Tumor Growth Suppression Through the Activation of p21, a Cyclin-Dependent Kinase Inhibitor

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Introduction

A. What is p21?

- p21 is a gene found on chromosome 6 at 6p21.2
- this gene produces a protein involved in cell cycle regulation
- its protein function is to act as a cyclin-dependent kinase inhibitor (Cdk inhibitor) to promote cell cycle suppression through the inhibition of phosphorylation by cyclin-dependent kinases (Cdk's)

Introduction Cont'd

B. How does p21 cause tumor suppression?

- tumor growth within tissues results from uncontrolled cell growth after DNA damage

- tumor suppression by p21 occurs in several steps:

1. Cellular DNA becomes damaged by chemical mutagenesis or radiation

2. Tumor suppressor gene, such as p53, is activated

Introduction Cont'd

3. p53 then binds to a response element on p21, which becomes activated

4. p21 then binds to either Cdk2, Cdk3, Cdk4, and Cdk6 to cause cell cycle arrest through its inhibition of cyclin-dependent kinases

5. Cell cycle arrest at Go/G1 continues until the DNA is repaired and p53 levels decline as a result of p21 inactivation

Structure and Function of p21

A. Function of p21

1. Role of p21 in cell cycle regulation

- acts as a Cdk inhibitor and promotes cell cycle arrest after DNA damage

2. Location of p21 actions in cell cycle

- the p21 protein product causes cell cycle arrest at Go/G1, which occurs just before S phases

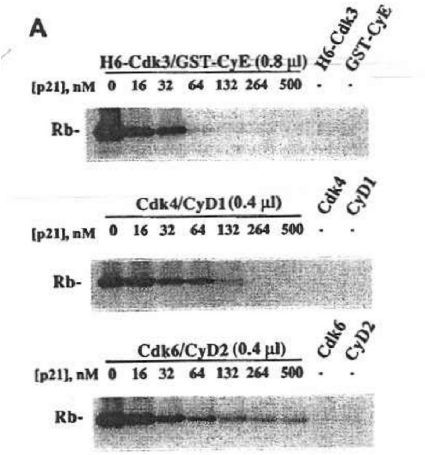
Structure and Function of p21 Cont'd

3. Effectors of p21 include tumor suppressor genes and in particular p53, which binds to the response element on p21

4. p21 is able to bind to Cdk2,Cdk3, Cdk4, and Cdk6 at the restriction point, Go/G1, in the cell cycle

- various concentrations of p21 are required for inhibition of different Cdk's (See next slide for data results on inhibition by p21)

GST-Rb kinase assay using sf9 extracts and p21 concentrations



Structure and Function of p21 Cont'd

5. p21 deficiency has been shown to cause tumor growth in various tissues, including the pituitary and thyroid gland, liver, and pancreas

6. The synergistic interaction of p21 and p18 can result in tissue specificity

- The loss of both p21 and p18 result in more rapidly forming tumors than p21 deficient mice, especially in the pituitary and lung
- No tumor growth is present when a functional Cdk inhibitor is present in the absence of p21, such as p18

Tumor Formation in p21-/- mice occurs in various tissues

TABLE 2. Spontaneous tumor formation and incidence in p18-, p21-, and p18/21-deficient mice over 1 year[a]

Organ	Wild type ($n = 17$)	$p18^{-/-}$ ($n = 18$)	$p21^{-/-}$ ($n = 22$)	$p18^{-/-}$-$p21^{-/-}$ ($n = 18$)
Pituitary				
Normal	17/17	2/18	18/21	1/17
Hyperplasia		8/18	3/21	1/17
Adenoma		7/18		15/17
Carcinoma		1/18[b]		
Stomach				
Normal	17/17	16/16	17/22	2/18
Neuroendocrine hyperplasia			5/22	16/18
Lungs				
Normal	17/17	15/15	20/21	9/17
Histiocytic pneumoniae			1/21	5/17
Bronchioalveolar adenoma				2/17
Bronchioalveolar carcinoma				1/17[c]
Liver				
Normal	17/17	15/15	22/22	14/16
Hepatic nodular hyperplasia				2/16
Adrenal				
Normal	15/15	7/17	19/19	1/13
Medullary hyperplasia		8/17		12/13
Pheochromocytoma		2/17[c]		
Thyroid				
Normal	17/17	12/18	22/22	15/17
C-cell hyperplasia		5/18		2/17
C-cell adenoma		1/18[d]		
Testis				
Normal interstitium	5/5	5/15	10/10	1/9
Interstitial hyperplasia		14/15		8/9
Interstitial adenoma		1/15		
Parathyroid				
Normal	17/17	15/15	16/16	12/14
Hyperplasia				2/14
Pancreas				
Normal	16/17	15/15	20/20	11/12
Islet cell hyperplasia	1/17			1/12
Intestine (duodenum), normal	17/17	15/15	18/18	15/15

P21 and Cyclin-Dependent Kinase Expression in Various Tissues

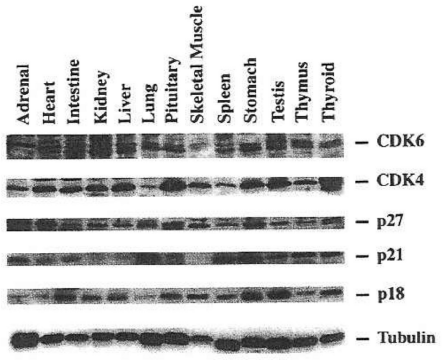


FIG. 5. Expression of CDK and CDK inhibitors in mouse tissues. Total cell lysates were prepared from the indicated wild-type tissues. Expression patterns of CDK4, CDK6, p27, p21, p18, and tubulin were determined by Western blot analysis. Tubulin expression was used to demonstrate equal loading of protein lysates.

Structure and Function of p21 Cont'd

B. Structure of p21

1. The promoter region and binding site for p53 can be shown through deletion analysis

- deletions performed on the first 164 amino acid residues of the promoter
- results indicate amino acid residues 1-80 are most important for inhibition of Cdk2 at the S phase restriction point

P21 Structure and Deletion Analysis

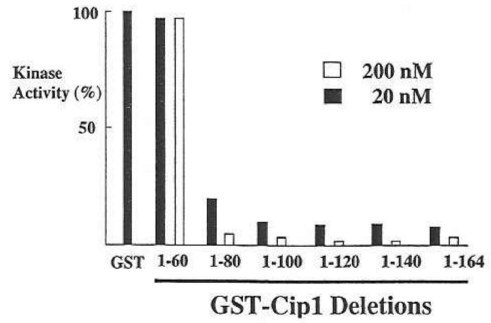


Figure 3. Deletion analysis of p21. The indicated C-terminal deletions of p21 were purified from *E. coli* as GST-fusion proteins and assayed for inhibition of Cdk2/cyclin A using histone H1 as substrate. Activities were quantitated by filter binding (solid symbol, 20 nM GST-fusion; open symbol, 200 nM GST-fusion).

Anti-Cancer Drugs and Their Interaction with p21

A. Daunomycin and its effects on p21 expression in HCT116 and MCF7 cells, which are human colorectal and breast cancer cells respectively

1. What is Daunomycin?

- a drug which causes cell cycle arrest through the indirect activation of p21

Anti-Cancer Drugs and Their Interaction with p21 Cont'd

2. Western Blot Analysis Results

- Increase in p21 expression with Daunomycin treatment in cancer cells

3. Analysis of p53 after Daunomycin treatment

- Drug causes a greater amount of cells arrested at Go/G1 after treatment

- Drug also causes an increase in cell apoptosis after treatment

Effects of Daunomycin on p21 expression in Human Cancer cells

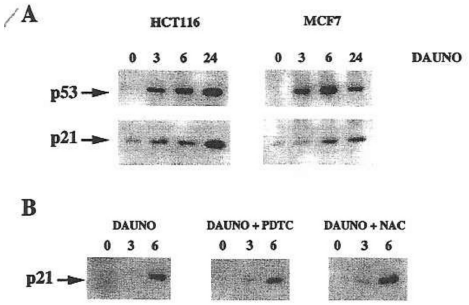


Fig. 1. Enhanced p21 expression in daunomycin-treated HCT116 and MCF7 cells. A, p53 and p21 protein accumulation after daunomycin treatment. HCT116 and MCF7 cells were treated with daunomycin (1 μM) for 3, 6, and 24 h. Nuclear protein extracts (10 μg) were analyzed by Western blotting using an anti-p53 or an anti-p21 antibody. B, effect of PDTC or NAC treatment on p21 induction by daunomycin. MCF7 cells were incubated with PDTC (60 μM) or NAC (10 mM) for 1 h before treatment with daunomycin (1 μM). Nuclear extracts were prepared after 3 and 6 h of daunomycin treatment and analyzed as described in A.

Role of p53 in cell cycle arrest and apoptosis after daunomycin

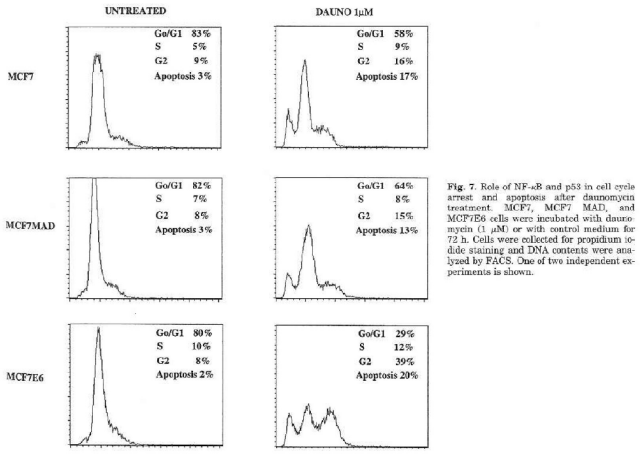


Fig. 7. Role of NF-κB and p53 in cell cycle arrest and apoptosis after daunomycin treatment. MCF7, MCF7 MAD, and MCF7E6 cells were incubated with daunomycin (1 μM) or with control medium for 72 h. Cells were collected for propidium iodide staining and DNA contents were analyzed by FACS. One of two independent experiments is shown.

Anti-Cancer Drugs and Their Interaction with P21 Cont'd

3. Overall effects and benefits of Daunomycin treatment

- Drug is a potent inducer of both p21 and p53 in human colorectal and breast cancer cells
- Use of daunomycin allows inhibition of cellular metastasis through activation of p21

Anti-Cancer Drugs and Their Interaction with p21 Cont'd

B. Effects of Apicidin on p21 activation in human prostate carcinoma cells

1. Apicidin
   - histone deacetylase inhibitor
   - activates expression of both p21 mRNA and protein (See next figure)
   - activation occurs through the accumulation of acetylated histones in the p21 chromatin

Induction of p21 mRNA and protein by Apicidin

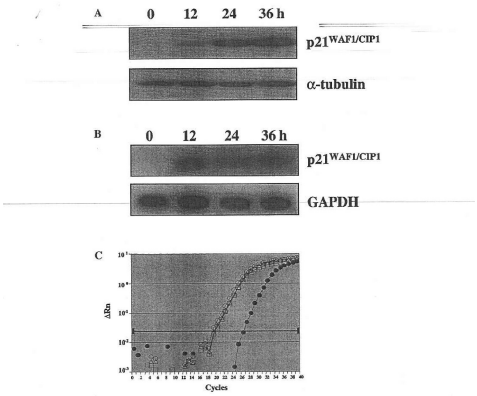


FIG. 2. Apicidin induces p21 protein and mRNA. (A) PC-3-M cells were incubated with apicidin (5 μM) for the time indicated and equal amounts of cell lysate (60 μg of protein) were separated on 12% SDS–polyacrylamide gels, transferred to nitrocellulose, and subjected to Western blot analysis using anti-p21 and anti-α-tubulin antibodies and ECL detection. (B) Cells were incubated with apicidin (5 μM) for the time indicated, total RNA was prepared and p21 mRNA was detected by Northern blot analysis. The same blot was probed with a cDNA to glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) to demonstrate comparable levels of mRNA per lane. (C) The quantification of *p21* gene expression was carried out using real-time fluorescence detection. Cells were incubated with apicidin (5 μM) for 0 h (closed circle), 12 h (open rectangular), 24 h (open triangle), and 36 h (open circle). p21 cDNA was prepared and *p21* gene was detected by real-time quantitative PCR using SYBR Green I dye described under Materials and Methods. The relative fluorescence value (ΔRn) is plotted as a function of cycle number. The threshold used for the calculation of relative initial template amounts is indicated by the dark horizontal line.

Anti-Cancer Drugs and Their Interaction with P21 Cont'd

2. Apicidin mechanism of action
   - Activates p21
   - Causes loss of viability in human prostate carcinoma cells in a concentration-dependent manner

Effects of apicidin on Human Prostate Carcinoma Cells

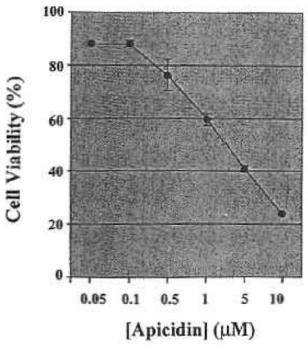


FIG. 1. Apicidin inhibits growth of PC-3-M cells. Cells were incubated with apicidin for 35 h and cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Anti-Cancer Drugs and Their Interaction with p21 Cont'd

3. p21 promoter activation by apicidin
   - Full-length vs. 93S p21 promoter luciferase reporter constructs (See next figure)
   - Results: 93S construct had higher amount of p21 induction
   - Conclusion: 93S section of p21 promoter is essential for p21 responsiveness to apicidin

P21 promoter activation by Apicidin

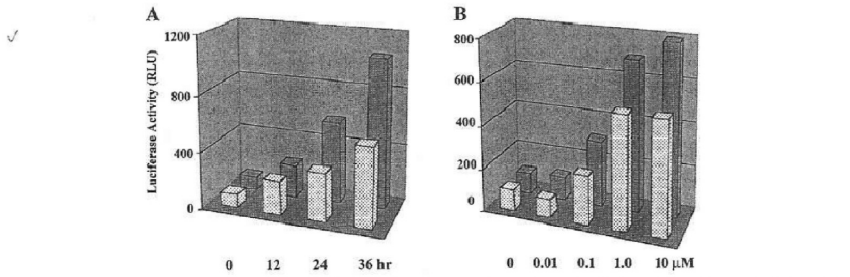


FIG. 3. Activation of the p21 promoter by apicidin. PC-3-M cells were transiently transfected with full length (dotted bar) and 93S (gray bar) p21 promoter-luciferase reporter constructs. The cells were incubated for the time indicated in the presence of 5 μM apicidin (A) or incubated for 36 h in different concentrations of apicidin (B). Cells were harvested and luciferase activity (RLU, relative luciferase units) was measured.

Anti-Cancer Drugs and Their Interactions with p21 Cont'd

4. Histone acetylation by apicidin
   - Levels of histone H3 and H4 aceytlation increased over time in pancreatic carcinoma cells
   - Immunoglobulin control showed no histone acetylation (See next figure)
   - Conclusion: inhibiting histone deacetylase allows apicidin to promote hyperacetylation of specific histones, H3 and H4, in the chromatin of the p21 gene

H3 and H4 accumulation induction by Apicidin

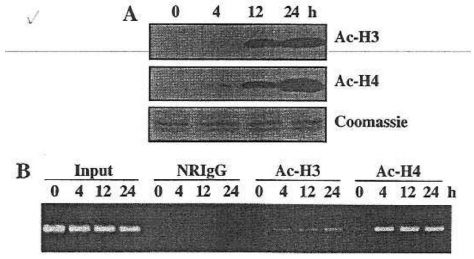


FIG. 4. Apicidin induces accumulation of acetylated histones H3 and H4. (A) Western blot analysis of acetylated histones H3 and H4 in PC-3-M cells. Histones were isolated by acid extraction from cells incubated with apicidin (5 μM) for the time indicated and detected by using anti-acetylated histones H3 and H4 antibodies and ECL detection. A parallel gel was stained with Coomassie blue for a loading control. (B) Crosslinked chromatin from each time point was incubated with acetylated histones H3 and H4 antibodies or normal rabbit IgG (NRIgG). Immunoprecipitates from each antibody were aliquotted and analyzed by normal PCR with primer specific for p21 promoter as indicated under Materials and Methods. PCR products were analyzed by 1.2% agarose/ethidium bromide gel electrophoresis.

Anti-Cancer Drugs and Their Interactions with p21 Cont'd

5. Overall effects and benefits of apicidin
   - Causes cell cycle arrest in pancreatic carcinoma cells through transcriptional activation of p21
   - Causes accumulation of acetylated histones H3 and H4 that are associated with the p21 promoter
   - p21 is a key target in growth inhibition by apicidin in several tissues besides pancreatic carcinoma cell lines

P21 Transfection in Carcinoma Cell Lines

A. Human Glioma Cells

- Occur by a malignant transformation of low grade astrocytoma

B. Northern Blot analysis

- increase in p21 mRNA after p21 transfection into glioma cells (See next figure)

Increase in p21 mRNA and Protein expression in p21 Transfectants

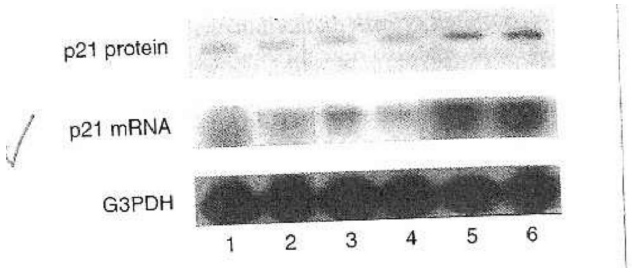


Figure 1. Western/Northern blot analysis. Control U251MG and T98G revealed low level of P21 protein and its mRNA expression. Each *p21* transfectant showed the increase of P21 protein and its mRNA. Lane 1: control U251MG, lane 2: control T98G, lane 3: U251MG transfected with pcDSR$\alpha\delta$, lane 4: T98G transfected with pcDSR$\alpha\delta$, lane 5: U251MG transfected with pcDSR$\alpha\delta$–p21, lane 6: T98G transfected with pcDSR$\alpha\delta$–p21.

p21 Transfection in Carcinoma Cell Lines Cont'd

C. p21 transfection vs. control cell lines

- p21 transfection promotes a decrease in tumor cell growth through the accumulation of p21 transfected cells in the Go/G1 phase (See next figure)

Effects of p21 transfection in Human Glioma Cells

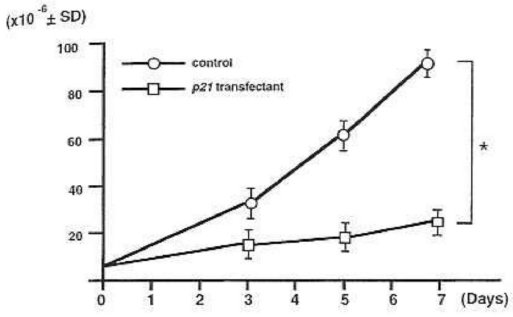


Figure 2. Growth curve of U251MG human glioma cells. The growth of *p21* transfectant was inhibited compared as control cells. The number of the cells at each point is the mean with standard deviation of four different wells. The asterisk represent significance at $p < 0.001$.

p21 Transfection in Carcinoma Cell Lines Cont'd

D. Conclusions

- Decrease in glioma tumor cell growth by p21 transfection
- Growth inhibition was followed by Go/G1 cell cycle arrest as the result of p21 activation

Conclusions

A. p21 is directly activated by p53 binding to its response element in the promoter region
   - Occurs after DNA damage

B. Cells accumulate at the Go/G1 checkpoint
   - Occurs at this stage to prevent further S phase replication of the damaged cell

C. p21 binds to various cyclins in Go/G1, such as cyclin B, to promote Cdk inhibiton and cell cycle arrest

Conclusions Cont'd

D. Apicidin and Daunomycin are currently used to treat patients that contain a p21 deficient gene

E. More current treatments also help to promote a reduction in tumor progression, including p21 transfection in cells containing a p21 gene deletion