p21\textsuperscript{WAF1}-derived Peptides Linked to an Internalization Peptide Inhibit Human Cancer Cell Growth\textsuperscript{1}

Marina Bonfanti, Stefano Taverna, Mario Saliona, Maurizio D'Incalci, and Massimo Broggini\textsuperscript{2}

Molecular Pharmacology Unit, Istituto di Ricerche Farmacologiche "Mario Negri" via Eritrea 62, 20157 Milan, Italy

Abstract

We tested the ability of synthetic peptides derived from p21\textsuperscript{WAF1}, fused to the internalization peptide sequence derived from Antennapedia, to inhibit the growth of cancer cells in two human ovarian cancer cell lines expressing wild-type p53 or not. Two fused peptides corresponding to p21\textsuperscript{WAF1} regions 17-33 and 63-77 inhibited cell growth in both cell lines while the same peptides without the internalization sequence were inactive. The fused peptides prevented growth at concentrations which inhibited cyclin-dependent kinase 2 and cdc2 activity, thus demonstrating that the peptides act by mimicking the action of p21\textsuperscript{WAF1} on kinases. This study illustrates the potential pharmacological use of small peptides fused with the Antennapedia internalization sequence in proliferative disorders. The approach may be extended to other diseases in which cell penetration of a peptide may be of therapeutic benefit. More stable drug-like molecules with better pharmacological properties could be designed based on the results obtained with peptides.

Introduction

Progression through the cell cycle is regulated by interactions among cyclins, cdk,\textsuperscript{3} and cdk inhibitors (1, 2). Of the latter, p21\textsuperscript{WAF1} is one of the most widely studied inhibitors of the activity of different cdk in vitro and in vivo (3-5). It has been reported to act as a tumor suppressor in vitro and in vivo (6-8). Schematically, p21\textsuperscript{WAF1} can be divided into two functional parts, the NH\textsubscript{2}-terminal which binds cyclins and cdk and the COOH-terminal which binds proliferating cell nuclear antigen (9-11). The specific regions interacting with cdk or cyclins have been established (3, 12-16).

No data exist on the ability of p21\textsuperscript{WAF1} peptides to arrest the growth of cancer cells in culture. The identification of a sequence in Antennapedia responsible for the internalization of proteins makes attractive the possibility to use this sequence to facilitate the cell entering of different peptides. Such an approach has been recently reported to be potentially successful (17, 18).

In this report, we describe the biological activity of peptides derived from the N-terminal region of p21\textsuperscript{WAF1} alone or fused to the Antennapedia internalization sequence. We evaluated the growth inhibition, the inhibition of cdk activity and apoptosis induced by these peptides in two human ovarian cancer cell lines expressing wild type or no p53.

Materials and Methods

Cell Lines and Evaluation of Growth Inhibition. Human ovarian cancer cell lines SKOV-3 and IGROV-1 were maintained in RPMI 1640 supplemented with 5% FCS. Growth inhibition was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in 96-well plates at different times after treatment with different concentrations of peptides. Concentrations inhibiting the growth by 50% (IC\textsubscript{50}) were calculated after 72 h.

Peptides. Peptides were synthesized by solid-phase chemistry on a model 430 synthesizer from Applied Biosystems (Foster City, CA) using N-(9-fluorenylmethoxycarbonyl) as the protective group for aminoic residues and 1-hydroxybenzotriazole, 2-(1H-benzotriazol-1-yl)-1,3,3-tetramethyloxironi- umhexafluorophosphate, and N,N-dicyclohexyl-carbodiimide as activators of carboxylic residues. Cleavage of peptides from the resin and purification were as described elsewhere (19). The amino acid sequence and the characteristics of the peptides are reported in Table 1.

Western Blotting Analysis. Total cell extracts were prepared from SKOV-3 and IGROV-1 cells after 4 h of peptide treatment according to standard procedures (20). One hundred \( \mu \)g of proteins for each sample were electrophoresed through 12% SDS-polyacrylamide gels and transferred to nitrocellulose. Filters were hybridized with monoclonal antibodies against cyclins A, B, and cdc2, polyclonal antibodies against p21\textsuperscript{WAF1}, CDK2, and CDK7 (Santa Cruz Biotechnology, Santa Cruz, CA), and detected with the enhanced chemiluminescence (Amersham, Milan, Italy) system after addition of antioimmun or antirabbit IgG (Santa Cruz Biotechnology).

Kinase Assay. Inhibition of kinase activity was measured by incubating 100 ng of total extracts containing insect cell-expressed cyclin E-CDK2 or cyclin A-CDK2 or 4 units of cyclin B-cdc2 (New England Biolabs, Beverly, MA) in the presence of different amounts of peptides at 4°C for 30 min. Kinase reactions were followed at 30°C for 20 min in a total volume of 25 \( \mu \)l of kinase buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% Triton X-100, 10 mM MgCl\textsubscript{2}, and 1 mM DTT) containing 2 \( \mu \)g of histone H1, 1 \( \mu \)M ATP, and 5 \( \mu \)Ci of [\( \gamma \)-32P]ATP (3000 Ci/mmole; Amersham); 25 \( \mu \)l of 2X SDS-loading buffer were added and the samples were boiled and loaded on 12% SDS-polyacrylamide gels. Histone H1 was loaded as a marker of molecular weight and separately stained with Coomassie blue.

Immunoprecipitation. Two hundred \( \mu \)g of proteins prepared as described above from untreated or peptide-treated cells were immunoprecipitated with antibodies specific for cdc2 and CDK2 (Santa Cruz Biotechnology) and collected by binding to protein A-Sepharose. After three washes in lysis buffer and two in kinase buffer, complexes were resuspended in 30 \( \mu \)l of kinase buffer. Half the sample was used for kinase assay as described above, and the other half was mixed with an equal volume of 2X SDS-loading buffer, heated at 100°C for 2 min, and loaded on 12% SDS-polyacrylamide gels. Proteins were electrotransferred and hybridized with antibodies against cyclins and cdk as described in "Western Blotting Analysis."

Cell Cycle Analysis. Cells were treated with the different peptides, harvested after 8 and 24 h, stained with propidium iodide, and analyzed using flow cytometry. Percentages of cells in the different cell cycle phases were calculated as described (21).

Results and Discussion

We determined the cell growth inhibitory activity of peptides derived from the NH\textsubscript{2}-terminal region of p21\textsuperscript{WAF1} (Table 1), fused to an internalization sequence derived from Antennapedia (22). AInt and CInt peptides caused dose-dependent inhibition in both IGROV-1 and SKOV-3 cells (Fig. 1), measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, while the basic A and C peptides did not show appreciable activity at concentrations below 500 \( \mu \)M. Similar results were obtained when cell growth inhibition was determined by cell counting. The Int peptide alone did not show appreciable activity at the doses tested. The BInt peptide was only slightly active in IGROV-1 but not in SKOV-3 cells and again B was not...
Table 1  Sequence and characteristics of the different peptides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>p21WAFF Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ACRRLFGPVDSEQLSRD</td>
<td>17-33</td>
</tr>
<tr>
<td>AInt</td>
<td>RQIKIFQQRMKKACRRLFGPVDSEQLSRD</td>
<td>17-33</td>
</tr>
<tr>
<td>B</td>
<td>WPFDVFETPLEGDFAW</td>
<td>49-65</td>
</tr>
<tr>
<td>BInt</td>
<td>RQIKIFQNRMMKKNDFDPFETPLEGDFAW</td>
<td>49-65</td>
</tr>
<tr>
<td>C</td>
<td>AKERVRGLGPKLY</td>
<td>63-77</td>
</tr>
<tr>
<td>CInt</td>
<td>RQIKIFQNRMMKKAENERVRGLGPKLY</td>
<td>63-77</td>
</tr>
<tr>
<td>Int</td>
<td>RQIKIFQNRMMKKAENERVRGLGPKLY</td>
<td>63-77</td>
</tr>
</tbody>
</table>

inactive in both cell lines. The activity of AInt and CInt peptides was not due to the sums of the effects of the two peptides, as suggested by experiments in which the addition of the two separate peptides in vitro did not inhibit cell growth (data not shown).

We calculated the IC50 for the different peptides from the data reported in Fig. 1. The IGROV-1 cell line was inhibited slightly more than SKOV-3 (Fig. 1). Possibly, the endogenous basal levels of p21WAFF in IGROV-1 but not in SKOV-3 (Fig. 2C) lowered the threshold amount of peptide needed to inhibit growth, although more studies are needed to verify this.

To check whether the presence of the Int peptide induced the internalization of the peptides, we fluoresceinated the AInt peptide through the cysteine residue with N-2{(iodoacetoxy)ethyl)-N-methyl}amino-7-nitrobenz-2-oxa-1,3-diazole (Molecular Probes) and observed intracellular fluorescence after treatment with 10 μM of this peptide, indicating that the AInt peptide does reach the putative intracellular targets (data not shown).

We analyzed the peptides' ability to inhibit CDK2 and cdc2 activity in cells treated with the IC50 of the different peptides after immunoprecipitation of the kinases with specific antibodies (Fig. 2, A and B). Again, the AInt and CInt peptides reduced the activity of both kinases in both cell lines; the A peptide and all of the other peptides tested, including the Int peptide, had no such effect. The decrease in activity after treatment with AInt and CInt peptides was not associated with a reduction in kinase levels, as shown by Western blot analysis on...
Fig. 2. Changes in histone H1 phosphorylation induced by the IC_{50} of the different peptides in SKOV-3 and IGROV-1 cells after immunoprecipitation with anti-CDK2 antibodies (A) or anti-cdc2 antibodies (B). Levels of CDK2 and cdc2 measured in CDK2 and cdc2 immunoprecipitates, respectively, are shown. Mix is the combination of A_int and C_int. CTR, control. C, Western blot analysis of cyclins A, B, and E, CDK2, CDK7, and cdc2 and p21^{WAF1} in SKOV-3 and IGROV-1 cells after a 4-h exposure to the IC_{50} of the different peptides. Mix is the combination of A_int and C_int. CTR, control.

No significant changes in total protein levels were observed with any peptide by Western blot analysis (Fig. 2C). Levels of cyclins A, B, and E, in particular, did not change after 4 h of treatment with active peptide concentrations. No differences were observed for the cdk tested, including CDK7 which positively regulates the activity of CDK2 and cdc2 (23).

We also tested the ability of the different peptides to inhibit CDK2 and cdc2 activity in vitro using recombinant CDK2/cyclin A, CDK2/cyclin E, and cdc2/cyclin B (Fig. 3). As in cells, A_int and C_int peptides were the most, although not the only, active in inhibiting the activity of both kinases. On increasing the amount of peptides added, some inhibition was found with A and B_int peptides too (data not shown), while both kinases were completely blocked with the A_int and C_int peptides.

Fig. 3. In vitro inhibition of recombinant cyclin A-CDK2, cyclin E-CDK2, and cyclin B-cdc2 kinase activities by different concentrations of the peptides. For details, see “Materials and Methods.”
CELL GROWTH INHIBITION INDUCED BY p21\textsuperscript{WAF1}-DERIVED PEPTIDES

Fig. 4. Flow cytometric analysis of IGROV-1 and SKOV-3 cells, untreated (A) or treated with 500 \mu M \textit{Int} peptide (B) or 30 or 50 \mu M \textit{AInt} peptide (C and D). Analysis was done after 8 and 24 h of treatment. At 24 h, the cells amounted to 98%, 84%, and 56% of controls for \textit{Int} (500 \mu M), \textit{AInt} (30 \mu M), and \textit{AInt} (50 \mu M) in SKOV-3 cells, respectively, and 100%, 80%, and 49% for \textit{Int} (500 \mu M), \textit{AInt} (30 \mu M), and \textit{AInt} (50 \mu M) in IGROV-1 cells, respectively.

These results confirm previous evidence that small p21\textsuperscript{WAF1}-derived peptides did not significantly reduce kinase activity in vitro, whereas the whole molecule or fragments of it did (3, 12–15). The presence of the internalization peptide may possibly stabilize the peptide cyclin/cdk complex and studies are in progress to verify this. The increase in potency observed in inhibiting the kinase activity in vitro for \textit{AInt} and \textit{CInt} peptides, however, is not sufficient to explain the striking difference in cell growth inhibition by these two peptides. The presence of the \textit{Int} sequence probably facilitated cell entry by the peptides, which also present increased potency, thus resulting in additive effects.

The fusion of the peptides we used is different from a previous study in which the internalization peptide was coupled to a peptide different from ours by a disulfide bond through the addition of a cysteine to both peptides (17, 18). Our results show that not only does the fusion of the peptides work, as shown by the increased internalization, but it also increases the potency of the peptide itself.

Flow cytometric DNA analysis did not bring to light any phase-specific arrest induced by active concentrations of \textit{AInt} and \textit{CInt} peptides, but rather a blockade of the progression of the cell cycle in all phases, as shown in Fig. 4 for \textit{AInt}. This is consistent with a generalized block of the activity of all the kinases involved in cell cycle progression and with data supporting the ability of p21\textsuperscript{WAF1} to block cells in both G\textsubscript{1} and G\textsubscript{2}, although we were expecting, in line with the findings of p21\textsuperscript{WAF1}-gene transfer experiments (7), a more pronounced G\textsubscript{1} block. However, on raising p21\textsuperscript{WAF1} levels through the introduction of wild-type \textit{tp53} in SKOV-3 cells, no clear G\textsubscript{1} block was reported (24).

We finally investigated whether the peptides induced apoptosis in the two cell lines: 24 h after treatment with the respective IC\textsubscript{50}, there was no evidence of DNA fragmentation, measured by gel electrophoresis, for any peptide in either cell line (data not shown). Similar results were obtained by microscopic examination of 4',6-diamidino-2-phenylindole-stained nuclei using the IC\textsubscript{50} of the different peptides: apoptosis was evident only in less than 5% of the cells examined (data not shown).
We have shown that small p21WAF1-mimicking peptides fused with the Antennapedia internalization peptide sequence enter the cells, inhibiting cdk activity and cell proliferation. These p21WAF1-mimicking peptides might have pharmacological potential for the therapy of proliferative disorders. In general, the results open the way to new therapeutic strategies based on the use of small peptides coupled with internalization sequences for the treatment of diseases for which intracellular penetration of a specific peptide may be beneficial. Synthetic molecules could be designed based on the structure of the peptides that prove active and this should result in stable, highly specific compounds.

References
p21\textit{WAF1}-derived Peptides Linked to an Internalization Peptide Inhibit Human Cancer Cell Growth

Marina Bonfanti, Stefano Taverna, Mario Salmona, et al.


Updated version: Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/57/8/1442

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.