Induction of Cell Death of Gastric Cancer Cells by a Modified Compound of the Annonaceous Acetogenin Family

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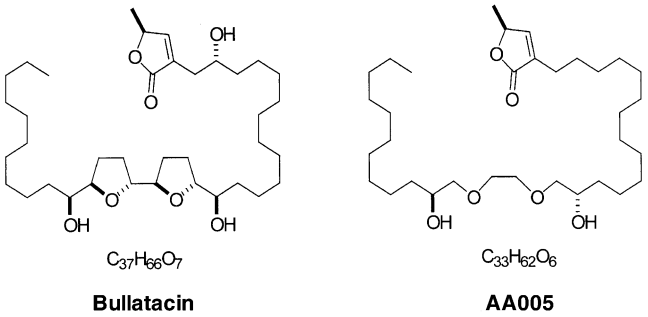
Annonaceous acetogenins are a family of natural products with antitumor activities. The polyether mimic AA005 has been synthesized. The cytotoxic mechanism of AA005 was investigated. It was found that AA005 can induce the cell death of gastric tumor cells. Most AA005-induced cell death is due to necrosis but partial and p53-independent apoptosis is also detected. An expanded study with gastric tumor cells and a cell-free system indicates that AA005 affects NADH:ubiquinone oxidoreductase (complex I; NADH = nicotinamide adenine dinucleotide, reduced form) in the mitochondrial electron transport system. These results show for the first time that the polyether mimic AA005 acts on the same biological target as the natural annonaceous acetogenins.

Introduction

Annonaceous acetogenins are a well-established family of natural products with significant bioactivities, especially as growth inhibitors of certain tumor cells.[1] For example, bullatacin, one of the most potent naturally occurring acetogenins,[2] can induce the cell death of various tumor cells.[3–6] Most members of the annonaceous acetogenin family contain two or three ring systems, including one/two tetrahydrofuran (THF) ring(s) in the middle of the alkyl chain, and an α,β-unsaturated γ-lactone at one end of the alkyl chain.

Apoptosis and necrosis are two distinct types of cell death, characterized by differences in cellular morphology and executive molecules. Necrosis is thought to be a passive cell death and is defined by swelling and rupture of the membranes of mitochondria and dissolution of organized structures.[7] Apoptosis, in contrast, is a controlled biological process to remove unwanted cells and, thus, is regulated in a sophisticated manner with antiapoptotic molecules, such as Bcl-2 and Bcl-X_L, or proapoptotic ones, such as Bax and Bak. During the processes of apoptosis, cells undergo distinct biochemical and morphological changes, which include membrane blebs and shrinking, nuclear condensation, DNA fragmentation, and disintegration of the dying cell into apoptotic bodies.[7–10]

During the last few years, we developed a series of molecules similar to the annonaceous acetogenins and based on polyether mimicry, and we found that the most of these molecules show comparable activity to the natural products.[11–14] Some were found to exhibit better selectivity between the tumor cells and normal human cells, a selectivity which is difficult to achieve with the natural acetogenins. In the present study, a new annonaceous acetogenin mimic, AA005, has been synthesized and investigated. The chemical structure of AA005 combines the advantages of bullatacin and those of other molecules from previous studies of annonaceous acetogenin mimicry.[7, 12] The chemical structures of AA005 and bullatacin are shown in Scheme 1. Previous publications showed that analogues of



Scheme 1. *The chemical structures of the annonaceous acetogenin bullatacin and its mimic AA005.*

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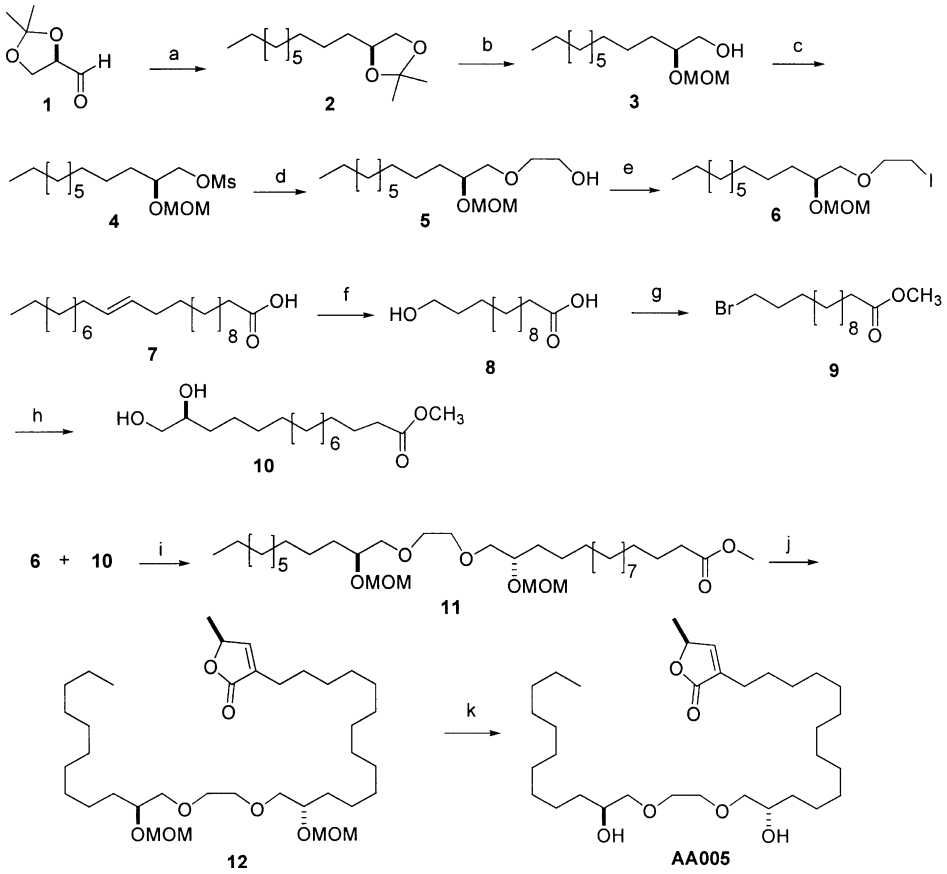
annonaceous acetogenins could induce apoptosis.[4, 15, 16] However, we have found that AA005 can induce only partial and p53-independent apoptosis of gastric tumor cells and most of the induced cell death was due to necrosis. A previous publication drew the preliminary conclusions that annonaceous acetogenins may induce apoptosis and inhibit the electron transport chain by measuring decylubiquinone as an artificial ubiquinone analogue in submitochondrial particles.[17] We have now expanded on the above finding with the gastric cancer cell line AGS and a cell-free system, and we have found indications that AA005 affects NADH:ubiquinone oxidoreductase (complex I; NADH = nicotinamide adenine dinucleotide, reduced form) in the mitochondrial electron transport system.



Scheme 2. The chemical synthesis of AA005. Reagents and conditions: a) 1. $C_8H_{17}CH{=}PPh_3$, 90%; 2. 10% Pd-C, H_2, CH_3OH, 95%; b) 2. 10% HCl, CH_3OH, 93%; 2. $HC(OCH_3)$, CSA, CH_2Cl_2, then DIBALH in toluene, CH_2Cl_2, 0 °C, 90%; c) MsCl, Et_3N, CH_2Cl_2, 100%; d) ethylene glycol, NaH, DMF, 72%; e) I_2, imidazole, PPh_3, 92%; f) 1. O_3, 0 – 5 °C, EtOH/cyclohexane (1:5); 2. KBH_4, 87% from erucic acid; g) 1. HBr, HOAc, 75%; 2. MeOH, $SOCl_2$; h) 1. PPh_3; 2. tBuOK, then (R)-glyceraldehyde acetonide; 3. H_2, EtOH, 5% Pd-C; 4. H^+, MeOH, 61% over 4 steps; i) 1. Bu_2SnO, $CHCl_3$/MeOH (10:1), reflux; 2. CsF, DMF; 3. MOMCl, iPr_2NH, CH_2Cl_2, 56% over 3 steps; j) 1. LDA, (S)-Me(OTHP)CHCHO; 2. 9% H_2SO_4, THF; 3. $(F_3CCO)_2O$, NEt_3, CH_2Cl_2, 60% over 3 steps; k) $BF_3 \cdot Et_2O$, Me_2S, 0 °C, 62%. CSA = (1S)-(+)-10-camphorsulfonic acid, DIBALH = diisobutylaluminum hydride, DMF = N,N-dimethylformamide, LDA = lithium diisopropylamide, MOM = methoxymethyl, Ms = mesyl = methylsulfonate, THP = tetrahydropyranyl.

Results and Discussion

Chemical synthesis of AA005

The synthesis of AA005 was accomplished by our previously reported procedure with slight modification (Scheme 2; see the Supporting Information for details).[12, 14] In brief, a Wittig reaction of aldehyde **1** followed by hydrogenation gave **2**. The alcohol **3** was obtained by selective protection of the secondary alcohol with the MOM group in a two-step procedure. Treatment of mesylate **4** with the mono sodium salt of ethylene glycol in DMF afforded alcohol **5**, which was further iodinated with I_2 and Ph_3P to give segment **6** in good yield. Segment **10** was prepared from commercially available material **7** through a sequence of routine reactions, as shown in Scheme 2. The coupling of segments **6** and **10** was achieved by Bu_2SnO and CsF, and the resultant alcohol was then protected as the MOM ether **11**. The α,β-unsaturated lactone unit of **12** was introduced by aldol condensation of **11** with D-*O*-THP-lactal, acid-catalyzed deprotection of *O*-THP, lactonization, and β-elimination of the hydroxy group. Finally, the MOM ether protecting groups were removed by $BF_3 \cdot Et_2O$ in dimethyl sulfide to afford the target molecule AA005.

Detection of cytotoxicity of AA005

To investigate the effects of AA005 on the cell viability, we administered AA005 to AGS gastric tumor cells and then analyzed cell viability with a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (see the Methods Section). The results demonstrated that AA005 induced the cell death of AGS cells in a dose-dependent manner (Figure 1 a). Furthermore, it was also shown that there was a time-dependent effect on cell cytotoxicity when AGS cells were exposed to AA005 for different durations (Figure 1 b). It was shown previously with a bicinchoninic acid assay that growth of breast cancer cells was inhibited by bullatacin, a natural compound of the annonaceous acetogenin family; this is concordant with our results.[5]

Detection of cell permeability and the change of mitochondrial potential in AA005-treated AGS cells

Dead cells lose the integrity of their cell membranes, so the cellular membrane permeability of the AA005-treated AGS cells was analyzed with a 4,6-diamidino-2-phenylindole (DAPI) assay, in which the nuclear DNA of permeabilized cells is stained with DAPI. The results showed that about half of the AGS cells treated

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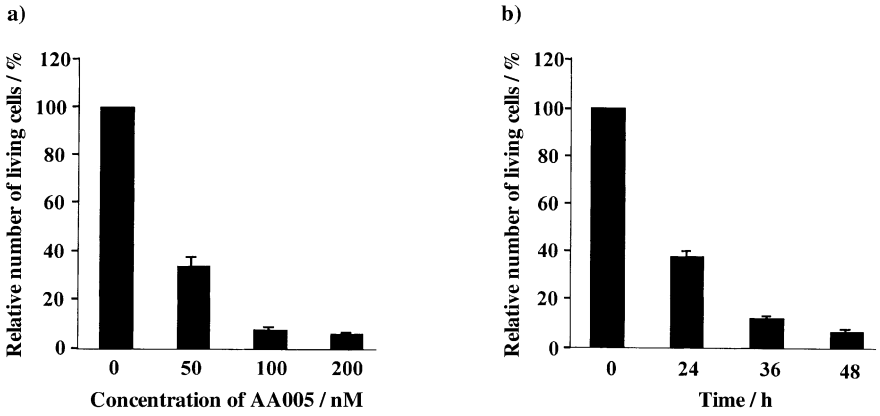

Figure 1. Induction of time- and dose-dependent cell death by AA005. a) Exponentially growing AGS cells were treated with the indicated dose of AA005 for 48 h. b) Exponentially growing AGS cells were treated with AA005 (100 nM) for the indicated time. The living cells were analyzed with an MTT assay and scored. At least three independent experiments were averaged and the mean data are shown (standard deviations are shown as bars).

with AA005 for 24 h were permeabilized, and after 48 h of treatment nearly all of the treated cells were permeabilized (Figure 2 a and c).

The change of mitochondrial potential ($\Delta\psi_m$) is also an indicator of dying cells. We detected the $\Delta\psi_m$ values of AGS cells by a 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1) assay (see the Methods Section). The data indicated that the loss of mitochondrial potential occurred when the cells were incubated with AA005 (Figure 2 b), and the amount of the cells losing potential was nearly the same as the amount of permeabilized cells (Figure 2 c). Taken together, these results indicate that AA005 causes the loss of AGS cellular integrity.

Partial induction of p53-independent apoptosis by AA005

There are two kinds of the cell death: apoptosis and necrosis. In the present study, flow cytometry analysis showed an increase in M30-positive cells and the sub-G1 DNA region (below the G0/G1 peak) when AGS cells were treated with AA005 (Figure 3 a and b),[18] a fact indicating that AA005 can induce apoptosis of AGS cells. However, the amount of apoptotic cells increased only slightly during the longer incubation with AA005 (Figure 3 a and b, compare columns at 24 h and 48 h), whereas the amount of dead cells in the sample treated for 48 h increased significantly over the amount after 24 h of treatment (see Table 1). These results suggest that the longer treatment with AA005 induces necrosis of AGS cells rather than apoptosis. Interestingly, a recent report showed that the natural partner of AA005, bullatacin, induced more apoptotic cells than necrotic cells;[4] this suggests that the cytotoxic mechanism of AA005 is not exactly the same as that of bullatacin.

To assess whether the cytotoxicity of AA005 correlates with the tumor-suppressor gene *p53*, we analyzed the AA005-induced apoptosis of a *p53*-mutated cell line (AER cells), which is a stable AGS cell line transfected with mutated *p53* (see the Methods Section). The results showed that the amount of AER apoptotic cells induced by AA005 was nearly the same as that of AGS cells (Figure 3 a and b). In addition, the amount of p53 protein found in the AGS and AER cells was not increased in the presence of AA005 (Figure 3 c), which suggests that AA005 does not activate p53 pathways. Taken together, these results indicate that AA005-induced apoptosis of gastric tumor cells is independent of p53.

Inhibition of respiration of AGS cells in the presence of AA005

On the basis of previous publications[5, 17] and our data on the change of the mitochondrial potential, $\Delta\psi_m$, we speculated that AA005 might affect the mitochondrial respiratory chain. To test this hypothesis, we measured the O_2 consumption of AGS cells in the presence of AA005. It was found that AA005 could completely inhibit the endogenous respiration of AGS cells (Figure 4 a, phase II), whereas AGS cells without the treatment of AA005 showed the normal profile of O_2 consumption (Figure 4 a, phase I). Moreover, when succinate, a substrate of succinate: ubiquinone oxidoreductase (complex II) in the respiratory chain, was added into the mixture, the endogenous respiration was restored immediately (Figure 4 a, compare phase I to phase III); this suggests that AA005 may affect NADH:ubiquinone oxidoreductase (complex I) in the mitochondrial electron transport chain. At the same time, a classical complex I inhibitor, rotenone, was used as a positive control, and a similar profile was detected (Figure 4 b). Therefore, we conclude that AA005 inhibits the respiration of AGS cells by affecting complex I in the mitochondrial electron transport chain. Since it has been reported that the

Table 1. The percentage of apoptotic cells in the population of cell death.

Drug treatment[a]	Cell death[b] [%]		Apoptosis [%]	
	cell permeability	mitochondria permeability	sub-G1 cells	M30-positive cells
24 hours	43.5	35.9 ± 2.248	21.11 ± 0.721	18.55 ± 3.441
48 hours	98	93.6 ± 1.873	25.79 ± 0.792	26.13 ± 1.850

[a] Cells were treated with 100 nM AA005. [b] The permeability of cells or mitochondria represents the cell death. See the text and the Methods Section for further details.

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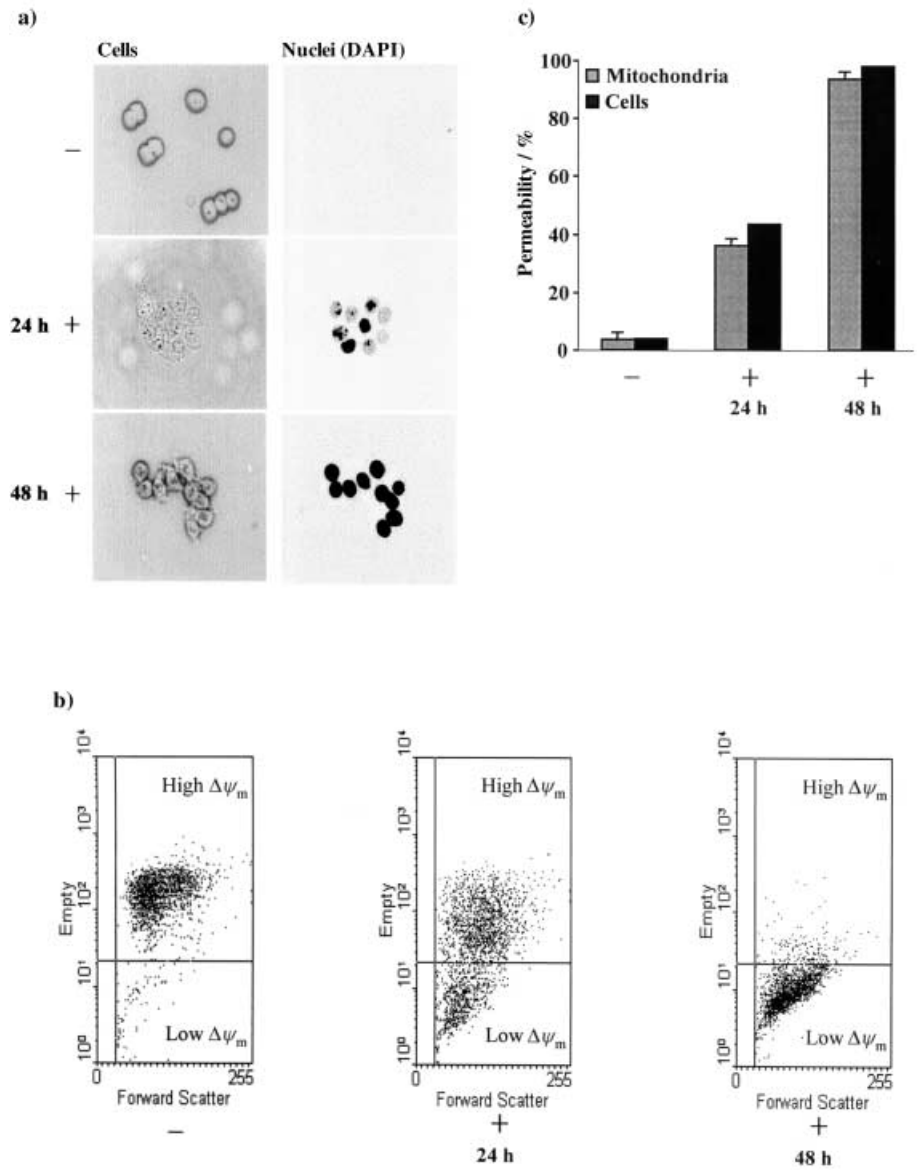

Figure 2. Induction of cell membrane permeability and loss of mitochondrial potential by AA005 treatment. a) AGS cells were treated with 100 nM AA005 for 24 and 48 h, and then the cells were stained with DAPI (1 μg mL^{-1}) and observed by fluorescence microscopy in visible (cells) and fluorescence (nuclei) light. b) The change of mitochondrial potential was analyzed by flow cytometry after the drug-treated cells were stained with JC-1. c) Statistical data of cell membrane permeability and breakdown of the mitochondrial potential. At least three independent experiments are averaged, and the mean data are shown (standard deviations are shown as bars).

typical natural annonaceous acetogenin, bullatacin, also inhibited adenosine triphosphate (ATP) production through its interaction with complex I in the mitochondrial electron transport chain,[6] at least one of the molecular targets of AA005 is the same as that of natural compounds in the annonaceous acetogenin family.

Inhibition of the complex I of the mitochondrial electron transport chain by AA005 in cell-free systems

As the absorption spectrum of NADH differs from that of its oxidized counterpart, we should be able to scan the absorption spectrum of the mitochondria and ascertain whether the nicotinamide adenine dinucleotide is in the oxidized or reduced state. To test further whether AA005 can directly inhibit NADH:ubiquinone oxidoreductase (complex I), we isolated mitochondria from AGS cells and analyzed the level of NADH with and without AA005 (see the Methods Section). After the mitochondria were treated with 100 nM AA005 for 2 h, a significant peak was detected at about 340 nm (Figure 5 a); this is thought to correspond to the specific absorbtion wavelength of NADH. This result indicates that AA005 inhibition of NADH:ubiquinone oxidoreductase (complex I) in the electron transport pathway leads to the enrichment of NADH. A similar result was observed when the cells were treated with rotenone (Figure 5 b).

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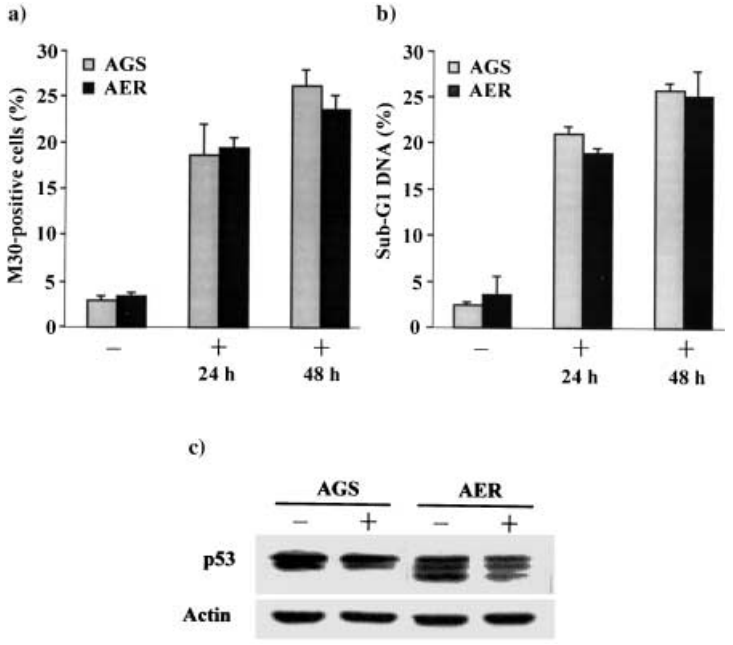

Figure 3. Induction of partial and p53-independent apoptosis of AGS cells by AA005 treatment. a) AGS and AER cells were incubated with 100 nM AA005 for the indicated time. Apoptotic cells were analyzed with an M30 assay. b) The percentage of sub-G1 cells was analyzed by flow cytometry. At least three independent experiments are averaged and the mean data are shown (standard deviations are shown as bars). c) AGS and AER cells were incubated with 100 nM AA005 for 48 h. The cell lysates were analyzed by Western blotting. The amount of proteins was controlled with an antibody against actin.

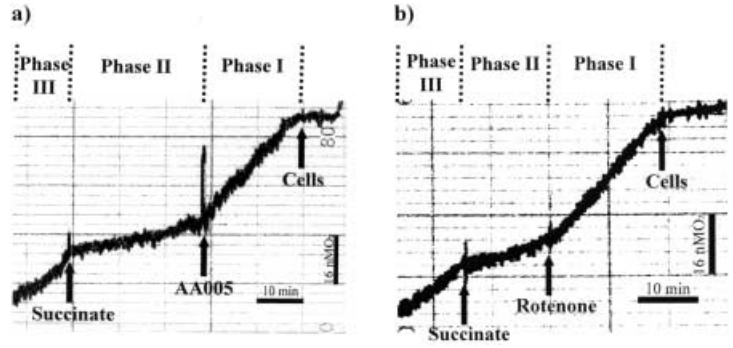

Figure 4. In vivo detection of inhibition of the mitochondrial respiratory chain by AA005. a) O_2 consumption of AGS cells in the presence of 100 nM AA005 was measured in a biological oxygen monitor (see the Methods Section). b) The typical inhibitor of mitochondrial respiratory chain, rotenone, was used as a positive control. Typical pictures are shown from the results of three independent experiments.

Since electron transport is tightly coupled to ATP synthesis, the inhibition of complex I by AA005 prevents ATP synthesis. It was reported that ATP depletion could block apoptosis.[19, 20] These previous data therefore support our results from the in vivo and in vitro experiments, which suggest that most of the cell death induced by AA005 came about through necrosis rather than apoptosis (see Table 1).

Methods Section

Synthesis of AA005: See the Supporting Information.

Cell culture: AGS human gastric tumor cells (provided from Dr. J. J. Y. Sung's laboratory in the Chinese University of Hong Kong) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL) at 37 °C in a 5% CO_2 atmosphere.

Cell death assays: After treatment of AGS cells with AA005, the viability of cells was measured by an MTT assay according to the manufacturer's protocol (Sigma). Meanwhile, apoptotic cells were analyzed either by measuring the sub-G1 DNA region (below the G0/G1 peak) with a flow cytometer (FACScan, Becton Dickinson) or by detecting M30-positive cells according to the manufacturer's instructions (Roche Molecular Biochemicals).

Construction of the mutated *p53* cell line: pEGFP-rehsTP53, a plasmid coding human mutant p53 with no DNA binding domain (deletion of amino acids 44 – 306), was constructed. AGS cells were infected with pEGFP-rehsTP53 by using LipoFECTIN 2000 (GIBCO BRL). A stable transformant of the plasmid, named the AER cell line, was selected in the presence of G418 (500 $\mu g\,mL^{-1}$; Roche Molecular Biochemicals) and maintained with G418 (200 $\mu g\,mL^{-1}$) at all times.

Western blotting: AGS and AER cells were harvested and lysed once with loading buffer (50 mM tris(hydroxymethyl)-aminomethane (Tris), 100 mM 1,4-dithiothreitol, 2% sodium dodecylsulfate (SDS), 10% glycerol, and 0.1% bromophenol blue; pH 6.8). The samples were subjected to 12% SDS-PAGE. Immunoblotting was carried out with an anti-p53 antibody (Santa Cruz Biotechnology). The proteins were detected by enhanced chemiluminescence (ECL, Amersham).

Assessment of cell permeability with DAPI staining: The cells were collected and suspended with phosphate-buffered saline containing DAPI (Sigma). The samples then were mounted on a slide and analyzed by fluorescence microscopy.

Assessment of the change in the mitochondrial potential ($\Delta\psi_m$) with JC-1 staining: Changes in the mitochondrial membrane potential were measured with a JC-1 assay.[21] In brief, after incubation with or without AA005 for the indicated times, 1×10^6 cells were resuspended in medium and incubated with JC-1 (Calbiochem-Novabiochem) at a final concentration of 5 $\mu g\,mL^{-1}$ for 20 min. The samples were then analyzed with by flow cytometry. A high FL-2 fluorescence indicates a normal $\Delta\psi_m$ value, whereas a gain in FL-1 fluorescence indicates a loss of potential.

Measurement of O_2 consumption of AGS cells: Determination of the O_2 consumption rate in intact cells was carried out as described by Bai et al.[22] In brief, $3.0 - 5.0 \times 10^6$ cells were harvested and suspended in buffer (0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris/HCl; pH 7.4) at 25 °C. The O_2 consumption measurements were carried out in a biological oxygen monitor in the presence of inhibitors (100 nM AA005 or 100 nM rotenone) and substrates (5 mM succinate).

Measurement of deoxidized NADH of isolated mitochondria: 6×10^7 cells were washed once in MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris/HCl, 1 mM ethylenediaminetetraacetate (EDTA); pH 7.5), resuspended in RSB solution (3 mL; 10 mM NaCl, 2.5 mM $MgCl_2$, 10 mM Tris/HCl; pH 7.5), and incubated on ice for 15 min. All the following procedures were carried out at 4 °C. The cells were Dounce

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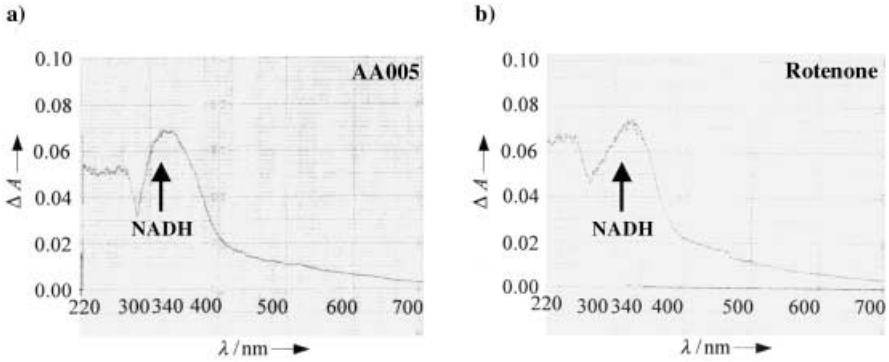

Figure 5. *In vitro measurement of the absorbance (A) spectrum of the mitochondrial electron transport chain after AA005 treatment. a) Isolated mitochondria of AGS cells were treated with 100 nM AA005 for 2 h, and then the absorbance spectrum of the mitochondria was recorded by a double-beam spectrophotometer (see the Methods Section). b) The inhibitor rotenone was used as a positive control. The arrows indicate the reduced state of NADH. Typical pictures are shown from the results of three independent experiments.*

homogenized and centrifuged (1000 *g*, 5 min) to remove the nuclei-containing pellet. The supernatants were centrifuged again (12 000 *g*, 30 min) to collect the mitochondria-containing pellet. The mitochondria-containing pellet was washed once with MS buffer and then resuspended in MS buffer. The mitochondrial fractions were treated with either 100 nM AA005 or 100 nM rotenone for 2 h. The samples were then analyzed with a dual-wavelength/double-beam recording spectrophotometer (UV3000, Shimadzu). The scan rate was 100 nm min^{-1}, the recording speed was 20 nm cm^{-1}, and the scan scope was 200 – 800 nm.

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