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Selective Inhibition of Human Lung Cancer Cell Growth by Peptides Derived from Retinoblastoma Protein

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Peptides containing retinoblastoma protein (RB) fragment 649-654 (LFYKKV) were tested for their ability to block the proliferation of RB-negative and RB-positive human non-small cell lung cancer (NSCLC) cells. These peptides potently restrained the growth of both types of tumor cells, as measured by metabolic (MTT) and cellular viability (trypan blue exclusion) assays. As such, and remarkably, the peptides were able to overcome the resistance of RB-positive cells usually observed with RB gene or protein replacement therapy. Compared to the overall performance of conventional chemotherapy tested in parallel, the peptides were more cytotoxic against RB-negative neoplastic cells and equipotent toward RB-positive tumor cells, yet less toxic toward normal human cells. Thus, these new molecules hold great promise to evolve into an efficient therapy for human lung cancer, a common malignancy still defying treatment and holding a poor prognosis, as well as for other human neoplasias.

Retinoblastoma protein (RB) is a nuclear tumor suppressor protein that plays a central role in blocking cell cycle progression in normal cells (1). As a result, it is able to prevent uncontrolled cell growth from occurring (1). Conversely, RB dysfunction, e.g. due to mutations in the RB gene, hyperphosphorylation of the RB protein or the latter's complex formation with oncoproteins of tumor viruses, has been implicated as a crucial step in the pathogenesis of a majority of human tumors (2). Therefore, RB inactivation could be well regarded as a "common denominator" of oncogenesis. Consistent with these insights, replacement of the RB gene (3) or addition of the RB protein (4) to RB-deficient tumor cells results in the normalization of the aberrant growth behavior of these cells *in vitro* and *in vivo* regardless of the specific tumor type.

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Moreover, RB is not simply a tumor suppressor protein among others, but *primus inter pares*. Its paramount importance is underscored by the fact that it is functionally mediating downstream growth-inhibitory effects of other important nuclear tumor suppressors such as p53 (1, 5) and p16, the latter being part of the so-called p16-cyclin D/cdk4-RB pathway (1, 6). Thus, RB could be regarded as the cell's ultimate safeguard in the decision between cellular quiescence and proliferation. This view is supported by experiments showing that the introduction of the p16 gene, protein or of peptides thereof into RB-defective tumor cells cannot interfere with these cells' abnormal growth behavior (6, 7), whereas RB replacement can normalize these cells (8).

Following the early recognition of RB's unique importance for cell growth control and hence of its potential to serve as a template for the engineering of optimal anti-cancer therapeutics, a potential RB-like peptide was initially designed (9). Subsequently, a derivative thereof was demonstrated (10) to physically associate with a fragment of the human papilloma virus (HPV) 16 E7 protein harboring the RB-binding motif LXCXE and with a peptide bearing an identical motif in human insulin, the presence of which had suggested that, intriguingly, insulin may interact with RB (11). This first experimental validation (10) of the initial concepts raised hopes of being close to having a small molecule at hand which should be able to imitate at least some of the crucial growth-inhibitory functions of RB outlined above.

Since the site of action of RB is the cell nucleus, we have now employed fusion polypeptides consisting of the previously described active site sequence $RB_{649-654}$ (9, 10, 11) and nuclear localization signals (NLS) in order to ensure the former's import from the extracellular space into the nucleus. Furthermore, we have chosen human non-small cell lung cancer (NSCLC) as a model for testing these peptides for two reasons. Lung cancer is yet a leading cause of cancer mortality in males and increasingly also in females. This is due

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to a high incidence of this malignancy coupled with a poor prognosis due to still insufficient treatment modalities. Secondly, human NSCLC represents a suitable model for testing RB replacement therapy since there are well-defined human NSCLC cells either lacking RB (the NCI-H596 cell line, briefly: H596) or containing RB (the A549 cell line) (4, 12). Past experience has shown RB replacement to be only effective in the first type of cells, i.e., in RB-negative cells (4). We present here data of our peptides' performance in both NSCLC cell types, contrasting it with their effect on normal human peripheral blood mononuclear cells (hPBMNC), and include the results of the testing of two other compounds, etoposide (VP-16)—which is a conventional chemotherapeutic currently used in the clinical treatment of lung cancer—and the differentiation agent all-*trans*-retinoic acid (ATRA), in the same cellular contexts. The present peptide data are also discussed in the light of the known performance of full-length RB protein in the same cells, finally leading to conclusions on these peptides' potential for clinical applications.

MATERIALS AND METHODS

Cells. Human PBMNC were isolated from the blood of healthy donors by means of a Ficoll gradient. The human NSCLC cell lines H596 as well as A549 were obtained from the U.S. National Cancer Institute (NCI) and were at passages 60–80 and 180–200, respectively, when tested. Non-synchronized tumor cells were maintained in RPMI 1640/10% FCS (Gibco, Paisley, UK) during the incubation with the various compounds.

Compounds. The peptides MCR-4, -8, and -10 were prepared by solid phase synthesis and purified to more than 95% homogeneity by HPLC and MS at Pichem, Inc. (Graz, Austria). Fluorescein isothiocyanate (FITC)-labeled peptides were equally prepared by Pichem, Inc. (Graz, Austria). VP-16 was obtained as Vepesid from Bristol-Myers Squibb, Inc. (Munich, Germany). The source for ATRA or tretinoin, respectively, was Sigma, Inc.

Fluorescence microscopy. For detection of FITC-labeled peptides and of control FITC, cells were visualized by employing a Leitz fluorescence microscope at ×128 magnification and images were photographed with a Leitz camera using a 400 ASA slide film and 1- to 3-minute exposure times.

Cellular proliferation. Tumor cells were detached with EDTA, washed with RPMI 1640/10% FCS and subsequently seeded at 10^5 cells/ml RPMI 1640/10% FCS in 96-well plates. The cells were then incubated in the presence or absence of the above substances for 72 h. Finally, the dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, briefly: MTT (Sigma, Inc.) was added to each well and its turnover determined by measuring the optical density (OD) in a Labsystems Multiscan RC ELISA reader.

Cellular viability. Tumor cells were detached with EDTA, washed with RPMI/10% FCS and subsequently seeded at 10^5 cells/ml RPMI 1640/10% FCS in 48-well plates. Tumor cells or normal hPBMNC, respectively, were then incubated in the presence or absence of the above substances for 72 h. Finally, viable cells were counted by means of the trypan blue exclusion method in a Neubauer chamber.

MCR-4 (all-D) NH_2- LFYKKVRQIKIWFQNRRMKWKK -COOH

MCR-8 (all-D) NH_2- TSLSLFYKKVYRLARQIKIWFQNRRMKWKK -COOH

MCR-10 (all-D) NH_2- TSLSLFYKKVYRLAGKRKRSQ -COOH

FIG. 1. Amino acid sequences of MCR-4, MCR-8, and MCR-10.

RESULTS

Design of MCR peptides. MCR-4 (Fig. 1) was engineered as a fusion of the $RB_{649-654}$ fragment LFYKKV (9, 10, 11) with a 16mer derived from the Antennapaedia homeodomain and capable of translocating into the nucleus (13). The sequence of MCR-8 (Fig. 1) is identical to that of MCR-4 except that LFYKKV was extended N- and C-terminally by each of those 4 amino acids that are naturally flanking the above RB hexapeptide in full-length RB, thus resulting in a 14-mer active site. It has been shown with endothelin receptor fragments that such extensions increase binding affinity considerably, maybe by inducing a natural (folding) environment in the larger fragment *vs.* the smaller one (14). MCR-10 (Fig. 1) resulted from coupling the active site of MCR-8 with the C-terminal NLS of human γ-interferon (15). All peptides were synthesized as all-D enantiomers in order to augment their protease stability.

Extracellularly given MCR peptides translocate into the cell nucleus of living H596 and A549 lung cancer cells. All three FITC-coupled peptides, i.e. FITC-MCR-4, -8 and -10 each at a concentration of 40 μM, internalized into both H596 and A549 tumor cells and translocated to the cell nucleus, whereas control FITC was found to be associated with cells only on the outside of the cell membrane (data not shown). The peptide translocation process was time-dependent, with the peptides rapidly reaching the nucleus already after 30 minutes of incubation at 37°C, yet the intracellular fluorescence signal being more intense at 2 h, suggesting that more FITC-peptides have reached the nucleus at that time (data not shown).

MCR peptides are more potent than etoposide in blocking the proliferation of H596 tumor cells. By employing the MTT assay, we could observe that the MCR peptides MCR-4, MCR-8, and MCR-10 were able to repress the cellular proliferation of the RB-negative human lung cancer cell line H596 in a dose-dependent fashion within a peptide concentration range of 10–50 μM compared to untreated tumor cell controls set as 100% (Fig. 2a). The order of MCR peptide growth-inhibitory potency at a 50 μM concentration was the following: MCR-8 was the most potent peptide with only 10% of tumor cell metabolic activity (corresponding to a 90% growth inhibition) left in its presence, followed by MCR-10 (20% MTT activity or 80% growth inhibition) and finally MCR-4 (60% MTT activity or

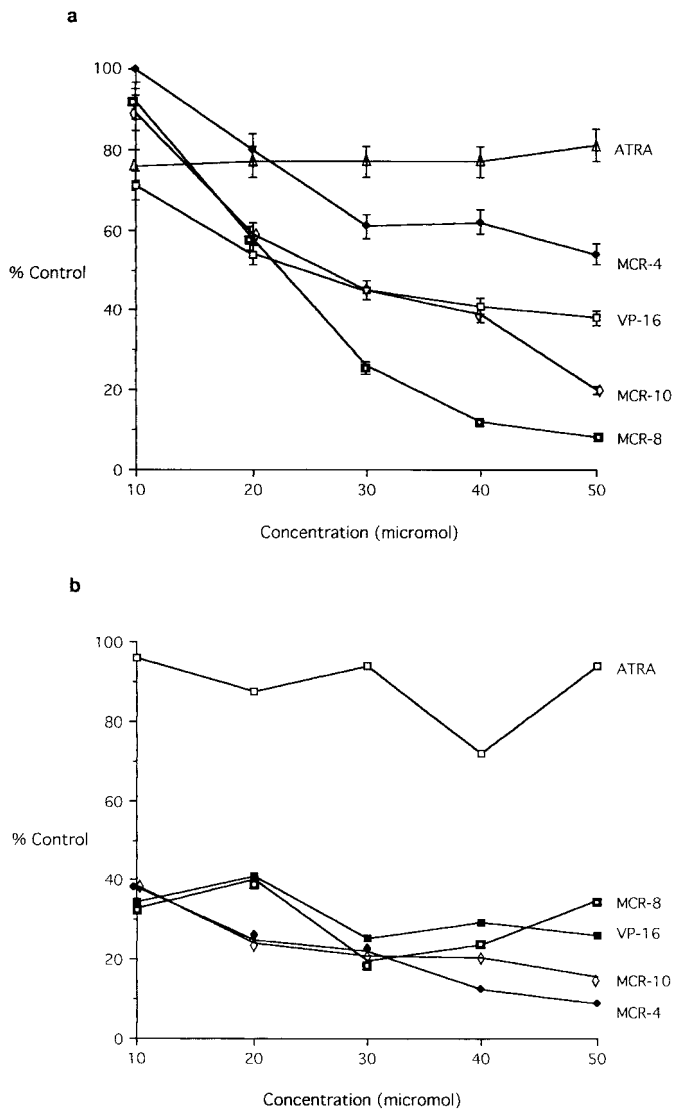

FIG. 2. (a) Proliferation of NCI-H596 human lung cancer cells in the presence of MCR peptides vs conventional agents (VP-16 and ATRA), whereby the proliferation of untreated controls has been set as 100%. This assay was done in quadruplicates and performed three times. The results (mean ± SEM) of one representative determination are shown. (b) Viability of NCI-H596 human lung cancer cells in the presence of MCR peptides vs conventional agents (VP-16 and ATRA), whereby the viability of untreated controls has been set as 100%. The median values from two independent determinations are shown.

40% growth inhibition) (Fig. 2a). Thus, two of the peptides, namely MCR-8 and MCR-10, were more potent at 50 μM doses than an equivalent concentration of etoposide which achieved about 60% growth inhibition in this assay (Fig. 2a). MCR-8 maintained this superiority also at lower concentrations, i.e., down to 30 μM (Fig. 2a). Moreover, the longer MCR-8 peptide was more potent that the shorter MCR-4 peptide at each tested concentration (Fig. 2a). In addition, the peptides were considerably more potent than ATRA which had only an insignificantly low activity in this MTT assay (Fig. 2a).

MCR peptides are more cytotoxic than etoposide toward H596 tumor cells. We also assessed the peptides' effects on the viability of H596 cells by trypan blue exclusion. In the dose range of 40–50 μM, MCR-10 and, in particular, MCR-4 was more tumoricidal than etoposide, leaving only 10–20% viable tumor cells *vs.* 30%, respectively (Fig. 2b). In the lower dose ranges, the MCR peptides were equipotent to etoposide. Interestingly, ATRA did not decrease tumor cell viability (Fig. 2b). Compared to the data obtained in the MTT assay, MCR-8 was here equipotent or even of weaker activity compared to MCR-4 (Fig. 2b).

MCR peptide-based inhibition of proliferation of A549 tumor cells compares to etoposide potency. This MTT assay revealed that, in the lower dose range (10–30 μM) etoposide, the chemotherapeutic agent, was more potent than the MCR peptides (Fig. 3a) when tested against the RB-positive human lung cancer cell line A549. However, at higher concentrations (40–50 μM), MCR-10 turned out to have a similar potency to etoposide for both compounds reduced tumor cell metabolism to 20–25% of controls (Fig. 3a). Moreover, at the highest concentration tested (50 μM), it was the MCR-8 peptide which displayed the strongest antiproliferative effect of all substances tested, blocking cancer cell metabolic activity down to less than 20% (Fig. 3a). Interestingly, the longer MCR-8 peptide had higher antiproliferative activity than the shorter MCR-4 peptide (Fig. 3a), as already observed with the H596 cells. ATRA displayed only a weak growth-inhibitory activity toward these tumor cells, i.e. it elicited a reduction of MTT activity to 60–80% of controls (Fig. 3a).

Similarly pronounced cytotoxicity of individual MCR peptides and etoposide toward A549 tumor cells. By trypan blue exclusion, we determined that, at lower concentrations, i.e., 20–30 μM, MCR-10 affected as profoundly as VP-16 the viability of the A549 tumor cells (Fig. 3b). In fact, MCR-10 displayed a tumor cell-directed cytotoxicity of over 80% in this concentration range (Fig. 3b). At higher doses, i.e., 40–50 μM, there were besides MCR-10 two other MCR peptides, MCR-4 and MCR-8, that were comparable in potency to VP-16, specifically these compounds were cytotoxic to 80–90% of the tumor cells (Fig. 3b). Notably and as already mentioned above with the H596 cells, ATRA did not decrease tumor cell viability; on the contrary, it even slightly increased it (Fig. 3b).

MCR peptides are much less toxic toward normal human cells than conventional antineoplastic agents. As a final step we chose to address the question of how the above peptides would affect the viability of normal cells such as human PBMNC as compared to etoposide

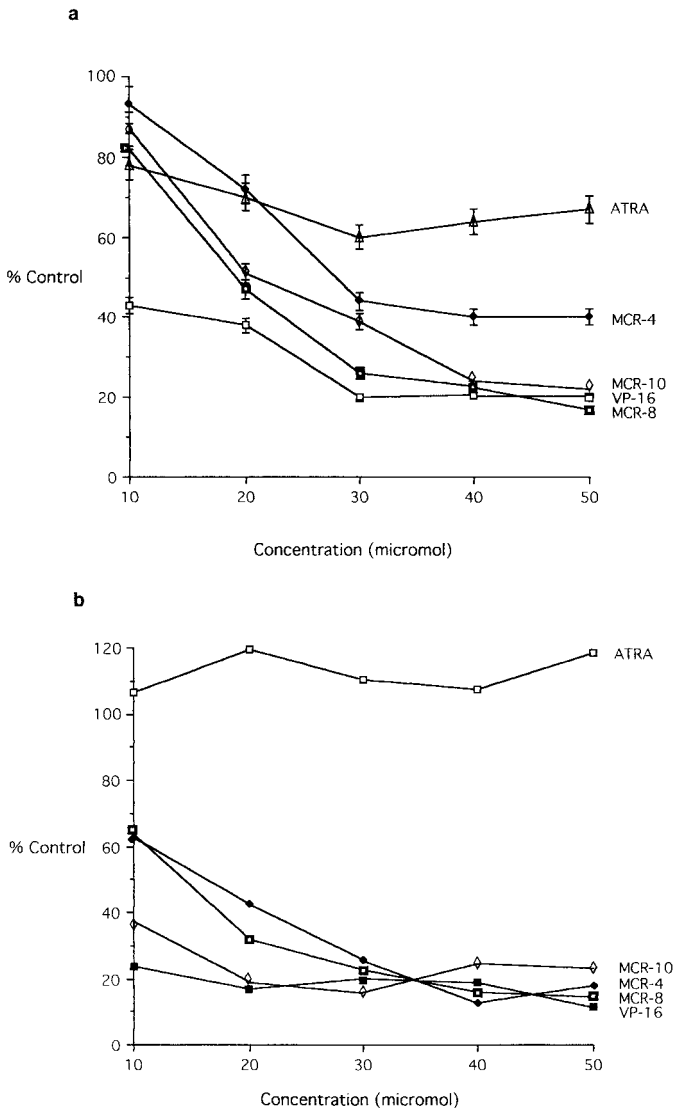

FIG. 3. (a) Proliferation of A549 human lung cancer cells in the presence of MCR peptides vs conventional agents (VP-16 and ATRA) whereby the proliferation of untreated controls has been set as 100%. This assay was done in quadruplicates and performed three times. The results (mean ± SEM) of one representative determination are shown. (b) Viability of A549 human lung cancer cells in the presence of MCR peptides vs conventional agents (VP-16 and ATRA) whereby the viability of untreated controls has been set as 100%. The median values from two independent determinations are shown.

and ATRA, respectively. Remarkably, the pattern of potency for the various substances toward tumor cells reported above markedly dissociated when normal cells were exposed to the same compounds (Fig. 4). As such, etoposide and ATRA were cytotoxic to the majority of normal human cells, leading to a cellular viability of 30–40% at 30–50 μM concentrations, whereas the MCR peptides were only slightly cytotoxic to the same cells, the cellular viability generally being 70–100% at equivalent peptide concentrations. Only at its highest concentration tested (50 μM) did MCR-4 reduce cellular viability to 60% compared to untreated controls (Fig. 4).

DISCUSSION

The purpose of the present study was to determine whether the previously observed complex formation between a polymeric RB fragment and peptides encompassing the RB-binding motif LXCXE in HPV16 E7 or insulin, respectively, (10) could be translated into a biologically relevant effect toward malignant cells vs normal cells. As such, several small peptides derived from the B region of the RB pocket were first coupled to cellular internalization sequences, yielding fusion polypeptides termed MCR peptides, and then their effect on the proliferation and viability of human lung cancer cells *vs.* normal cells was measured and compared with the action of conventional antiproliferative agents such as ATRA and etoposide.

Interestingly, this study showed that MCR peptides display antineoplastic activity against both RB-negative and RB-positive tumor cells, thus revealing a wider and therefore better activity spectrum than full-length RB protein itself (4). Moreover, the presumable increase in binding affinity expected to occur as a result of the extension of the active site structure of MCR-4, leading to the structure of MCR-8 (cf. above), did indeed correlate with a higher antiproliferative activity of MCR-8 vs MCR-4 (Figs. 2a and 3a).

Based on previous data with related MCR peptides (16, 17), the presumable cause for the antineoplastic activity displayed by the present MCR peptides is a block of cell cycle progression and probably also the induction of apoptosis, as both previously and now

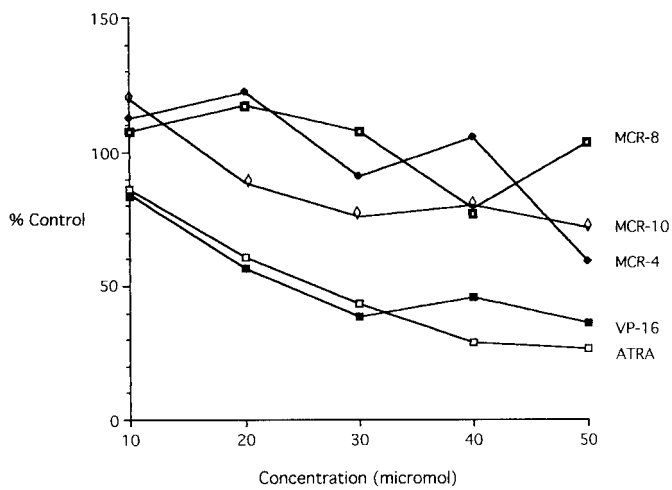

FIG. 4. Viability of normal human peripheral blood mononuclear cells in the presence of MCR peptides vs conventional agents (VP-16 and ATRA) whereby the viability of untreated controls has been set as 100%. The median values of one representative determination are shown. This experiment was repeated twice.

suggested by changes in cellular morphology such as nuclear condensation and fragmentation occurring upon MCR peptide treatment. As far as the more precise mechanism of action in the RB-negative H596 NSCLC cells is concerned, one can presently assume that the MCR peptides might have physically associated with nuclear proteins containing the LXCXE motif since a similar peptide (10) has already been shown to be capable of recognizing the LXCXE motif. Among such proteins, cyclin D is a particularly likely binding partner candidate for the MCR peptides. As a result of this interaction, LXCXE-motif-mediated cyclin D binding to the RB-related proteins p107 and p130, which can partially substitute for the lacking growth-inhibitory function of RB in RB-negative cells (18, 19), could have been abrogated and as such the p107/p130 inactivation prevented. By comparison, in the RB-positive A549 NSCLC cells, which are basically resistant to full-length RB replacement therapy (4), the MCR peptides were also active, most likely through a similar mechanism as in RB-negative cells, i.e., by sequestering oncoproteins bearing the LXCXE motif.

Moreover, it should be noted that the RB-like MCR peptides were active at concentrations around 20 μM, whereas full-length RB achieved similar activity at concentrations that were 2 log lower (4). However, in contrast to the MCR peptides, full-length RB was only active in RB-negative cells, not in RB-positive cells (4). In this context, it is interesting to mention that, similar to MCR peptides, another (yet much longer) fragment of full-length RB, an N-terminally truncated RB, had been shown to be superior to full-length RB itself by being active not only in RB-negative, but also in RB-positive cells, specifically by causing tumor cell apoptosis (20). This study thus confirmed the results of a previous investigation demonstrating that full-length RB is a relatively weak tumor suppressor (21). Based on these data, it is tempting to speculate that, within a tumor suppressor protein, growth-inhibitory segments may be functionally attenuated or even antagonized by self-binding (growth-stimulatory) regions. Consistent with this concept, the A region of the RB pocket harbors several domains homologous to growth-promoting viral and cellular oncoprotein sequences known to associate with (segments in the B region of) the RB pocket (R. T. Radulescu, unpublished observation).

Also, individual MCR peptides revealed themselves to be equally potent (towards RB-positive A549 human NSCLC cells) or even of superior potency (towards RB-negative H596 human NSCLC cells) compared to conventional chemotherapy (etoposide). In this context, it should be noted that (a) in the cellular viability assays (trypan blue exclusion tests), MCR-10, already at a 10 μM concentration, displayed considerable potency corresponding to a tumor cell-directed cytotoxicity of 60% (Figs. 2b and 3b), implying that its IC_{50} should be at a lower dose; (b) etoposide was tested in concentrations previously proven to be highly active (10–50 μM) as a result of its interference with IGF/IGF receptor-induced cell proliferation (22).

The marked anti-tumor potency of the MCR peptides presented here, already promising in itself, is even more remarkable in the light of the fact that these compounds, when tested in the same concentrations shown to be cytotoxic towards tumor cells, were largely innocuous toward normal human cells (hPBMNC), affecting the viability of only a small fraction of normal cells (Fig. 4). In contrast, etoposide was toxic to the majority of normal cells (Fig. 4). This effect is well-known and, moreover, not surprising because etoposide targets a topoisomerase protein that is indispensable not only in tumor cells, but also in normal cells for DNA repair processes. Interestingly, ATRA turned out to be a negative control in these assays. This is consistent with other reports showing that ATRA cannot affect human lung cancer cell proliferation even at high concentrations (23).

The superior therapeutic index of the MCR peptides revealed in this study is indeed notable since it could be seen as a first important step toward complying with the long-sought "Holy Grail" of anti-cancer drug development which has still not been achieved (24) and consists in developing selective anti-tumor agents that are cytotoxic toward tumor cells, yet harmless toward normal cells.

In terms of peptide length, it should be noted that synthetic peptides similar in length to MCR peptides have already been shown to be active in blocking cell cycle progression of transformed cells at similar concentrations as those of the MCR peptides shown here, i.e., at 10–50 μM concentrations (7, 25). The (in terms of peptide synthesis effort and cost) not insignificant length of 20–40 amino acids of all these peptides derives from the fact that they are bimodular: i.e., they consist of an active site module of 6 to about 25 amino acids and of a second module of up to 16 amino acids responsible for internalization and nuclear localization. This bifunctional structure is necessary for activity since single modules, i.e. the active site or the translocation sequence alone, have provingly no effect on tumor cell cycle progression (data not shown and Ref. 17).

Interestingly, naturally occurring peptide hormones of paramount importance for normal metabolism such as ACTH, insulin, endothelin or calcitonin have a similar length, suggesting that this length has been evolutionarily selected as an optimal one. Although naturally occurring and circulating counterparts to the above synthetic cell cycle regulatory peptides have not been found as yet, their presence should not be excluded.

Taken together, the MCR peptides presented here can be regarded as promising candidate agents for the treatment of human neoplasia, particularly of human lung cancer. These compounds should be active and display satisfactory bioavailability without further

structural modifications in *in vivo* settings too. Preliminary evidence from experiments demonstrating antineoplastic effects of MCR peptides against syngeneic lung tumors implanted subcutaneously in mice suggests that this goal is achievable (26). Therefore, this potential merits to be further explored and refined. At the end of such a drug development road, synthetic inducible biological response amplifiers, briefly: SIBRAs (27), i.e., peptides combining antineoplastic properties with the ability to stimulate the promoter activity of endogenous tumor suppressor genes that are functional and normally undergoing positive autoregulation, may come true. Thus, a sustained *in vivo* action necessary for the reversal of the malignant process would be ensured.

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