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Iterative Design and in Vivo Evaluation of an Oncolytic Antilymphoma Peptide

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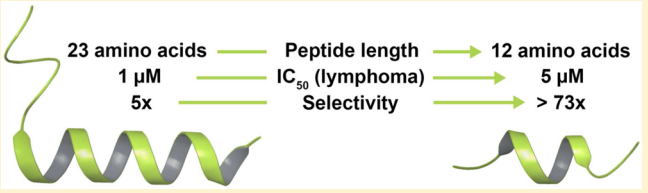
Supporting Information

ABSTRACT: Oncolytic peptides represent a promising new strategy within the field of cancer immunotherapy. Here we describe the systematic design and evaluation of short antilymphoma peptides within this paradigm. The peptides were tested in vitro and in vivo to identify a lead compound for further evaluation as novel oncolytic immunotherapeutic. In vitro tests revealed peptides with high activity against several lymphoma types and low cytotoxicity toward normal cells. Treated lymphoma cells exhibited a reduced mitochondrial membrane potential that resulted in an irreversible disintegration of their plasma membranes. No caspase activation or ultrastructural features of apoptotic cell death were observed. One of these peptides, **11**, was shown to induce complete tumor regression and protective immunity following intralesional treatment of murine A20 B-lymphomas. Due to its selectivity for lymphoma cells and its ability to induce tumor-specific immune responses, **11** has the potential to be used in intralesional treatment of accessible lymphoma tumors.



INTRODUCTION

In 2015, the number of United States citizens living with, or in remission from, lymphoma was above 760,000, making lymphoma the sixth most common cancer in the country.[1−3] Among adolescents (15−19 years), lymphoma is the most prevalent form of cancer accounting for 23% of all cases.[3] The two main types of lymphomas, Hodgkin's (HL) and non-Hodgkin's (NHL), both show a strong correlation with age. NHL is generally associated with older patients, whereas HL is mostly diagnosed in young adults.[2] Owing to modern treatments (chemotherapy, radiation, or a combination of both), the prognosis for lymphoma has greatly improved in the last years. However, several side effects are still associated with treatment, many of which are long-term and serious (e.g., formation of second cancers, infertility, etc.). Although many of these side effects, for example, the loss of fertility, might be an acceptable trade-off for older patients, they have a severe impact on the life of younger patients. Efforts are, therefore, ongoing to develop new drugs and treatment strategies that will improve the long-term prognosis for patients even further. Of these new strategies, immunotherapy is arguably the most promising. The anti-CD20 monoclonal antibody, rituximab, is currently the standard of care for all B-cell lymphomas.[4]

It has been shown that certain antimicrobial peptides can also have an anticancer effect.[5,6] During the past decade, our group has been developing a novel strategy for the intralesional treatment of solid tumors using α-helical cationic amphipathic peptides (CAPs).[7−11] The rationale underlying this strategy is the exploitation of immunogenic cell death (ICD).[12] By treating a tumor with an ICD-inducing peptide, an extended number of tumor-specific antigens and danger signals are released, resulting in a subsequent tumor-specific immune response (i.e., in situ autologous tumor cell vaccination). We have previously demonstrated that intralesional treatment of solid tumors with CAPs can lead to ICD, complete tumor regression with abscopal effects, and the initiation of a protective immune response.[13−15] Recent studies have also shown strong synergic effects when L-lysyl-L-lysyl-L-tryptophanyl-L-tryptophanyl-L-lysyl-L-lysyl-L tryptophanyl-β,β-diphenyl-L-alanyl-L-lysinamide (LTX-315, **12**) is used in combination with immune checkpoint inhibitors.[16]

The oncolytic peptide approach has two important advantages over immunotherapeutic strategies currently being applied in the clinic. First, the success of the treatment is not dependent on a single antigenic marker, as is the case with anticancer monoclonal antibodies. Second, it is believed that the rapid destruction of the cell membranes would prevent the development of drug resistance.

However, the treatment of lymphomas with CAPs may be more challenging than with other types of solid tumors. Whereas most solid tumors present a well-defined target, lymphomas are often more diffused in character and contain large numbers of normal lymphocytes. CAPs with limited selectivity could therefore lead to unacceptable levels of collateral damage to normal lymphocytes, other immune cells,

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as well as the surrounding healthy tissue. Oncolytic peptides with improved selectivity are therefore required.

There are two potential targets that can be used to selectively increase the interaction between cancer cells and cationic peptides. First, cancer cell membranes are often enriched with negatively charged macromolecules due to the overexpression of, among others, phosphatidyl serine[17] and proteoglycans.[18,19] This increase in anionic character makes cancer cells more amenable to electrostatic interactions with positively charged CAPs than their noncancerous counterparts. The initial electrostatic interaction is followed by membrane disruption (various models have been proposed)[20] and ultimately the semiselective lysis of the cancer cells.

Second, the mitochondrion has long been considered as a promising therapeutic target.[21] Cancer mitochondria are often hyperpolarized[22] and as such are electrostatically favorable targets for cationic compounds.[23] Further, having a similar composition as prokaryotes,[24] the mitochondrial membrane has been shown to be sensitive to antimicrobial peptides (AMPs).[25,26] This led to the hypothesis that cationic AMPs, if transported across the cell membrane, could induce cancer cell death by means of mitochondrial membrane disruption.

The overall aim of this study was to generate cationic ICD-inducing peptides with high selectivity for lymphoma cells versus normal cells. To achieve this, the peptides were designed to simultaneously target overexpressed extracellular proteoglycans and hyperpolarized mitochondria.

Using a previously published proapoptotic mitochondrial targeting chimeric peptide[27] as a starting and reference point, several novel peptides were designed, synthesized, and tested for activity in vitro and in vivo.

RESULTS

Chimeric Proapoptotic Peptides Exhibited Activity against Lymphoma B Cells. In the study of Law et al.,[27] a chimeric peptide composed of a 14-mer model α-helical AMP,[28] linked to a short 7-mer arginine cell penetration peptide (CPP) (Figure 1) exhibited activity toward cancer cells

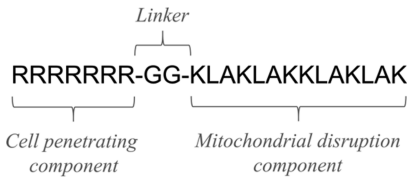

Figure 1. A proapoptotic peptide consisting of an AMP linked to a CPP with an amidated C-terminus. After crossing the cell membrane, the AMP will disrupt mitochondrial membranes resulting in cell death.

in vitro. When this peptide (designated as **1a** in the present study) was synthesized and tested against A20 lymphoma B cells, high activity (IC_{50} = 1 μM) was observed. However, consisting of 23 amino acids, **1a** is a relatively long peptide. Thus, **1a** was considered ideal as a starting point for creating new, shorter, and more selective antilymphoma peptides.

The Sequential Position of the AMP Component Was Not Critical for Activity. For reasons explained below, it was decided to investigate if the position of the AMP component in the chimeric peptide is important. An analogue of **1a** (designated **1b**) with the AMP positioned at the N-terminus, instead of the C-terminus, was synthesized and tested. Only a marginal reduction in activity was observed with an IC_{50} value of 2 μM. This indicated that the AMP component could be placed either at the C- or N-terminus of a chimeric antilymphoma peptide, with a negligible penalty in activity.

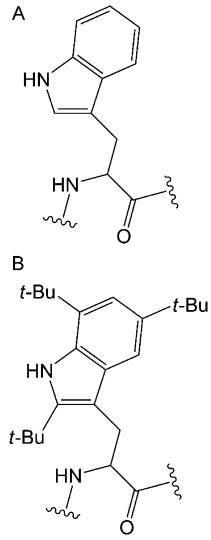

Figure 2. (A) Structure of normal tryptophan. (B) Tbt is an ultrahydrophobic tryptophan analogue containing three tertiary butyl (*t*Bu) groups on the indole ring.

Only a Very Short AMP Component Is Needed for Antilymphoma Activity. A key design objective was to determine the minimum number of amino acids critical for activity. To achieve this, all redundant elements in the reference peptide had to be identified and removed. As a first step, the AMP part of **1b** was replaced with a series of short AMP sequences consisting of alternating tryptophan and arginine residues (1−6 amino acids) resulting in peptides **2**−**7**, see Figure 3. Positioned at the N-terminus, the AMP component gains an additional charge which might be critical in the case of very short AMPs.[29]

A surprisingly large difference in activity (ΔIC_{50} = 58 μM) was obtained between peptides **2** (10-mer) and **4a** (12-mer) suggesting that a critical level of hydrophobicity and charge is necessary for high activity (Figure 3). Interestingly, in a previous study,[29] the AMP component of **4a** (i.e., WRW) was found to encompass the minimum requirements for activity against *S. aureus*. In the same study, it was also shown that the weak antimicrobial activity of WRW could be improved by increasing its hydrophobicity through benzylation. The relationship between hydrophobicity and antilymphoma activity was therefore further explored with hydrophobic analogues of **4a**. When one or both tryptophans of **4a** were replaced with the ultrahydrophobic β-(2,5,7-tri*tert*-butylindol-3-yl)alanine (Tbt) amino acid,[30] see Figure 2, (**4a** → **4b** → **4c**), only a small increase in activity (ΔIC_{50} = 2−3 μM) was seen. It thus appears that a minimum level of hydrophobicity is required for antilymphoma activity. However, any increase in hydrophobicity beyond two tryptophans had little effect on activity.

The incorporation of further amino acids had only minor effects on activity. A third tryptophan (**5** → **6a**) or tenth arginine (**6a** → **7**) resulted in insignificant increases in potency. Thus, with the further elongation of the AMP component unlikely to yield significant improvement in activity, the upper limits for the relationship between antilymphoma activity and hydrophobicity/charge were considered established.

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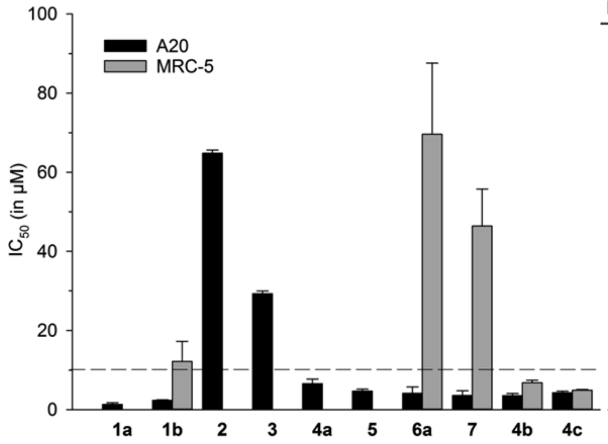
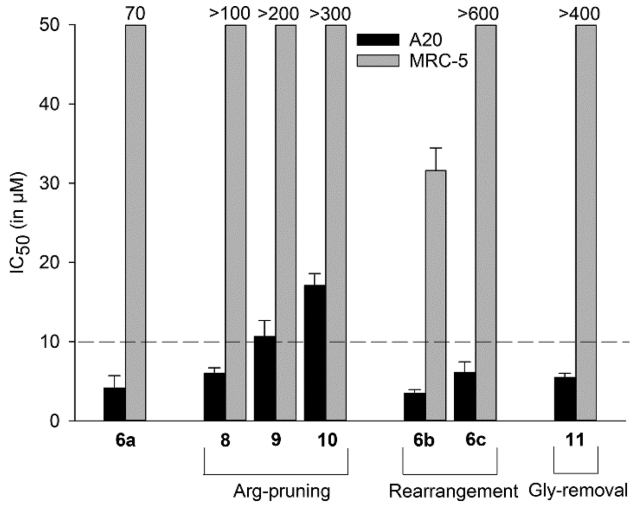


Peptide	Sequence	#AA	IC_{50} (μM) A20	MRC-5	SI-1
1a	RRRRRRR-GG-KLAKLAKKLAKLAK	23	1±0	n.d.	n.d.
1b	KLAKLAKKLAKLAK-GG-RRRRRRR	23	2±0	12±5	5
2	W-GG-RRRRRRR	10	65±1	n.d.	n.d.
3	RW-GG-RRRRRRR	11	29±1	n.d.	n.d.
4a	WRW-GG-RRRRRRR	12	7±1	n.d.	n.d.
5	RWRW-GG-RRRRRRR	13	5±0	n.d.	n.d.
6a	WRWRW-GG-RRRRRRR	14	4±2	70±18	17
7	RWRWRW-GG-RRRRRRR	15	4±1	46±9	12
4b	WR(Tbt)-GG-RRRRRRR	12	4±1	7±1	2
4c	(Tbt)R(Tbt)-GG-RRRRRRR	12	4±0	5±0	1

Figure 3. First activity/selectivity screen with chimeric peptides against A20 cells. All peptides were prepared as C-terminal amides. Five peptides with high activity were selected for testing against MRC-5 cells to determine selectivity. The IC_{50} values (with standard deviation error bars) shown are averages of three independent cell viability experiments (MTT assay) done in triplicate. The dashed line at 10 μM represents the arbitrary boundary between high and moderate cytotoxicity. (#AA = number of amino acids; SI-1 = IC_{50}A20/IC_{50}MRC-5; n.d. = not determined.)



Peptide	Sequence	#AA	IC_{50} (μM) A20	MRC-5	SI-1
6a	WRWRW-GG-RRRRRRR	14	4±2	70±18	17
8	WRWRW-GG-RRRRRR	13	6±1	124±16	21
9	WRWRW-GG-RRRRR	12	11±2	285±85	23
10	WRWRW-GG-RRRR	11	17±2	388±43	>23
6b	WWW-GG-RRRRRRRRR	14	3±0	32±3	9
6c	WRRRGRRWRGRRRW	14	6±1	618±0	>100
11	WRRRRRWRRRRW	12	5±1	502±82	>73

Figure 4. Second activity/selectivity screen for analogues of **6a** against A20 lymphoma and MRC-5 fibroblast cells. Three rounds of modifications were done to shorten the peptide and increase selectivity without decreasing antilymphoma activity. All peptides were prepared as C-terminal amides. The IC_{50} values (with standard deviation error bars) shown are an average of three independent cell viability experiments (MTT assay) performed in triplicate. The dashed line at 10 μM represents the arbitrary boundary between high and moderate cytotoxicity. (#AA = number of amino acids; SI-1 = IC_{50}A20/IC_{50}MRC-5.)

Tryptophan-Containing Chimeric Antilymphoma Peptides Showed Selectivity for Lymphoma Cells. A second design objective was to investigate whether high (i.e., IC_{50} < 10 μM), but still selective, cytotoxicity against lymphoma cells could be obtained. In order to quantify and compare the selective cytotoxicity of the different peptides, two different selectivity indexes (SI-1 and SI-2) were used. For the initial screening process, SI-1 values were used to estimate a peptide's selectivity for lymphoma cells. The SI-1 value was defined as the ratio between a peptide's IC_{50} values against normal human fibroblasts (MRC-5) and A20 lymphoma cells (i.e., SI-1 = IC_{50} MRC-5/IC_{50} A20).

Five of the most active peptides (i.e., **1b**, **4b**, **4c**, **6a**, and **7**) were selected from the A20 lymphoma screening round, and their SI-1 values determined. The results indicated that the AMP component of the chimeric peptide plays an important role in selectivity. The Tbt-containing peptides, **4b** and **4c**, exhibited almost no selectivity at all, while the 14-mer model AMP peptide, **1b**, displayed low selectivity. In contrast, both tryptophan-containing peptides, **6a** and **7**, showed high selectivity with SI-1 values higher than 10. Based on the criteria of peptide length and high activity and selectivity, **6a** was selected for further study, since it is one amino acid shorter and has a better SI-1 than peptide **7**.

Covalent Linkage Between the AMP and CPP Components Was Essential for Activity. To confirm that the antilymphoma activity was not due only to a single component of the chimeric peptide **6a**, its AMP and CPP components, WRWRW and RRRRRRR, were tested against A20 cells both separately and together in an equimolar mixture. Neither of the two components, alone or in combination, did induce any effects against the A20 lymphoma cells (results not shown). This result also demonstrates that the trifluoroacetic acid (TFA) content (≥30%) of the lyophilized peptides (see Table S1 in Supporting Information) was not responsible for activity.

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Removing Arginines from the CPP Component Resulted in Lower Activity. Next, it was attempted to shorten **6a** further by pruning arginines from the C-terminus. Since arginines do not need to be in an uninterrupted sequence for efficient membrane transduction,[31] it was believed that the two arginines from the AMP component of **6a** could contribute to membrane transduction.

A steady decrease in activity against both A20 and MRC-5 cells was seen for each arginine being removed in peptides **8**–**10** (Figure 4). Taking our design criteria into consideration, this strategy does not represent a real improvement in respect to **6a**. With an unchanged SI-1 and decreased activity, any gains made in length reduction were lost. These results indicate that nine arginines are the optimum for effective membrane interaction with lymphoma cells. Similar results have been obtained for CPPs, where the number of arginines had a direct influence on membrane translocation efficiency.[32−34]

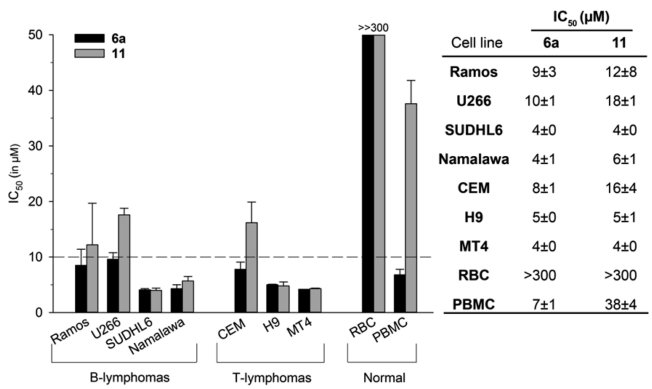
Arginine and Tryptophan Distribution Influenced Selectivity. In a second attempt to improve on **6a**, the role of amino acid sequence was explored. Two analogues where the hydrophobic tryptophans and cationic arginines were clustered (**6b**) or spread across the peptide (**6c**) were synthesized and tested (Figure 4). As the IC_{50} values against A20 cells were not affected significantly by these rearrangements, it appears unlikely that a highly structured conformation is required for the activity of these peptides. In contrast, lytic peptides specifically designed to have α-helical conformations often lose their ability to disrupt membranes when their sequences are changed.[10]

The rearrangements, however, had a profound effect on cytotoxicity against MRC-5 cells. The SI-1 value for **6b** was markedly lower than for **6a**, whereas a sharp increase in SI-1 was seen for **6c**. These results show that although toxicity against normal fibroblasts is sequence dependent, the activity against A20 B-lymphoma is not.

The Glycine Linker Between the AMP and CPP Components Was Redundant. It is reasonable to deduce from the rearrangement results that the antilymphoma activity observed was not based on a mitochondrial disruption component (i.e., AMP) transported by a cell penetration component (i.e., CPP) to its target. Rather, it seemed to be the overall hydrophobicity and cationic charge of the peptide that underlay cytotoxicity. This further indicated that the two glycines used to link the AMP and CPP components were redundant. The removal of the two glycines (**6c** → **11**) resulted in a slight increase in activity and slight reduction in SI-1. Despite containing an equal number of tryptophans and arginines, peptides **6a** and **11** represent two different designs. Peptide **6a** is based on an AMP-CPP construct, while **11** does not consist of components with presupposed functions. Thus, in order to ascertain which design is best in terms of activity across a broad range of different lymphoma types, both peptides were studied further.

Activity against Various B- and T-Lymphoma Cell Lines. Next, **6a** and **11** were tested against a panel of human lymphoma cell lines, see Figure 5. Including the murine A20 cell line, used in the preceding screening rounds, a total of eight lymphoma cell lines were tested.

Both peptides showed high activity against the majority of the B- and T-lymphoma cell lines tested. Peptide **6a** exhibited high activity (IC_{50} < 10 μM) against all eight cell lines tested. Similar results were observed for **11**, with the exception of the U266 and CEM cell lines, which were slightly less sensitive



Cell line	IC_{50} (μM) 6a	11
Ramos	9±3	12±8
U266	10±1	18±1
SUDHL6	4±0	4±0
Namalawa	4±1	6±1
CEM	8±1	16±4
H9	5±0	5±1
MT4	4±0	4±0
RBC	>300	>300
PBMC	7±1	38±4

Figure 5. Panel screen against several B- and T-lymphoma cell lines and normal blood cells (RBCs and PBMCs) to determine overall activity and selectivity of peptide **6a** and **11**. The IC_{50} values (with standard deviation error bars) shown are an average of three independent cell viability experiments (MTT assay) performed in triplicate, except for RBC and PBMC (two independent experiments). The dashed line at 10 μM represents the arbitrary boundary between high and moderate cytotoxicity.

toward this peptide. Importantly, both peptides exhibited high activity against the most common types of B-lymphoma, diffuse large B-cell (SU-DHL-6), and Burkitt's lymphoma (Ramos and Namalawa). When tested against the three T-cell lymphoma lines, both **6a** and **11** displayed high activity against the H9 and MT4 cell lines (IC_{50} values <5 μM).

Peptide 11 Has Low Cytotoxicity against Normal Blood Cells. The design of new antilymphoma peptides was based on effects against the A20 B-lymphoma cell line and normal skin fibroblasts. The effect on the fibroblast also gave an indication on the potential of unwanted cytotoxicity toward the healthy tissue surrounding the tumor. However, antilymphoma peptides should also be less cytotoxic to normal lymphocytes. Since the isolation of B- and T-lymphocytes was deemed too costly and labor intensive for screening purposes, **6a** and **11** were tested against peripheral blood mononuclear cells (PBMCs) instead. In addition to B- and T-lymphocytes, PBMCs consist of several other types of immune cells (i.e., NK cells, monocytes, and macrophages). Hence, an advantage of testing the peptides against PBMCs is that a broader indication of unwanted toxicity against normal cells could be obtained. By comparing IC_{50} values obtained from the different lymphoma cell lines and PBMC screening, a second selectivity index, SI-2, was calculated (i.e., SI-2 = IC_{50} PBMC/IC_{50} lymphoma cell line).

Peptide **6a** exhibited similar toxicity against the PBMCs (IC_{50} = 6.8 μM) than to the other human lymphoma tested, and therefore almost no selectivity (SI-2 < 2). In contrast, peptide **11** displayed lower toxicity against the healthy blood cells (IC_{50} = 37.6 μM), resulting in a SI-2 values ranging from 2 to7 when compared to the different lymphoma cell lines.

When tested against red blood cells (RBCs), neither of the peptides induced significant hemolysis. In both cases, a IC_{50} value could not be determined as only 2.3% hemolysis was seen for **6a**, at the maximum concentration (309 μM) tested. For **11**, only 1.1% hemolysis was seen at maximum concentration (320 μM).

Low Activity toward Nonlymphoid Cancer Cells. To determine whether the observed cytotoxic activity was restricted to lymphoma cells, peptides **6a** and **11** were tested against several other cancer cell lines of different origins (Figure

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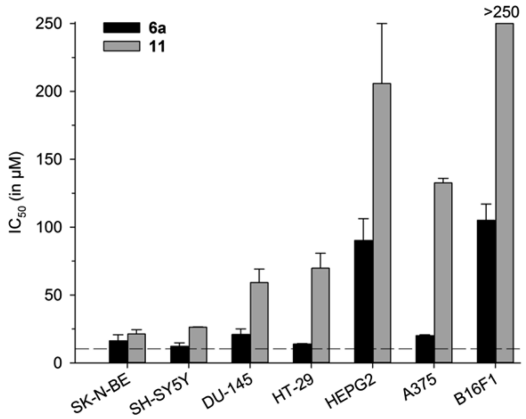

Cell line	IC_{50} (μM) 6a	11
SK-N-BE	16±6	21±4
SH-SY5Y	12±4	26±0
DU-145	20±5	59±14
HT-29	14±0	70±16
HEPG2	90±23	206±63
A375	20±1	133±5
B16F1	105±17	>250

Figure 6. Panel screen against several nonlymphoma cell lines. The peptides were tested against neuroblastoma (SK-N-BE and SH-SY5Y), prostate carcinoma (DU-145), colon carcinoma (HT-29), liver cancer (HEPG2), and melanoma (A375 and B16F1). The IC_{50} values (with standard deviation error bars) shown are an average of at least two independent cell viability experiments (MTT assay) performed in triplicate. The dashed line at 10 μM represents the arbitrary boundary between high and moderate cytotoxicity.

6). When comparing these IC_{50} values with those of Figure 5, it appears that **6a** and especially **11** are less active against nonlymphoid cancer types. In fact, **11** had low activity (i.e., IC_{50} > 25 μM) against the majority of cell lines and moderate activity (i.e., 10 μM < IC_{50} < 25 μM) only against the neuroblastoma cell lines. Peptide **6a** exhibited moderate activity against most of the cell lines tested. It thus appears that the penalty for **11**'s high selectivity might be that its activity is restricted to lymphoma cells. On the other hand, although less selective, **6a** seems to have cytotoxic activity against a broader range of cancer types. However, a more comprehensive panel screen is needed to make a more accurate assessment.

The Peptides Reduced the Mitochondrial Membrane Potential. Next, the mechanism by which **6a** and **11** exert their cytotoxic effect was investigated. Mitochondrial disruption and caspase-dependent apoptosis were the originally intended mechanisms of action, and to assess if these goals were met, changes to mitochondrial membrane potential ($\Delta\Psi_m$) as well as changes to the composition and integrity of cell membranes were examined by using flow cytometry assays.

The A20 cells were treated with peptides **6a** and **11** (at IC_{50} concentrations) and the $\Delta\Psi_m$ measured at 60, 120, 180, and 240 min using tetramethylrhodamine ethylester perchlorate (TMRE) as dye. Both peptides induced a decrease in the membrane potential. The changes were induced earlier by **6a** than **11**, but after 4 h of treatment, the effect on $\Delta\Psi_m$ was the same for both peptides (Figure 7A). It is important, though, to note that this reduction in membrane potential does not necessarily mean that the peptide had a direct effect on the mitochondrial membrane.

Caspase-Independent Cell Death Characterized by Necrosis. To test for apoptosis and necrosis, the amount of phosphatidylserine (PS) translocated to the outer cell membrane and cell membrane integrity was simultaneously quantified by dual labeling with annexinV-FITC and propidium iodide (PI). The A20 cells were treated with the peptides for 1 h. Although a significant number of the affected cells stained positive for PS, a majority of the cells (≈ 60%) showed signs of disrupted cell membranes (Figure 6B). However, it is not possible to deduce from these results whether cell membrane disruption was due to primary or secondary necrosis.

Caspases are proteases that play essential roles in apoptosis (programmed cell death). To explore whether apoptosis was induced, the treated cells were screened for activation of caspases using fluorescently labeled substrates. Neither initiator nor effector caspases were activated, as shown in Figure 7C.

Next, the effect of **6a** on A20 cells was investigated using electron microscopy. Micrographs were taken at different time points and peptide concentrations. No evidence supporting classical apoptosis (e.g., membrane blebbing, condensed chromatin, disrupted mitochondria, etc.) was seen, thereby confirming the results from the caspase screen. Instead, signs of extensive necrosis were observed with transmission electron microscopy (TEM) (Figure 8B−E). Despite observing discontinuities in the outer membrane of mitochondria, in the majority of cases, the mitochondria of affected cells were still intact (Figure 8F). This suggests that the peptide did not disrupt the mitochondrial membrane directly, but that mitochondrial membrane disruption was a result of a preceding event that caused cellular stress, which then affected mitochondrial function and morphology.

The scanning electron microscopy (SEM) analysis revealed images of affected cells with massive amounts of intracellular material extruding from the cells (Figure 8I,J). This effect was observed over a wide concentration range (1−10× IC_{50}), suggesting that the causative event is not restricted to a specific peptide:cell ratio. However, although the final morphological outcome was the same, a quicker onset of the effect was observed with higher peptide concentrations. For example, the first signs of intracellular material extruded from the cells were observed at 90 min for 1× IC_{50}, at 45 min for 2.5× IC_{50}, and 15 min at 10× IC_{50} (data not shown).

The question that arises is whether the observed necrosis is due to direct disruption of the cell membrane or whether a lethal intracellular event preceded cell lysis.

Intralesional Treatment Resulted in Complete Regression of Lymphoma. Next, the ability of the peptides to induce an anticancer effect in vivo was assessed using the well-established murine A20 B-lymphoma model. Mice were inoculated with A20 lymphoma cells, and when tumor size was sufficient, peptide treatment commenced. The mice were injected either with saline (Group 1) or a peptide solution of **6a** (Group 2) or **11** (Group 3). A significant tumor growth inhibition effect, increased median survival time (MST), and overall survival (OS) in all animals from Groups 2 and 3 were seen when compared with the control animals. At the end of

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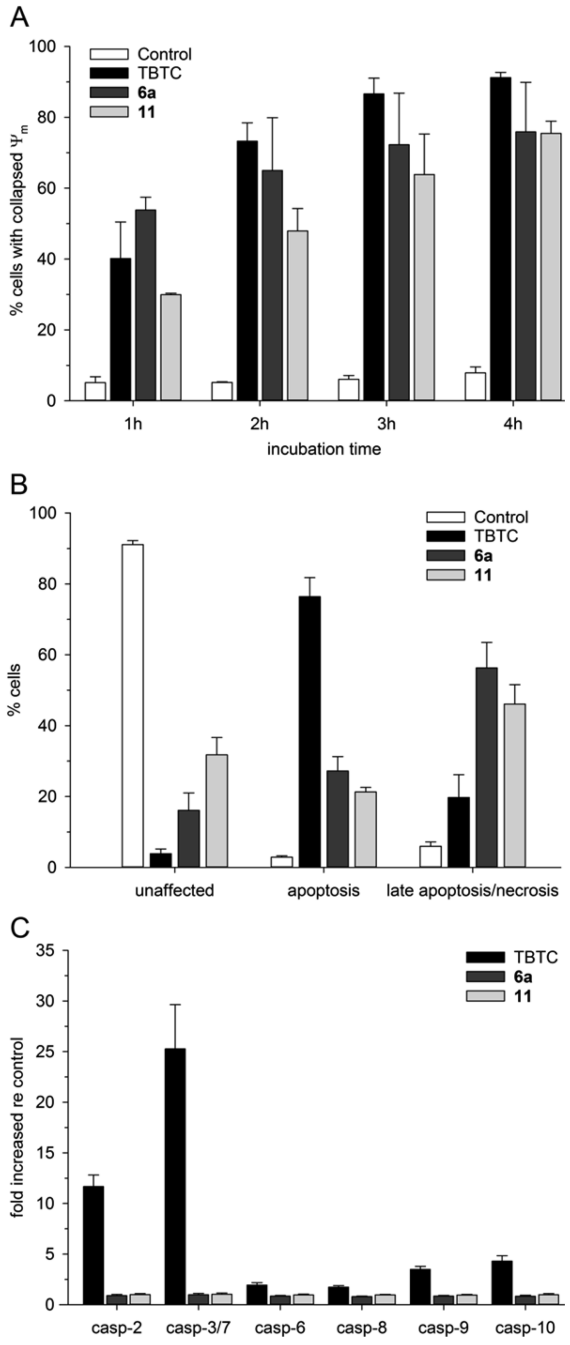

Figure 7. Mechanism of action studies for **6a** and **11**. A20 cells were treated with peptides (at IC_{50} concentration) and TBTC used as positive control. (A) The percentage of cells with collapsed mitochondrial membrane potential (Ψ_m) following peptide treatment using the mitochondrial dye TMRE. (B) The presence of translocated phosphatidylserine and membrane integrity investigated by staining with annexinV-FITC and PI. (C) Screening for the activation of caspase using fluorescently labeled substrates.

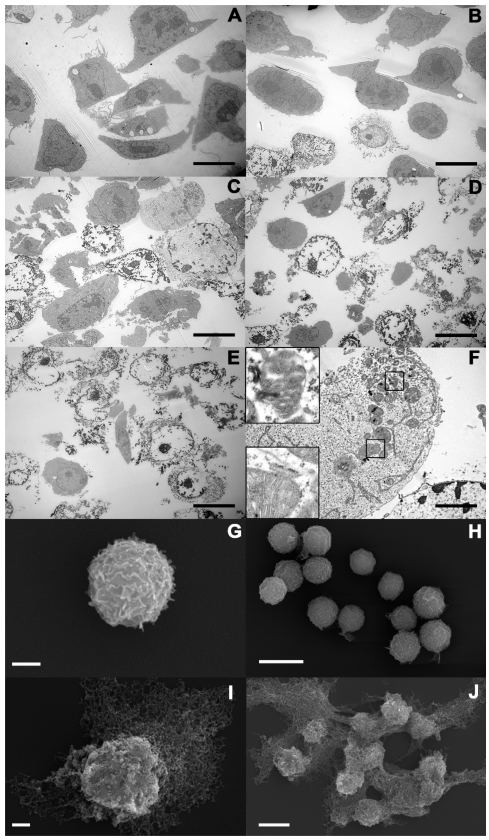

Figure 8. Representative electron micrographs of lymphoma cells treated with **6a**. (A) TEM micrographs of untreated Ramos cells after 4 h incubation. (B–E) Time study conducted with Ramos cells treated with **6a** at a concentration of 2× IC_{50} for 30 min (B), 60 min (C), 120 min (D), and 240 min (E). (F) Ultrastructures of a Ramos cell treated with **6a** for 60 min showing intact mitochondria and a disintegrated cell membrane. Inserts showing affected mitochondria. (G and H) SEM micrographs of untreated A20 cells after 90 min incubation. (I and J) Treatment of A20 cells with **6a** resulted in similar morphological effects over a broad concentration range using 1× IC_{50} for 90 min (I) and 10× IC_{50} for 45 min (J). (Magnification is 3000× for A–F, 7000× for G, 2200× for H, 4500× for I, and 1500× for J; scale bar represents 10 μm in A–E, H, and J; 2 μm in F, G, and I).

study (55 days), complete tumor regression was obtained in the majority of mice treated with **11** (OS = 67%) and in half of the mice treated with **6a** (MST = 48 days; OS = 50%), see Figure 9A. In contrast, tumor growth in the mice of Group 1 was uninhibited, and all animals were euthanized within 35 days (MST = 22 days).

Peptide Treatment Induced a Protective Immune Response. To assess whether peptide treatment has resulted in a tumor-specific immune response, the cured mice were rechallenged with A20 lymphoma cells 4 weeks after the completion of the peptide treatment study (Figure 9B). The mice previously cured by **6a** and **11** as well as a control group of naive mice were inoculated with viable A20 lymphoma cells.

Within 10 days after the tumor rechallenge, five of the six **11** cured mice developed measurable tumors. The tumor growth, however, was significantly inhibited compared to tumor growth in the control animals. Eventually the tumors in the majority of mice receded before going into complete regression until the end of the study of 55 days (OS = 83%).

Similar results were initially observed for the animals cured with **6a**, and 10 days after tumor rechallenge, three out of the four animals developed tumors. However, only one mouse in this group remained tumor free until the end of the study (MST = 47 days, OS = 25%). The naive mice quickly developed tumors. and within 18 days all were euthanized (MST = 15 days).

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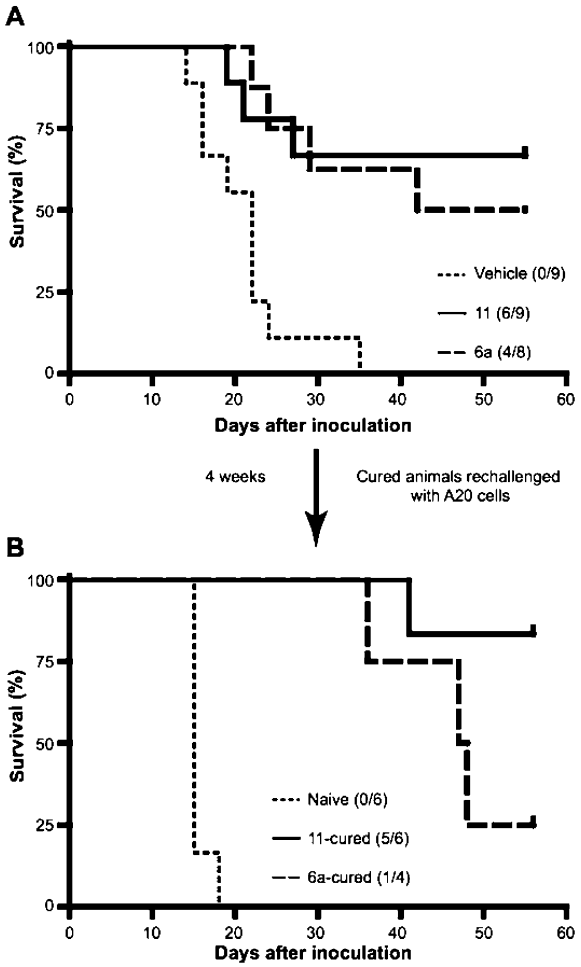

Figure 9. In vivo studies showing the effect of **6a** and **11** after intralesional treatment of A20 lymphoma tumors in mice. The mice were treated with intralesional injections solution (50 μL of a 25 mg/mL solution) once a day for 3 consecutive days. (A) Peptide treatment study showing the direct antitumor effect of the peptides. (B) The rechallenge study showing a protective immune response. Cured animals were rechallenged with A20 cells 4 weeks after completion of the preceding peptide treatment study. All survival curves were compared by the log-rank test.

These in vivo results clearly indicate that, in terms of primary antitumor and protective immune effects, **11** is the most effective of the two peptides tested.

DISCUSSION AND CONCLUSIONS

The aim of this study was to investigate the possibility of designing a short antilymphoma peptide with improved selectivity and the ability to induce ICD. Starting with a 23-mer reference peptide (**1a**), a process of AMP replacement, arginine pruning, and reshuffling resulted in peptide **11**. This peptide was significantly shorter than the initial reference peptide, displayed high activity and selectivity for lymphoma cells, and induced a protective immune response following intralesional treatment of lymphoma tumors.

The optimization process was guided by three design objectives that are related to expected therapeutic issues. Keeping peptide length to the minimum was the first objective. The rationale being that longer peptides are more likely to induce unwanted immunogenicity, and they have higher production costs. High activity against a wide range of different lymphoma types was our second objective. This was important since there are over 60 different lymphoma subtypes, and a peptide active against only one subtype will be considered clinically, and therefore commercially, less interesting. The third objective was to design a new generation of oncolytic peptides with improved selectivity for the lymphoma cells in relation to nonmalignant cells.

With peptide **11**, all three objectives were achieved, since it is (i) almost 50% shorter than the reference peptide, (ii) had high activity against most of the B- and T-cell lymphomas tested with IC_{50} values similar to other CAPs, and (iii) almost no activity was seen against normal fibroblasts, RBCs, as well as low activity against normal lymphoid cells.

Interestingly, activity was not due to the intended CPP-linker-AMP principle. Rather, activity was governed by the amount of charge and hydrophobicity of the peptide, while selectivity was determined by the arrangement of the hydrophobic tryptophan residues. These observations played a key role in the optimization process that eventually resulted in peptide **11**.

It is noteworthy that the amino acid sequence did not play a role in the cytotoxicity toward lymphoma cells. This is in contrast to peptides like Brevinin-2R, where the peptide's high activity was lost when its sequence was scrambled.[35] This result strongly indicates that activity is unlikely to be receptor-mediated, nor relying on a specific secondary structure, as was the case with the oncolytic peptide **12**.[36]

However, unlike activity, selectivity was found to be sequence dependent and was significantly increased when the tryptophans were dispersed. This observation correlates with a previous study with arginine- and tryptophan-rich CPPs.[37] The selectivity of **11** (SI-1 > 73) represents a significant improvement on our group's first-generation oncolytic peptides (SI-1 for LTX-302 $\approx$ 8 and **12** $\approx$ 4).[14,36]

The results from the lymphoma panel screen suggest that the role played in activity by the overexpressed proteoglycan, heparan sulfate (HS), was less important than initially anticipated. Activity was not entirely relying on HS overexpression, since no correlation between IC_{50} values and HS expression[19] of the lymphoma cell lines tested against could be found. These findings do not exclude HS as a facilitator for the peptides' mode of action but suggest that other anionic cell membrane components might also be involved.

The mechanistic basis for **11**'s selective cytotoxicity toward lymphoma cell lines remains unresolved. But, it is most likely due to an inherent property associated with transformed lymphoid cells. One possible explanation might be the fact that lymphoid cells are well endowed with microvilli. This results in an increased cell surface area, which could allow for a higher ratio of interaction between peptides and cells. Equally, intracellular differences (e.g., metabolic pathways) specific to lymphoid cells could be the basis for the cytotoxicity. It is clear, though, that the level of selectivity of **11** can only be understood once its mechanism of action is fully elucidated.

In the context of cationic anticancer peptides, caspase-dependent apoptosis or the direct lysis of the cell membrane (i.e., primary necrosis) is the most commonly observed mode of action. However, in this study, no evidence for caspase-dependent apoptosis was observed, and there is reason to believe that primary necrosis might not be the mode of action either. Although TEM clearly shows disrupted cell membranes, SEM micrographs are distinctly different from those seen in previous studies by our group (see Figure S1 in Supporting

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Information). In addition, simple predictive (i.e., molecular modeling) and comparative (i.e., grand average hydropathy calculations and idealized helical wheel projections) studies raise further doubts about the ability of **11** to directly disrupt membranes (see Figures S4 and S5 and Table S3 in Supporting Information). The preliminary bioinformatics suggest that peptide **11** might be closer related to cell penetration peptides than to membranolytic peptides. Nonetheless, structural and membrane disruption studies are needed to confirm either way.

An alternative, though much less common, mode of action for **11** is a type of programmed cell death independent of caspase activation. Several CAPs have been reported to cause cell death via secondary necrosis after interaction with an intracellular target. Even though the evidence pointing toward this is currently limited and circumstantial, there are several arguments that make this a conceivable hypothesis worth consideration.

First, the cell penetration capability of **11** was not empirically determined in this study, but it is not unreasonable to assume that it can cross the cell membrane. Both the TMRE assay and TEM images suggest that an intracellular target was affected during peptide treatment. Further, two very similar peptides, RW_{mix} and RW_9, consisting only of arginines and tryptophans have been shown to have excellent cell penetration capabilities.[37,38] The similarities in amino acid composition, overall charge and hydrophobicity suggest that **11** might also share the ability of RW_{mix} and RW_9 to traverse cellular membranes.

Second, the route followed into the cell will, to a large extend, influence the peptide's ultimate destination and effect. It is known that cationic peptides can be trafficked to many intracellular organelles, e.g., nucleus, Golgi complex,[39] or endoplasmic reticulum (ER).[40] Several CAPs have been shown to induce caspase-independent cell death with Ca^{2+} and reactive oxygen species (ROS) as mediators.[35,41,42] Following their entry into the cell, these peptides are thought to induce a massive Ca^{2+} release from the ER. The increased cytosolic Ca^{2+} concentration then in turn leads to a reduction in mitochondrial membrane potential, and subsequently an increase in mitochondrial ROS production. This oxidative stress results in the permeabilization of lysosomal membranes, leading to a release of lethal amounts of proteolytic enzymes into the cytoplasm, ultimately resulting in a necrotic-type of cell death. Peptide **11** was shown to induce a reduction in mitochondrial membrane potential without caspase activation, but advanced mode of action studies are needed to test this hypothesis.

However, from a therapeutic point of view, it may be less important how cell death is initiated (e.g., caspase- or ROS-dependent) than the way cell death is concluded (i.e., whether the cell dies in a manner that can elicit an immune response, or not).

It is predicted that the exploitation of ICD by oncolytic agents such as viruses[43] and peptides[11] will become a future cornerstone of oncolytic immunotherapy.[44] Oncolytic peptides developed in our group[36] have shown great promise in preclinical studies[16] and our lead compound, **12**, is currently being evaluated as an oncolytic immunotherapeutic in clinical trials.[45] We envisaged that oncolytic peptides will be used in combination with other immunotherapeutic modalities such immune checkpoint inhibitor (e.g., anti-PD-1, anti-CTLA-4) to improve clinical outcomes. The in vivo results obtained for **11** are similar to that obtained in previous studies with LTX-peptides, which bodes well for further development as an antilymphoma drug within the oncolytic immunotherapy paradigm.

In conclusion, following a systematic approach, a short arginine- and tryptophan-rich peptide with selective cytotoxicity toward B- and T-cell lymphomas has been generated. Preliminary in vivo results suggest that peptide **11** has the potential to be further developed as a novel oncolytic immunotherapeutic agent for the treatment of various types of lymphomas. Follow-up studies to determine **11**'s exact mode of action as well as preclinical assessments in canine models are currently being planned.

EXPERIMENTAL SECTION

Chemicals and Reagents Used. Annexin V-FITC (Biovision), propidium iodide (Sigma-Aldrich), tributyltin chloride (Sigma-Aldrich), RPMI-1640 (Sigma-Aldrich), tetramethylrhodamine ethyl ester perchlorate (Invitrogen), Hank's balanced salt solution (Sigma-Aldrich), and Caspase Fluorometric Substrate Set II Plus kit (Biovision)

Peptide Synthesis. All peptides were synthesized on a Tribute (Protein Technologies Inc. Tucson, AZ, USA) instrument using standard Fmoc protocols and amino acid derivatives. The Fmoc derivative of Tbt was prepared as previously described.[46]

After synthesis, the peptides were cleaved from the Rink amide resin (Novabiochem) using a cocktail containing 95% TFA, 2.5% water, and 2.5% triisopropylsilane for 3 h. The TFA was removed under vacuum and the fully deprotected and C-terminal amidated peptides precipitated with diethyl ether.

Peptide Purification and Characterization. The crude material was purified on a Waters 600E semipreparative RP-HPLC system using a C_{18} (20 × 250 mm, 5 μm, 100 Å) Inersil ODS-3 column from GL Sciences. Milli-Q water (Solvent A) and acetonitrile (Solvent B), both modified with 0.1% TFA, were used as solvent system. The default gradient used for purification was 10−40% Solvent B over 30 min at a flow rate of 15 mL/min and with peak detection set at 214 nm. For very hydrophilic peptides, a gradient of 0−30% B over 30 min was used. The purity of peptides was determined with analytical RP-HPLC (Waters 2695 system) using a C_{18} (4.6 × 250 mm, 5 μm, 100 Å) Sunfire column (Waters Corporation, Milford, MA, USA). The same solvent system and detection settings were used as for purification, but on a gradient of 0−50% Solvent B over 30 min with a flow rate of 1 mL/min. All peptides used in biological assays had a purity of >95%.

The molecular mass of the purified peptides were determined by MALDI-ToF (MALDI micro MX, Waters Corporation, Milford, MA, USA). The mass spectra were obtained with positive ion (reflector) mode with α-cyano-4-hydroxycinnamic acid as matrix. Results from the MS analysis can be seen in Table S1 (Supporting Information). The purified peptides were then freeze-dried (Labconco FreeZone 4.5 Plus, Kansas City, MO, USA) and stored at −20 °C until further use.

Cell Culture. The cell lines used in this study were tested for mycoplasma and cultured at 37 °C, in 5% CO_2 humidified atmosphere (Hera Cell 150 incubator, Thermo Electron Corp., Lanselbold, Germany). Lymphoma cells and the MRC-5 fibroblast were cultured in RPMI-1640 and MEM growth media, respectively. All media were modified with 10% FBS and 1% L-glutamine, while for A20 lymphoma cells, the growth media further was supplemented with 1.5 g/L sodium bicarbonate, 2.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol.

PBMC Isolation. Buffy coat samples, from healthy donors, were obtained from the Blood Bank at Tromsø University Hospital. PBMC were isolated by centrifugation in Lymphoprep (1.077 g/mL; Nycomed Pharma, Oslo, Norway). Briefly, buffy coat was diluted 1:4 in 0.2% PBSA (0.2% bovine serum albumin in PBS) without calcium or magnesium, 35 mL of the diluted buffy coat was layered over 15 mL of Lymphoprep, followed by 15 min centrifugation at 800*g*, without breaks, at room temperature. The interface was collected

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and mixed gently with 40 mL 0.2% PBSA. The sample was centrifuged two times at 400*g*, for 6 min, at room temperature, eliminating the supernatant in between centrifugations. The pellet contained the PBMCs. The cells were resuspended in RPMI 1640 medium and immediately used in cell viability assays.

RBC Isolation and Hemolysis Assay. Blood samples taken from two healthy donors were heparinized and washed. The RBCs were then resuspended in PBS and diluted to 10% hematocrit. The RBCs were treated immediately with the peptides for a period of 1 h at 37 °C. After treatment, the cells were spun down, and 100 μL of the supernatant transferred to a 96-well plate. The absorbance was read at 405 nm.

Cell Viability Assay. The first screens against A20 and MRC-5 were done manually, while the lymphoma panel screens were done at Marbio (Tromsø) using an automated platform (Biomek NX, Beckman Coulter, Hialeah, FL, USA).

Cells were automatically counted (Z1 Coulter Particle Counter, Beckman Coulter, Hialeah, FL, USA), and the cells seeded out in 96-well plates. The plates with adherent cells were incubated for at least 16 h before the start of the assay.

For adherent cells, growth media was removed, and the cells washed once with 100 μL assay media (RPMI-1640). All cell types were then treated with 100 μL peptide solution (concentration varying from 500 to 10 μg/mL) for 4 h. Normal assay media and assay media containing 0.1% Triton were used as negative and positive controls, respectively. After a 4 h incubation period, 10 μL of a MTT solution (5 mg/mL) was added to each well, and the plate incubated for a further 2 h. Following this, 70 μL of the media was carefully removed, and 100 μL acidified isopropanol added. The formed formazan crystals were dissolved by agitating the cells with a micropipette and by shaking (30 min). The absorbance of the reduced MTT (formazan) was read on Versa Max Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 590 nm. All assays, unless mentioned otherwise, were performed in triplicate.

The net peptide content of the peptides tested where not empirically determined, but the significant weight contribution of TFA-counterions (see Table S1, Supporting Information), was taken into account during the calculation of IC_{50} values. The IC_{50} values of peptides previously published by our group[14,36] were corrected to account for theoretical TFA content in order to make more meaningful comparisons.

Annexin V-FITC/PI Assay. The assay was adapted from Tørfoss et al.[47] Briefly, A20 cells were used in a final concentration of 3×10^5 cells/mL, and the cells were seeded in RPMI-1640 medium containing compounds **6a** and **11** (at IC_{50} concentrations). As control for apoptosis induction, cells treated with 0.5 μM TBTC were used. Untreated cells were used as negative control. After 1 h incubation, the cells were collected, centrifuged, and resuspended to 1×10^6 cells/mL in 10 mM HEPES/NaOH buffer, containing 0.14 M NaCl and 2.5 mM $CaCl_2$.

The cells were subsequently stained with annxin V-FITC and PI and incubated for 10 min in the dark at room temperature. The stained cells were analyzed using the FL-1 and FL-3 channel of a FACSCalibur (Becton & Dickenson, San Jose, CA, USA) flow cytometer.

Mitochondrial Membrane Potential ($\Delta\Psi_m$) Assessment. The assay was adapted from Ausbacher et al.[48] Briefly, the A20 cells (3×10^5 cells/mL) were seeded in 12-well plates and incubated for 1−4 h with **6a** and **11** at their determined IC_{50} values. As positive control, TBTC (0.1 μM) treated cells were used and untreated cells as negative control. TMRE was added to a final concentration of 100 nM 20 min before the respective experiment end points. Subsequently, the cells were collected and resuspended in Hank's balanced salt solution to a final concentration of 1×10^6 cells/mL and analyzed with the FL-2 channel of a FACSCalibur flow cytometer.

Caspase Activity Screening. To detect involvement of caspase 2, −3/7, −6, −8, −9, and −10, an activity screen was performed using the Caspase Fluorometric Substrate Set II Plus kit (Biovision Research Products, Mountain View, CA, USA) according to Ausbacher et al.[48] Briefly, A20 cells (3×10^5 cells/mL) were treated with 1× IC_{50} of **6a**, 1× IC_{50} of **11**, or 0.5 μM TBTC for 1 h. Subsequently the cells were lysed, and the lysate was supplemented with reaction buffer as well as DL-dithiothretiol solution. The 7-amino-4-trifluoro methylcoumarin-labeled caspase substrates were added, incubated at 37 °C for 2 h, and analyzed on a fluorescence plate reader (SpectraMAX Gemini EM, Molecular devices, Sunnyvale, CA, USA). Caspase activity was determined by comparing the fluorescence intensities of treated and untreated control samples.

Transmission Electron Microscopy (TEM). Ramos cells (2×10^5 cells/mL) were suspended in peptide containing serum-free RPMI-1640 medium and transferred in culture flasks (NUNC Easy flask 25 cm^2, Thermo Fischer Scientific, Langenselbold, Germany). The peptide concentrations were chosen according to the previously determined IC_{50} values. All cells were prefixed with Karnovsky's cacodylate-buffered (pH 7.2) formaldehyde-glutaraldehyde fixative at 4 °C overnight. The fixative was replaced by Karnovsky's buffer and postfixated with 1% osmium tetroxide. After dehydration in a graded series of ethanol, samples were infiltrated with a 1:1 mixture of acetonitrile and Epon resin (AGAR 100, DDSA, MNA and DMP-30) overnight. Pure resin was added the following day and then polymerized for 24 h. Ultrathin sections were prepared and placed on Formvar, carbon-stabilized copper grids. The samples were stained and contrasted with uranyl acetate (5%) and Reynold's lead citrate. Samples were analyzed on a JEOL-1010 transmission electron microscope (JEOL, Akaishima, Japan). An Olympus Morada side-mounted TEM CCD camera (Olympus soft imaging solutions GmbH, Münster, Germany) was used for image acquisition.

Scanning Electron Microscopy (SEM). The same incubation procedures as for the TEM experiment were performed in the SEM studies. All cells were fixed with McDowell's fixative at 4 °C, overnight. For postfixation, 1% osmium tetroxide was used, and dehydration was accomplished with a graded series of ethanol. Hexamethyldisilazane was applied for chemical drying. Specimens were mounted on aluminum stubs and prior to examination sputter coated for 90 s. Samples were analyzed on a JEOL JSM-6300 scanning electron microscope (JEOL, Akaishima, Japan), and image acquisition was carried out via an EDAX Phoenix EDAM III data acquisition module (EDAX Inc., Mahwah, NJ, USA).

Animals. Female BALB/c mice, 6−8 weeks of age, were purchased from Charles River, Germany. All mice were housed in cages in a specific pathogen-free animal facility according to the local and European Ethical Committee guidelines. All animal experiments were approved by the Norwegian National Animal Research Authority (NARA) (approval ID: FOTS ID-5267), and the protocol was approved by the committee on animal ethics at UiT The Arctic University of Norway.

Intralesional Treatment of A20 Lymphomas. A20 tumor cells were harvested, washed in RPMI-1640, and inoculated (i.d.) into the left side of the abdomen in BALB/c mice (5×10^6 cells in 50 μL RPMI-1640 per mouse). When the tumor size reached 20 mm^2, the mice were split into three groups. Group 1 ($n = 9$) were treated with vehicle (saline, 0.9% NaCl in sterile H_2O) and served as a control group. Mice in Groups 2 ($n = 8$) and 3 ($n = 9$) were treated with peptides **6a** and **11**, respectively. Both peptides were dissolved in saline to a concentration of 25 mg/mL and sterilized by means of filtration. The treatment regime was intralesional injections (50 μL of above-mentioned peptide solutions) performed once a day for three consecutive days.

Tumor size was measured three times a week using an electronic caliper and expressed as the area of an ellipse [(maximum dimension/2) × (minimum dimension/2) × 3.4. Animals were euthanized when the tumor exceeded 100 mm^2. Animals were also euthanized if the tumor had not reached that size but presented ulcerations or the animals developed metastasis.

Secondary Tumor Challenge. Animals with complete regression of A20 tumors after treatment with **6a** and **11** were rechallenged with A20 lymphoma cells 4 weeks after the completion of the peptide treatment study. The cells (5×10^6) were inoculated (i.d.) on the right side of the abdomen (contralateral to the first tumor site) 4 weeks after the animals were cured.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00839.

MW and calculated TFA content of peptides, HPLC data for purified peptides, cell lines used, additional electron micrographs, flow cytometry results, preliminary bioinformatics, animal study information, and statistical information (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AMP, antimicrobial peptide; CAP, cationic amphipathic peptide; CPP, cell penetration peptide; HL, Hodgkin's lymphoma; HS, heparan sulfate; ICD, immunogenic cell death; MST, median survival time; NHL, non-Hodgkin's lymphoma; OS, overall survival; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; PS, phosphatidylserine; RBC, human red blood cells; ROS, reactive oxygen species; SEM, scanning electron microscopy; SI, selectivity index; Tbt, 2,5,7,-tri(*tert*-butyl)tryptophan; TBTC, tributyltin chloride; TEM, transmission electron microscopy; TMRE, tetramethylrhodamine ethyl ester perchlorate

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