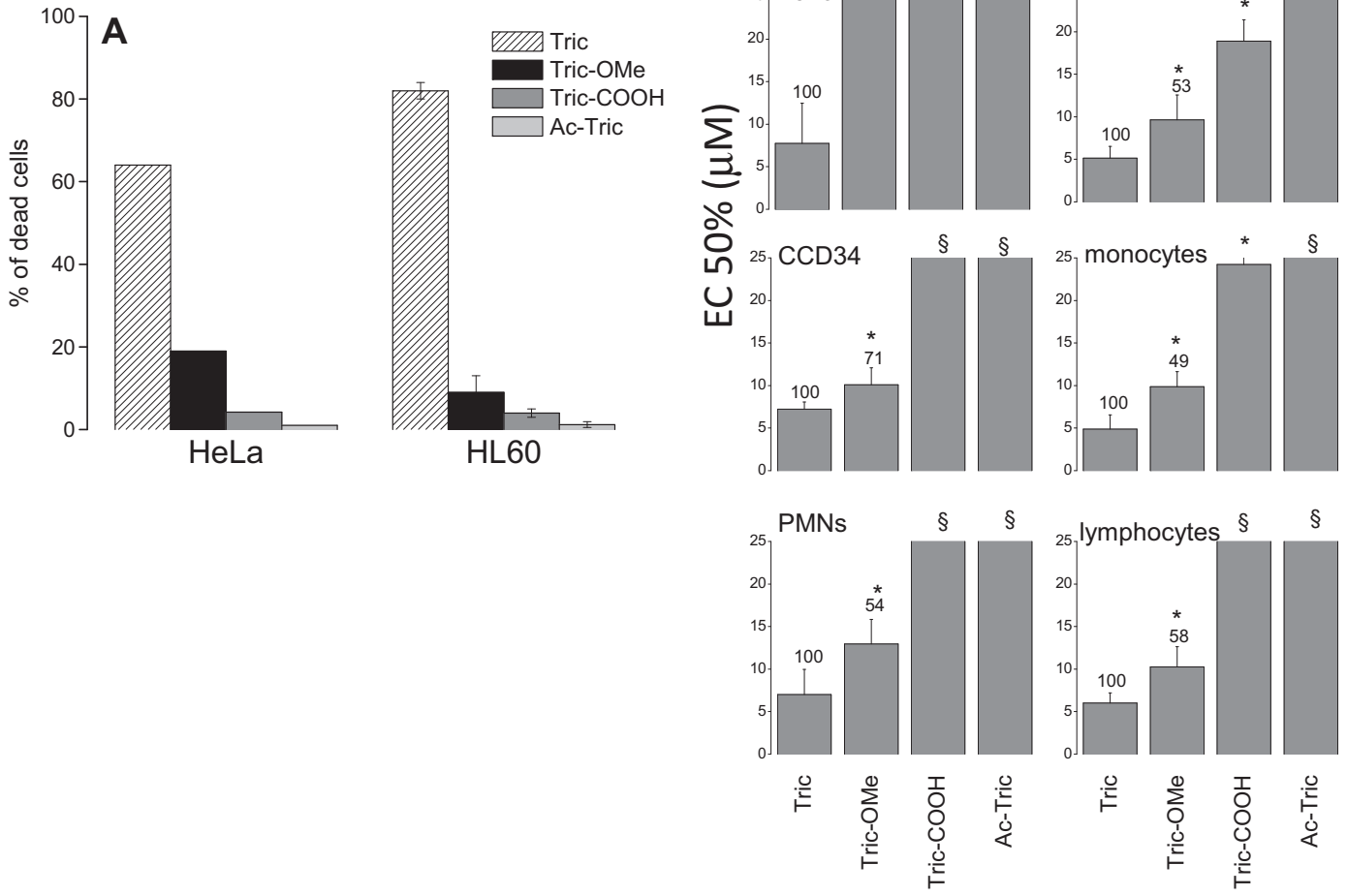

Fig. 2. Cytotoxic effects of Tric and its analogs with either a N- or C-terminal modification. A) HeLa and HL60 cells, treated with 20 μM of the peptides for 24 h, were stained with AnnexinV-FITC and propidium iodide and subsequently subjected to FACS analysis. Results were expressed as percentage of AnnexinV$^+$ plus PI$^+$ cells with respect to the total number of cells acquired by FACS. B) EC_{50} values (such as peptide doses resulting in a 50% of the maximum effect using the MTS assay) were calculated as explained in the Materials and methods section and expressed as means ± SD of four experiments run in duplicate. *<0.05 with respect to trichogin-treated cells; § indicates no activity up to 25 μM. Numbers above histograms indicate the percentage of cytotoxic activity with respect to wt Tric, whose activity was fixed to 100%. Similar data were obtained with primary human macrophages and dendritic cells (not shown).

medium containing 10% FCS. To study the effects of endosomal acidification on Tric uptake, bafilomycin A1 (BafA1) was used as an inhibitor of endosome acidification. Stock solution of BafA1 was prepared in dimethyl sulfoxide (DMSO) at concentrations of 200 μM. To examine the energy-dependence of Tric uptake, sodium azide (Sigma) and 2-deoxy-D-glucose (DOG, Sigma) were used. Inhibitors were dissolved on the day of each experiment in Dulbecco-PBS (D-PBS, Gibco). Cells were then incubated in the presence of inhibitors for 30 min at 37 °C in D-PBS containing 10% FCS (100 nM BafA1 or 10 mM sodium azide and 5 mM DOG); thereafter, cells were washed three times in PBS, and were incubated for 2 h with 5 μM of fluorescent trichogin analogs in D-PBS buffer supplemented with 10% FCS with or without each inhibitor. Cells were then washed in PBS and resuspended in FACS buffer (PBS, 1% FBS) and 10,000 events per sample were analyzed using a FACSCanto® analyzer (Becton Dickinson). Dead cells were excluded from FACS analysis by adding propidium iodide.

2.9. Confocal microscopy

Macrophages (2×10^6 cells/well) and HeLa cells (1×10^5 cells/well) were seeded on cover glasses and incubated with 5 μM K6-FITC for 2 h at 37 °C or at 0 °C. Unfixed cells were briefly rinsed in PBS and directly analyzed under a confocal microscope (SP2 Leica). Untreated cells were used as control. Images were acquired with different fluorescence filters; representative pictures were collected as Tiff files and processed with standard imaging programs.

2.10. Mathematical and statistical analyses

All mathematical and statistical analyses were performed using the analysis and statistic packages of the Microcal Origin 8 software. EC_{50} values of cytotoxicity and hemolysis were obtained after fitting dose–response data non-linearly with logistic curves [$Y=A2 + (A1-A2)/(1+(x/x_0)^p)$] from statistically significant convergent curves. MTS assay data were normalized as % of control (A2 = 100, A1 = 0). LDH and hemoglobin release assay data were normalized as % of maximal extracellular release induced by distilled water (A2 = background % release, A1 = 100%). Normal (Gaussian) distribution of data was checked and confirmed by the Shapiro–Wilk test (0.05 level). Significance of the differences between means (0.05 level) was evaluated by two samples or paired t test, when applicable, and by ANOVA analysis plus Bonferroni, Scheffe and Tukey tests.

3. Results

3.1. Permeation of membranes

To assess the possible role of the C-terminal alcohol group in model membrane damage, we quantified the efficacy of various Tric synthetic analogs to induce the leakage of carboxyfluorescein (CF) entrapped in small unilamellar vesicles (SUVs). SUVs were made of zwitter-ionic phospholipid and cholesterol (DOPC/Ch 7:3), considered a proxy of the eukaryotic lipid monolayer facing the extracellular space. We also measured the release of hemoglobin from erythrocytes (Fig. 1A, B).

Liposomal leakage, in agreement with previous evidence, was not induced by peptides lacking the N-terminal *n*Oct moiety. On the other hand, replacement of the primary alcohol ($-CH_2OH$) by a methyl ester group (Tric-OMe) or by a carboxylic acid (Tric-COOH) showed a partial or a complete activity inhibition, respectively (Fig. 1A). As previously reported, the hemolytic effect of Tric was much reduced as compared to the liposomal leakage (threshold: 8 μM, 25% hemoglobin release at 32 μM). However, in spite of these different efficacies in the two systems, we observed a similar reduction of the activity of the other Tric analogs. In fact, the membrane destabilization of erythrocytes by Ac-Tric and Tric-COOH was totally absent, and that induced by Tric-OMe was ~60–70% inhibited (threshold: 16 μM; 8% hemoglobin release at 32 μM) compared to Tric (Fig. 1B).

Far-UV CD spectra in 100 mM SDS aqueous solution, a membrane-mimetic environment (Fig. 1C), show two negative maxima at about 205 and 222 nm of moderate intensities and a positive maximum at about 195 nm [30] for all peptides investigated. This observation indicates that they all adopt a right-handed, mixed α-/3_{10}-helical

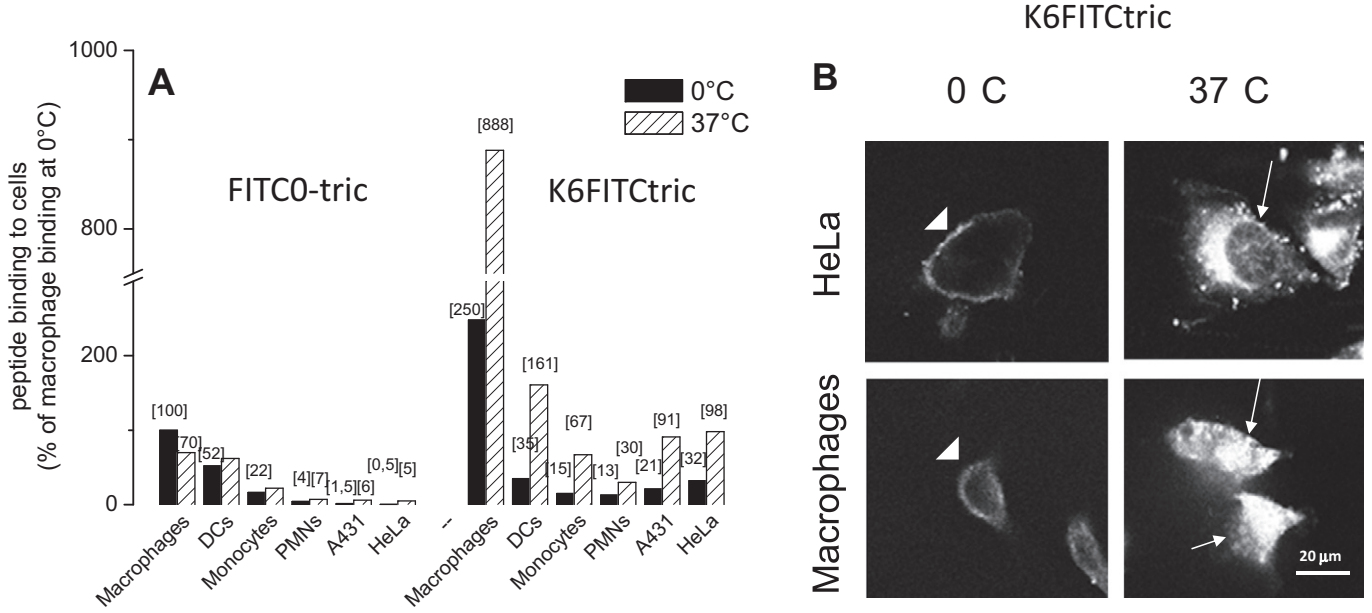

Fig. 3. Binding and internalization of FITC-labeled trichogin analogs to human cells. A) Different cells were incubated at 37 °C and at 0 °C with 5 μM of FITC0-tric or K6FITC-OMe and the associated fluorescence was measured by FACS. Numbers above histograms indicate the percentage of MFI with respect to MFI of FITC0-tric-treated macrophages at 0 °C, that was fixed to 100%. B) Confocal microscopy on HeLa and macrophages. Cells, previously seeded onto cover glasses, were incubated for 2 h at the indicated temperatures with a 5 μM solution of K6FITC-OMe, washed with PBS and then directly analyzed by confocal microscopy. Arrowheads indicate the plasma membrane, and arrows indicate intracellular vesicles.

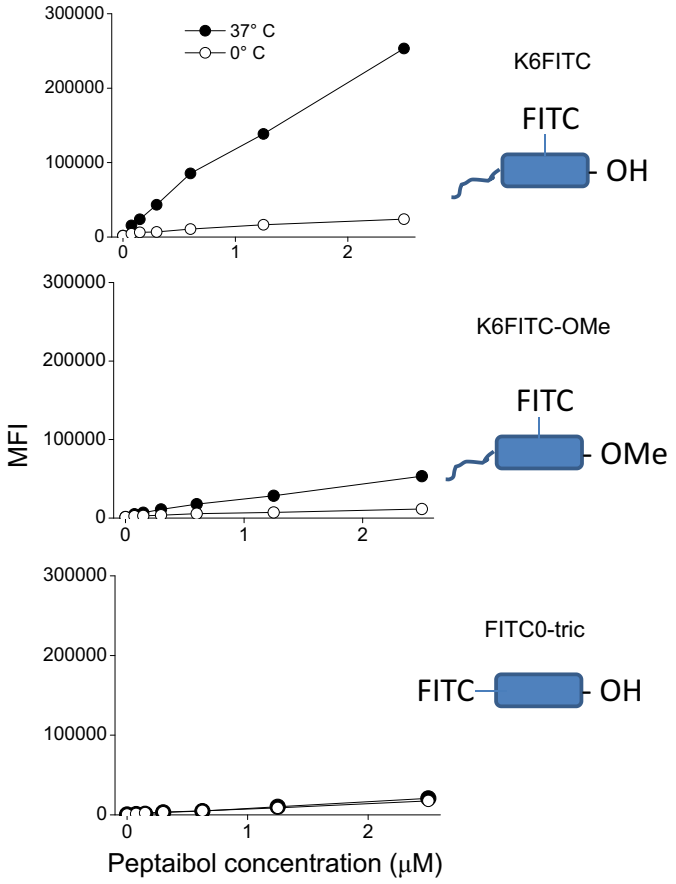

Fig. 4. Dendritic cell binding and internalization of FITC-labeled trichogin analogs. Dendritic cells were incubated for 2 h with each FITC-labeled analog (up to 5 μM) indicated in the figure, both at 37 °C and 0 °C; cells were then analyzed by FACS, maintaining the same instrument settings.

conformation [14], comparable to that of the native peptide [31]. Thus, the different behaviors with liposomal and erythrocyte membranes are reasonably ascribed to the moieties located at the peptide extremities, rather than to the peptide conformation.

3.2. Cellular effects of Tric and its analogs modified at the N- and/or C-termini

Having established the relative potencies of Tric and its analogs to destabilize membranes, we engaged in assessing their toxic effects on bacteria and eukaryotic cells. First, we observed that the peptides display no antibiotic activity up to 64 μM (not shown). This apparent lack of effects on prokaryotic cells, in line with previous observations [16], poses the problem of the real activity and selectivity of Tric. To fill this gap, we initially tested the killing of two human cancer-derived cell lines (HeLa and HL60) after 24 h by a dose of Tric able to fully permeabilize the model liposome membrane (20 μM). Surprisingly, FACS analysis showed that Tric effectively induced a necrotic phenotype in HeLa cells, characterized by the permeabilization of their plasma membrane to PI, and an abortive/late apoptotic one in HL60, indicated by both leakage to PI and Annexin V labeling of surface-exposed PS (Fig. 2A). In good correlation with their membrane permeation potencies, Tric-COOH and Ac-Tric were very weak or not cytotoxic (90–95% and >98% inhibition in both cell types, respectively), while Tric-OMe showed a marked reduction of its cytotoxicity on both cell types (~70–90% inhibition compared to Tric). These data further support the intriguing possibility that not only the N-terminus *n*Oct but also the C-terminus $-CH_2-OH$ of Tric may play a specific action in the interaction with and destabilization of eukaryotic cells and, consequently, in its intoxication mechanism.

To consolidate and extend this indication we analyzed the cytocidal effects of Tric and its derivatives on a wider range of human cells, both healthy and neoplastic: (i) three primary human blood cells (lymphocytes, PMNs, monocytes), (ii) two types of *in vitro* differentiated, monocyte-derived, antigen presenting cells (macrophages and dendritic cells), (iii) the *in vitro* stabilized line CCD34Lu (from the human normal lung), (iv) the monocyte-like HL60 cell line from human myeloid leukemia, and (v) three tumor derived non-leukocyte cell lines: HeLa (human ovary sarcoma), A431 (human epidermoid carcinoma) and A549 (human lung carcinoma). Toxicity was evaluated quantitatively by dose–response curves up to 20 μM peptide concentration after a 24 h incubation using as parameter the decrease of MTS reduction rate by mitochondria or the extracellular release of the cytosolic enzyme LDH. Data showed that Tric has a similar cytotoxic effect on both cancer-derived and non-tumoral cells with mean (±SD) EC 50% values of 5.8 (±0.41) μM for the MTS assay and ~9 (±2) μM for the LDH assay (Fig. 2B and Fig. S1). Again, Tric-COOH

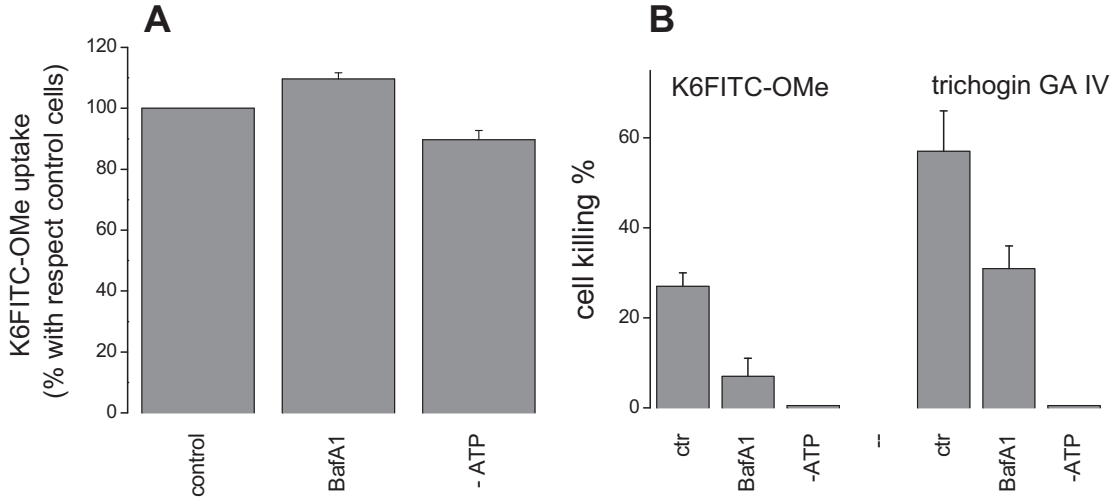

Fig. 5. The effects of BafA1 and ATP depletion by sodium azide and DOG on Tric association to and cytotoxicity towards cells. A) HeLa cells were pre-treated or not for 30 min at 37 °C with BafA1 or sodium azide plus DOG as described in Materials and methods, then cells were washed and incubated for 2 h with 5 μM of K6FITC-OMe, both at 37 °C and 0 °C (not shown). Then, MFI associated with cells was measured by FACS; the results were expressed as percentage of MFI with respect to the control cells, whose MFI was fixed to 100%. B) HeLa cells were incubated as previously described and the residual number of cells in the samples was estimated by FACS count, fixing the time of acquisition at 60 s; results were expressed as the percentage of dead cells with respect to not treated with the indicated peptides. Data are the means ± SE of three experiments run in duplicate.

and Ac-Tric were not cytotoxic. Eventually, the replacement of the C-terminal $-CH_2OH$ with a methyl ester (Tric-OMe) determined a partial but significant inhibition of Tric cytotoxic action in all cells (mean inhibition = 54–60%), resulting in shifts of the mean EC 50% values to 13 ± 2.2 for the MTS assay and to 22 ± 3 for the LDH assay.

3.3. Cell binding and endocytosis of Tric and its analogs

The data above highlight the novel finding that Tric is indeed cytotoxic to many human cells at doses completely devoid of antibacterial or hemolytic effects. Moreover, in agreement with CF liposomal leak, it is evident that the C-terminal alcoholic function plays a crucial role in cell killing.

To address this intriguing issue, we explored the mechanism of human cell intoxication by Tric. We first investigated the role of the crucial N-terminal group on peptide–cell interactions by measuring cell capture of two fluorescent Tric analogs (Table 1): FITC0-tric, in which *n*Oct is substituted by the fluorescent probe FITC, and K6FITC-OMe, in which *n*Oct is present and the fluorescent label is located on the side chain, linked to a Lys ε-amino group. This allowed us to quantitatively compare cell association by FACS analysis and to assess the contribution of the *n*Oct to it. Experiments were performed in HeLa cells, A431 cells, monocytes, PMNs, lymphocytes, macrophages, and dendritic cells at two critic temperatures: 0 °C, allowing binding but not compatible with membrane endocytosis and 37 °C, the physiological temperature also permitting membrane endocytosis. Our results (Fig. 3A) show that cell association of the *n*Oct-bearing Tric analog (K6FITC-OMe) after a 2 h incubation is always improved at 37 °C (mean fold increase ± SD = 3.85 ± 1.1), although characterized by different intensities depending on the cell type. On the contrary, cell association of the *n*Oct lacking FITC0-tric in the same conditions was relatively smaller and similar at either 0 °C or 37 °C. Confocal microscopy of HeLa cells and macrophages confirmed that K6FITC-OMe signal is faint and mostly located on the cell plasma membrane at 0 °C, while much stronger and localized in intracellular compartments, resembling endosomes, at 37 °C (Fig. 3B). These data suggest that the peptide backbone of Tric does not mediate an effective binding and internalization to human cells. On the contrary, the *n*Oct is necessary to ensure the efficient association in all tested cells, especially at 37 °C. To assess the influence of the C-terminal alcohol on the trichogin–cell interaction we compared peptide accumulation in dendritic cells of K6FITC and K6FITC-OMe (Fig. 4). While the reduced cell binding of the two analogs at 0 °C was similar, and comparable to the *n*Oct lacking FITC0-tric, the cell association increase at 37 °C was remarkably more intense in the presence of the C-terminal alcohol. Collectively, these data suggest that simple binding to the cell plasma membrane at 0 °C is mainly mediated by the peptide backbone of Tric, and poorly modulated by the *n*Oct and the terminal primary alcohol. On the other hand, cellular internalization triggered at 37 °C essentially requires the *n*Oct but is further up-modulated by the C-terminal $-CH_2-OH$.

Table 2
MICs (μM ± SE; N ≥ 4; peptides dose range: 0–64 μM) after 24 h against two Gram$^+$ and two Gram$^-$ bacterial strains.

Compounds	*S. aureus*	*S. epidermis*	*P. aeruginosa*	*E. coli*
Tric	>64	>64	>64	>64
K2	3 ± 1.4	4.3 ± 2	58 ± 16	24 ± 17.1
K2-OMe	6 ± 2.8	6 ± 2.8	>64	32 ± 0
K2-COOH	32 ± 0	48 ± 22	>64	>64
K6	3 ± 1.7	4 ± 2	>64	16 ± 0
K6-OMe	4 ± 1	6 ± 2.8	32 ± 0	32 ± 0
K9	5.3 ± 2.1	4.7 ± 1.6	57 ± 16	32 ± 17.5
K9-OMe	3 ± 1.4	1 ± 0.5	>64	32 ± 0

3.4. Effect of endocytosis blockade and of endolysosomal neutralization on Tric cytotoxicity

Having observed that Tric binds more efficiently and is strongly internalized by human cells at 37 °C and that both the *n*Oct and the

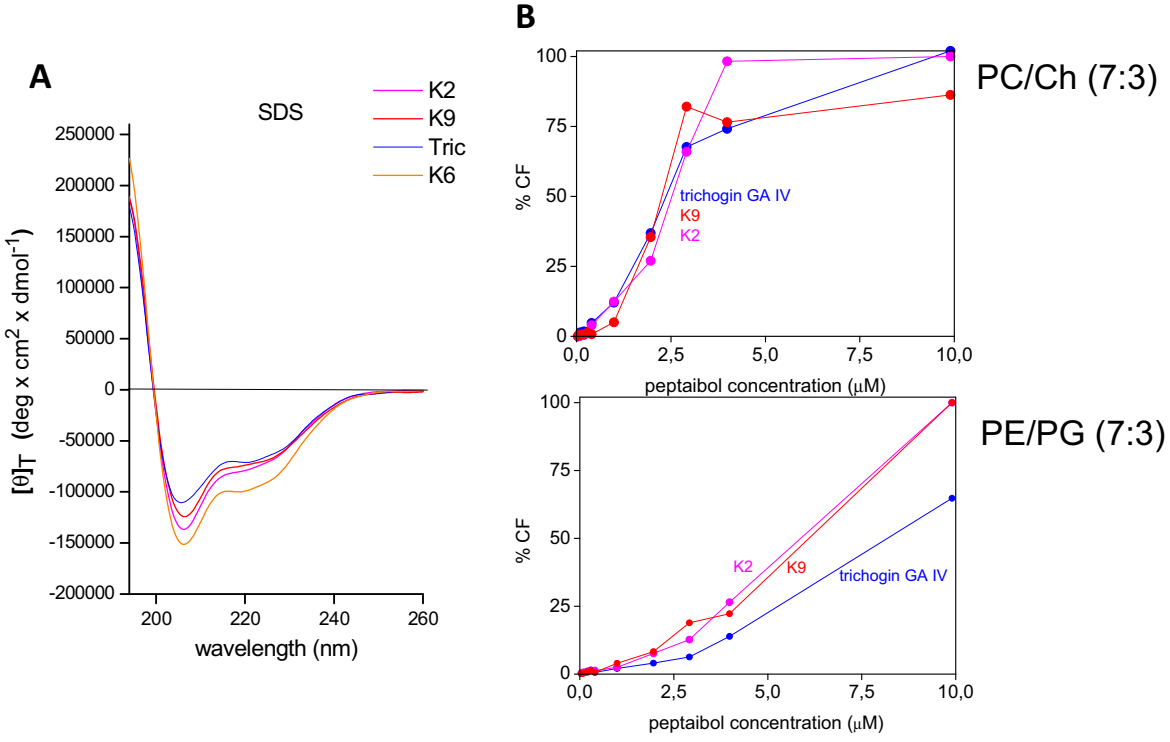

Fig. 6. Interaction of K-substituted Tric analogs with model membranes. A) Far-UV CD spectra of trichogin GA IV and its analogs in 100 mM SDS aqueous solution (peptide concentration: 0.1 mM). B) CF leakage from SUVs (DOPC/Ch 7:3 or PE/PG 7:3 — lipid concentration 60 μM) induced by increasing peptide concentrations.

primary terminal alcohol are important for such effect, we investigated whether the cytotoxic effect requires or not endocytosis. To test this we used BafA1, a drug which selectively neutralizes the lumen of endosomes by blocking the V-ATPase, so affecting their function and maturation [32]. In addition we depleted cellular ATP by DOG plus sodium azide incubation to prevent cellular internalization [33]. Fig. 5A shows that, as previously observed, after a two hour incubation K6FITC-OMe can associate effectively to cells at 37 °C and that blocking endocytosis or endolysosomal acidification did not affect such binding. Strikingly, however, the cytotoxic action of K6FITC-OMe and of the native Tric, measured in parallel by counting the cell number by FACS, was prevented by endocytosis blockade and partially inhibited by neutralization of endolysosomes (Fig. 5B). These observations indicate that the increased internalization of Tric at 37 °C is the consequence of an increased association of the peptide to the cell plasma membrane at this temperature. In addition, they demonstrate that the peptide externally bound to the plasma membrane does not damage the lipid bilayer. On the contrary, cell intoxication and eventual cell lysis require Tric internalization in acidic endosomes.

3.5. Modulation of cytotoxic and antibacterial activities of Tric by increasing the cationic amphipathic properties of its helical peptide backbone

It has been previously shown that the antibacterial effects of Tric can be improved by the substitution of glycines with cationic lysines [28]. With the aim of further testing the cell selectivity of Tric, we determined how the insertion of a Lys residue affects structure, liposomal leakage, hemolysis and human cell killing and association.

Single substitution of Gly for Lys in position 2, 6 or 9 of Tric did not alter significantly its helical structure, according to CD spectra in membrane-mimetic SDS micelles (Fig. 6A).

Interestingly, dose–response analysis of CF leak from liposomes mimicking either eukaryotic (DOPC/Ch) or prokaryotic (PE/PG) membranes revealed important differential effects (Fig. 6B). First, Tric was significantly more effective on PC/Ch liposomes (EC 50% effect ~2 μM) than on PE/PG ones (EC 50% effect ~9 μM). Second, while the leakage effect was not significantly modified by Lys mono-substituted Tric analogs in eukaryotic like-liposomes, the introduction of positively charged residue improved the CF leak induced by the peptide analogs in negative, prokaryotic like-membranes (EC 50% ~6 μM).

In agreement with liposomal data, we show that Lys-monosubstituted Tric analogs effectively block the growth of Gram$^+$ *Staphylococcus aureus* and *Staphylococcus epidermidis* (MIC range: 1–6 μM), while are less active on Gram$^-$ *Escherichia coli* (MIC range 16–32 μM) and *Pseudomonas aeruginosa* (MIC range: 32–64 μM) (Table 2). In addition, we here also demonstrate that replacement of the C-terminal $-CH_2-OH$ with $-COOMe$ is irrelevant for the anti-Gram$^+$ effect of Lys-substituted Tric, while its substitution with $-COOH$ strongly reduces the antibacterial effects on Gram$^+$.

Surprisingly, we found (Fig. 7A and Fig. S2) that, on the contrary, the Lys-monosubstituted Tric analogs were equally or slightly less effective than Tric in killing human nucleated cells, again in good agreement with data obtained with "eukaryotic-mimetic" liposomes. Once again, the substitution of the C-terminal $-CH_2-OH$ with $-COOH$ or a methyl ester was found to strongly or significantly reduce cytotoxicity. PI/Annexin V labeling experiments confirmed that an optimal cytotoxic action of Lys-monosubstituted Tric on human cells still requires the alcohol (Fig. 7B). Eventually, we showed a similar inhibition of the destabilization of red blood cell plasma membrane induced by LysTric at concentrations >8 μM by the substitution of the C-terminal primary alcohol with a COOH or a methyl ester (total ablation and ~60% inhibition, respectively) (Fig. 7C). A schematic summary to better appreciate the differential modulation of the cytotoxic and antibacterial activities of Tric by chemical modifications interesting its α-helix and the C-terminus is shown in Fig. 7D.

4. Discussion

We here present the novel observation that the short peptaibiotic Tric from *T. longibrachiatum*, normally assumed to be an antimicrobial peptide, is, on the contrary, an effective cytotoxin at doses devoid of antibacterial effects. Several primary and cancer-derived human cell types were indeed lysed by trichogin with similar efficacies. This represents a new paradigm, challenging the previous suggestions that peptaibols are just antimicrobial agents, at least in the case of trichogin.

In addition, based on structure–function correlation analysis, we underscore that not only the N-terminal *n*Oct chain of Tric, but also the C-terminal $-CH_2-OH$ moiety plays a major role in human cell killing. We in fact showed for the first time that the presence of this distinctive and unique feature of peptaibiotics significantly improves the efficacy of Tric to permeate artificial (liposomes) and natural (erythrocytes) membranes. Even more importantly from the biological point of view, the $-CH_2-OH$ group significantly contributed to the exertion of optimal cytotoxic effects of Tric on human cells.

Our data are consistent with a recent study in model membranes [34] indicating that the C-terminal OH group of Tric mostly interacts with the glycerol moiety, and to a minor extent with the phosphate groups *via* H-bonding interactions. These interactions may contribute, together with the ones driven by N-terminal *n*Oct, to the Tric induced bilayer deformation and thinning eventually leading to membrane permeabilization. In fact, the substitution of the C-terminal alcohol with a methyl ester (Tric-OMe) eliminates the possibility of establishing these interactions. Moreover, the presence of a C-terminal COOH group instead of the $-CH_2-OH$ (Tric-COOH), negatively charged at physiological pH, would determine an electrostatic repulsion with the lipid phosphate groups, strongly disfavoring membrane interaction and effects, as observed in this study.

The existence of eukaryotic-specific intoxication mechanisms of Tric is further supported by our observation that the artificial improvement of the amphipathic and cationic features of the peptide α-helix of Tric by introducing K residues, known to abruptly increasing Tric anti-bacterial effects, particularly of the two Gram$^+$ bacteria here analyzed (*S. aureus* and *S. epidermidis*), does not improve the sensitivity of any human cell type tested to Tric. Moreover, while the OMe analogs of Lys-monosubstituted Tric are as effective as the $-OH$ containing ones in antibacterial assays, they displayed a further mitigated killing efficacy of human cells and a reduced hemolytic action.

Overall, these data point to a peculiar role of the terminal OH in the interaction of Tric with the eukaryotic cell plasma membrane, irrelevant in the interaction with the bacterial one. To understand in more detail the mechanism of human cell killing by Tric, we analyzed its cell binding and endocytosis and assessed the importance of these two processes for the induction of the eventual cell lysis. We found that the backbone of trichogin is not enough to ensure effective association to all human cell tested and that, on the contrary, the presence of both the N-terminal *n*Oct and the C-terminal alcohol is always necessary for its effective binding to the cell plasma membrane and for the subsequent endocytosis at 37 °C. Prevention of endocytosis by energy depletion not only revealed that both the nOct and the C-terminal alcohol mediate the temperature dependent binding to cell plasma membrane, but also proved that this initial interaction is not enough to determine its destabilization and the cell death. The neutralization of endosome acidification did not alter the total Tric cell capture but partially inhibited its cytotoxic effect. Again, this observation indicates that the transport of Tric in internalization vesicles is important for the induction of cytotoxic effects. These data may suggest that cytotoxicity of Tric is stronger in human eukaryotic cells, compared to bacteria, thanks to the endocytic step. Indeed, the current models of membrane destabilization by Tric suggest a high cooperative interaction between peptide molecules leading to the formation of toxic structures, possibly transmembrane permeabilizing aggregates, after reaching a critical membrane concentration [35]. It may therefore be that the critical concentration of

trichogin necessary for membrane damage is not obtained in the plasma membrane at the investigated peptide concentrations, but is only reached after endocytosis and accumulation in endolysosomes. The eventual disruption of endo-lysosomal compartments may then secondarily trigger necrosis or apoptosis, as observed. This may explain the partial inhibitory effect obtained by neutralizing the acidity of an intracellular compartment on Tric cytotoxic action, since it is known that maturation of the endosomes is slowed in these conditions. In this view the membrane permeation of human cells leading to cell lysis, shown in this study, would not be the direct consequence of the Tric plasma membrane destabilization, but rather a secondary non-specific effect typical of the necrosis (or abortive apoptosis) triggered by cell-internalized Tric (see model in Fig. 8). Our model suggests the hypothesis that the general presence of a C-terminal $-CH_2OH$ in peptaibiotics may be justified by its need for an optimal interaction with eukaryotic membranes, being necessary for effective binding to the plasma membrane and for the subsequent membrane destabilization after a critically needed internalization step. This may shed a new light on the role of Tric, and possibly other peptaibiotics in nature, and suggests that this peptide may be a pathogenic factor in human and other mammal opportunistic infections by *T. longibrachiatum*.

5. Conclusions

i) The membrane-active peptaibiotic Tric from the opportunistic mold *T. longibrachiatum* is a wide-spectrum anti-human cytotoxin, acting at concentrations lacking any antibacterial activity; our results suggest that this molecule might be a pathogenic/virulence factor in human opportunistic infections by *T. longibrachiatum*.

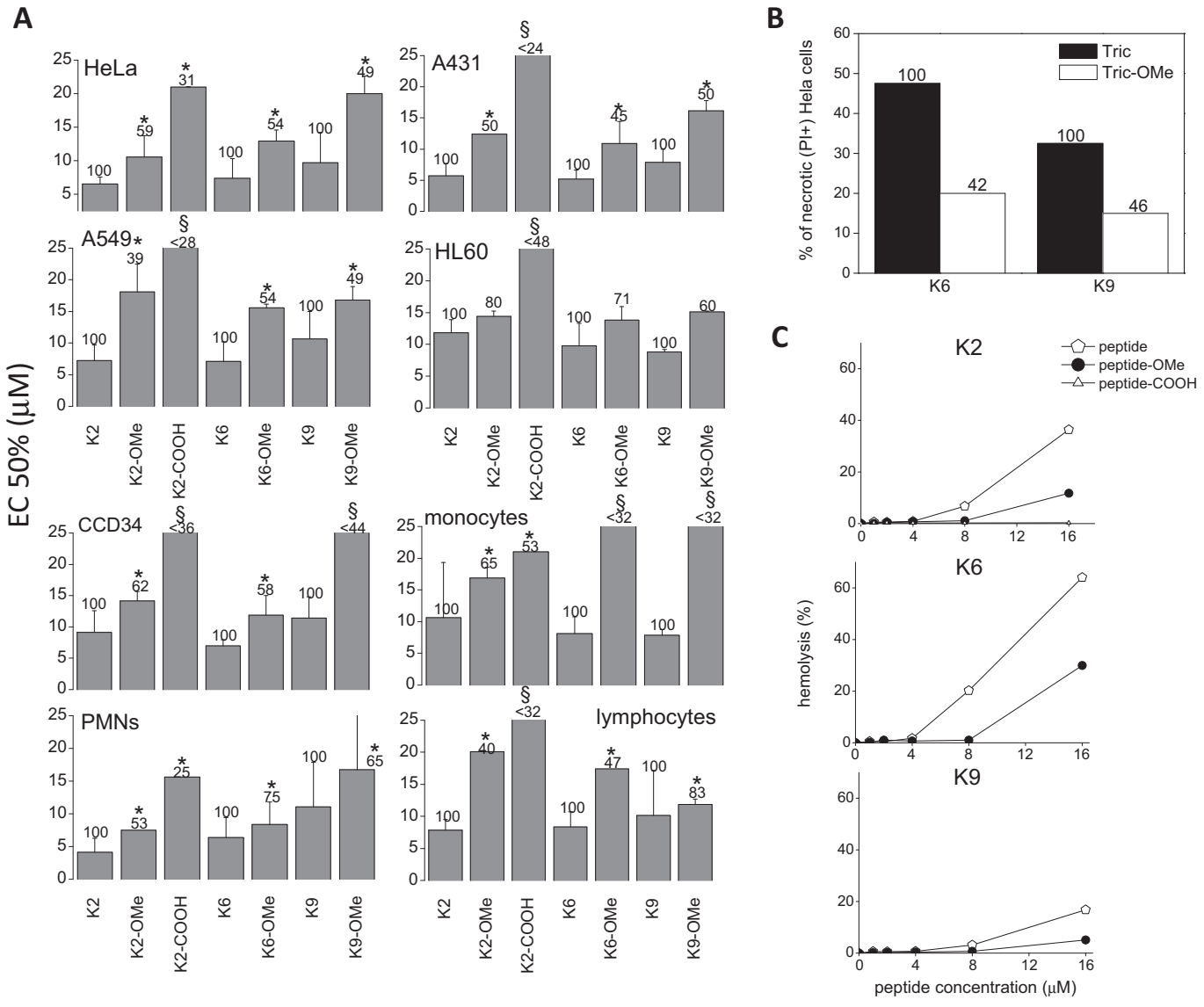

Fig. 7. The effects of K-substituted Tric analogs towards human cells. A) Cells were treated as previously described and EC_{50} values (means ± SD of four experiments run in duplicate) were calculated as explained in the Materials and methods section. Numbers above histograms indicate the percentage of cytotoxic activity with respect to the relative K-substituted analog, not modified at the C-terminus, whose activity was fixed to 100%. *<0.05 with respect to K-substituted analog-treated cells; § indicates no activity up to 25 μM. B) HeLa cells, treated for 24 h with 20 μM of K6- or K9-Tric, were stained with AnnexinV-FITC and PI and subsequently subjected to FACS analysis. Results were expressed as percentage of PI^+ (necrotic) cells with respect to the total number of cells acquired by FACS. Numbers above histograms indicate the percentage of necrotic cells after the treatment with the methyl ester K-substituted analog with respect to the equivalent K-analog, whose activity was fixed to 100%. C) Induction of hemolysis by different K-substituted analogs after 2 h incubation with fresh red blood cells; results were expressed as percentage with respect to total hemolysis induced by water. Data are the means ± SD of three experiments run in duplicate. D) Summary of the modulation of cytotoxic action on human cells and of the antibacterial efficacy of Tric and of Tric analogs modified at the level of the peptide helix by Lys substitution (K-Tric) and at the level of the C-terminus (−OMe or −COOH).

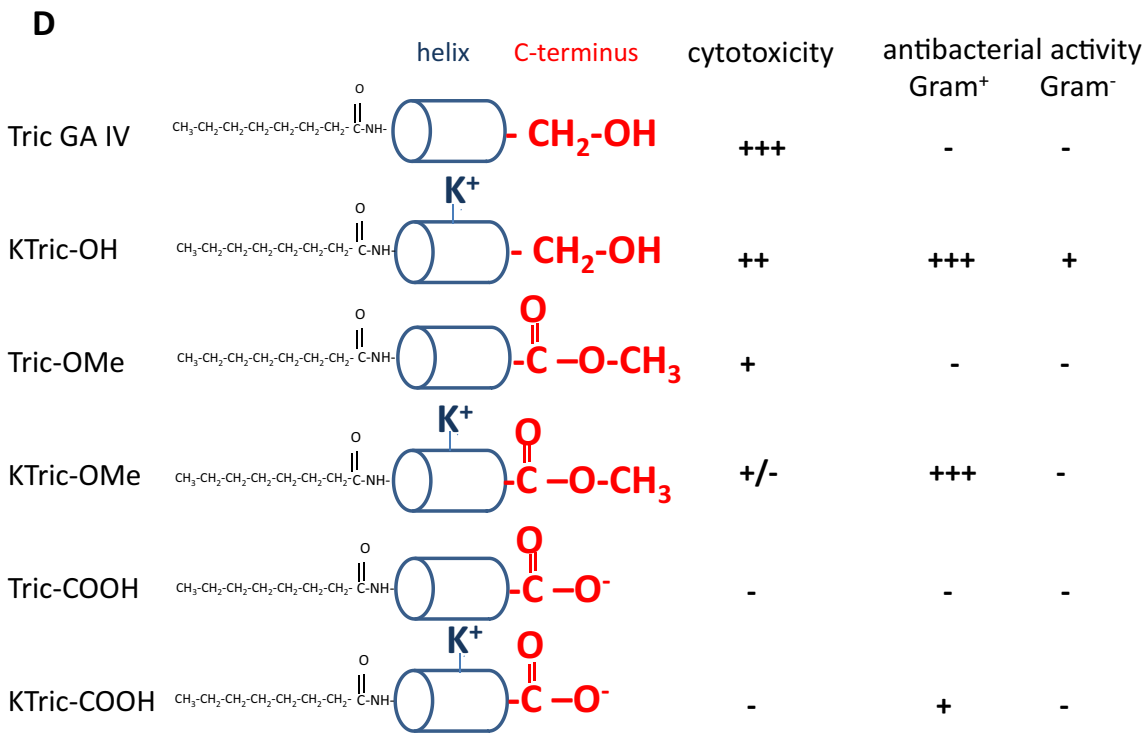

Fig. 7 (*continued*).

ii) Although the membrane damage activity of Tric essentially requires the N-terminal *n*Oct in both model and cellular membranes, the C-terminal $-CH_2OH$ group is also found to be necessary for efficient membrane association in a temperature dependent fashion (present at 37 °C), followed by endocytosis and killing of human cells.

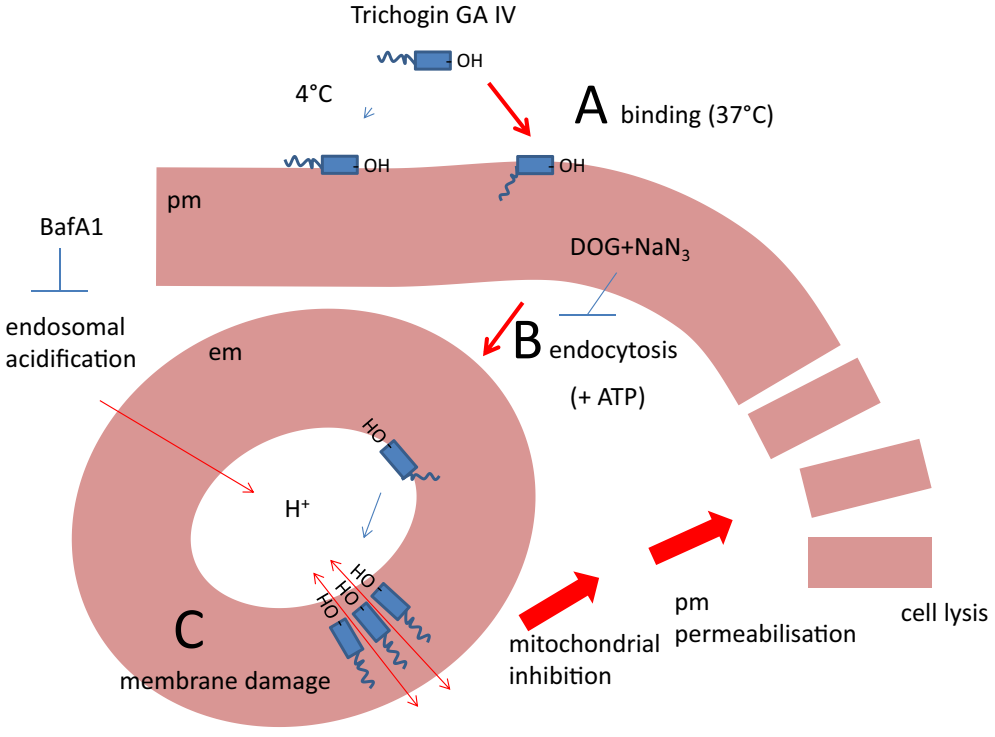

Fig. 8. Model of human cellular killing by Tric. The temperature dependent trichogin binding to the cell plasma membrane (step A) requires both the N-terminal Octyl and the C-terminal primary alcohol, and is followed by its endocytosis (step B). Prevention of endocytosis by energy depletion does not affect the plasma membrane binding but protects cells from the Tric cytotoxic effects. After endocytosis, it is proposed that Tric can reach the critic density allowing the formation of aggregates able to damage endosomal/lysosomal membranes (step C), a phase that requires the acidic condition in the lumen of these compartments and that also requires both the *n*Octyl and the primary alcohol. Plasma membrane damage is indirect with permeation to PI and leak of larger cytosolic components, and eventually cellular lysis follows the intracellular action of Tric and is preceded by mitochondrial activity inhibition, similarly to necrotic cell death.

iii) The internalization in acidic endosomes is required for the induction of a human cell lytic death by Tric.
iv) Taking advantage from this information, we demonstrated that the improvement of the cationic and amphipathic feature of the Tric backbone (by Lys insertion) together with the $-CH_2OH$ replacement (by COOMe) strongly boosts its scanty anti-Gram$^+$ activity, mitigating at the same time adverse cytotoxic effects on human cells.
v) Thus, the remarkably tunable bioactivity of Tric may open the way to a new generation of potentially interesting antibiotic and, possibly, antitumor agents.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.10.005.

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