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# Napyradiomycin A1, an inhibitor of mitochondrial complexes I and II

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We have previously proposed a cell-based screening method 'EGF-induced (epidermal growth factor) filopodium protrusion assay' to identify mitochondrial electron transport inhibitors or glycolysis inhibitors. Filopodia are spike-like cell membrane projections that contribute to tumor metastasis. Previously, we have reported that mitochondrial electron transport inhibition resulted in the inhibition of EGF-induced filopodium protrusion in human adenocarcinoma A431 cells only when their glycolytic pathways were restricted.[1] By using the inhibition of filopodium protrusion as an indicator, we identified napyradiomycin A1 (Figure 1a; isolated from *Streptomyces antimycoticus* NT17),[2] which was previously identified as an antibacterial antibiotic,[3] as a candidate of mitochondrial electron transport inhibitor. A431 cells were treated with napyradiomycin A1 with or without 10 mM 2-deoxy-D-glucose (Sigma-Aldrich, St Louis, MO, USA) for 30 min, followed by 30 ng ml$^{-1}$ EGF (Sigma-Aldrich) stimulation and observation under a microscope. As shown in Figure 1b, 20 μM napyradiomycin A1 inhibited EGF-induced filopodium protrusion in A431 cells only in the presence of the glycolytic enzyme hexokinase inhibitor 2-deoxy-D-glucose. Mitochondrial electron transport inhibitor rotenone (Sigma-Aldrich) also inhibited filopodium protrusion only in the presence of 2-deoxy-D-glucose. Furthermore, it was reported that co-treatment with a mitochondrial electron transport inhibitor and a glycolytic inhibitor markedly decreased intracellular ATP levels.[1] We then tested whether napyradiomycin A1 decreased ATP levels in A431 cells in which glycolytic pathways were restricted. As intracellular ATP levels were not affected by EGF stimulation (data not shown), we measured intracellular ATP levels under the condition where A431 cells were treated with napyradiomycin A1 with or without 10 mM 2-deoxy-D-glucose for 30 min in the absence of EGF. After incubation, intracellular ATP levels were measured using a Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA) with a luminometer (Wallac; Perkin-Elmer, Waltham, MA, USA). As shown in Figure 1c, napyradiomycin A1 treatment did not decrease cellular ATP levels; however, 20 μM napyradiomycin A1 markedly decreased cellular ATP levels in the presence of 2-deoxy-D-glucose in A431 cells. Furthermore, ATP levels in HeLa cells also decreased under co-treatment with napyradiomycin A1 and 2-deoxy-D-glucose (data not shown). These results suggested that napyradiomycin A1 inhibited mitochondrial electron transport in cancer cells.

Next, we examined whether napyradiomycin A1 actually inhibited mitochondrial electron transport *in vitro* by using submitochondrial particles (SMP) obtained from the bovine heart. In order to prepare SMP, bovine hearts were homogenized in MSH buffer (210 mM mannitol, 70 mM sucrose, 1 mM DTT, 1 mM EGTA, 0.1% BSA and 10 mM HEPES pH 7.4) with a Potter-Elvehjem homogenizer (Nippon genetics, Tokyo, Japan). Homogenates were centrifuged at 1000 *g* for 10 min, and the resulting supernatant was further centrifuged at 8000 *g* for 20 min. Pellets were suspended in MSH buffer and obtained as SMP.[4]

The mitochondrial electron transport chain consists of four discrete multisubunit complexes: NADH–ubiquinone oxidoreductase (complex I), succinate–ubiquinone oxidoreductase (complex II), ubiquinol–cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV). Therefore, we evaluated which complex was the target of napyradiomycin A1.

Mitochondrial complex I activity was measured by monitoring the absorbance change of NADH at 340 nm in the presence of antimycin A (Sigma-Aldrich) and KCN (Sigma-Aldrich), an inhibitor of complex III and complex IV, respectively.[5,6] The enzyme assay was performed at 30 °C in a buffer containing 50 mM phosphate (pH 7.4), 250 mM sucrose, 0.1 μg ml$^{-1}$ antimycin A, 2 mM KCN, 50 μM decylubiquinone (DB; 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone, an exogenous hydrophobic quinone that acts as electron acceptor; Sigma-Aldrich),[7] 0.2 mM NADH, and 12 μg ml$^{-1}$ SMP with or without napyradiomycin A1. Rotenone was used as a positive control of complex I inhibitor. We found that napyradiomycin A1 inhibited complex I activity with an $IC_{50}$ value of 20 μM (Figure 2a).

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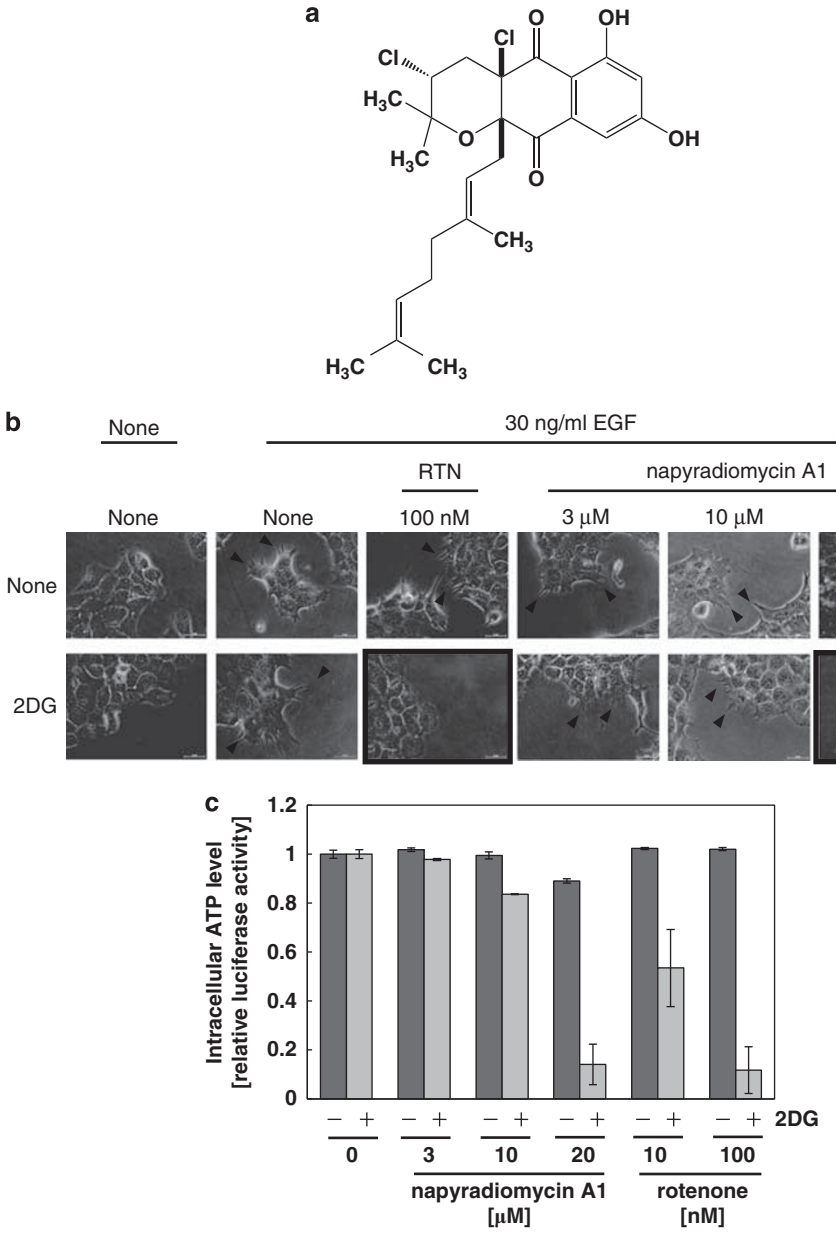


**Figure 1** Napyradiomycin A1 decreased intracellular ATP levels. (**a**) The structure of napyradiomycin A1.[3] (**b**) Napyradiomycin A1 inhibited EGF-induced filopodia protrusion only in the presence of 2-deoxy-D-glucose (2DG, 10 mM). The assay method was as described previously.[1] Mitochondrial electron transport inhibitor rotenone (RTN 100 nM) was used as a positive control. Arrowhead indicates filopodia, and the framed photos show filopodium inhibition. (**c**) Napyradiomycin A1 decreased intracellular ATP levels in 2DG-treated A431 cells. A431 cells were treated with napyradiomycin A1 with or without 10 mM of 2DG for 30 min. After incubation, intracellular ATP levels were measured using a Cell Titer-Glo Luminescent Cell Viability Assay Kit with a luminometer.

Mitochondrial complex II activity was measured by monitoring the absorbance change of 2,6-dichlorophenolindophenol (Sigma-Aldrich) at 600 nm in the presence of rotenone and KCN.[8] The enzyme assay was performed at 30 °C in a buffer containing 50 mM phosphate (pH 7.4), 0.1 μM rotenone, 2 mM KCN, 40 μM 2,6-dichlorophenolindophenol, and 12 μg ml$^{-1}$ SMP with or without napyradiomycin A1. Theonoyl trifluoroacetone (Sigma-Aldrich) was used as a positive control of complex II inhibitor.[9] We found that napyradiomycin A1 inhibited complex II activity with an $IC_{50}$ value of 9.7 μM (Figure 2b).

Mitochondrial complex III activity was measured by monitoring the absorbance change of the reduction of oxidized cytochrome *c* at 550 nm in the presence of rotenone, KCN and an electron donor decylubiquinol. Decylubiquinol was obtained by the reduction of DB with sodium borohydride.[10] The enzyme assay was performed at 30 °C in a buffer containing 50 mM Tris (pH 7.6), 1 mM $MgCl_2$, 0.1 μM rotenone, 2 mM KCN, 40 μM cytochrome *c*, 50 μM decylubiquinol and 12 μg ml$^{-1}$ SMP with or without napyradiomycin A1. Although antimycin A inhibited complex III activity, napyradiomycin A1 did not, even at 20 μM (Figure 2c)

Mitochondrial complex IV activity was measured by monitoring the absorbance change of the oxidation of reduced cytochrome *c* at 550 nm. The enzyme assay was performed at 30 °C in a buffer containing 50 mM Tris (pH 7.6), 1 mM $MgCl_2$, 0.1 μg ml$^{-1}$ antimycin A, 0.1 μM rotenone, 20 μM reduced cytochrome *c* and 12 μg ml$^{-1}$ SMP

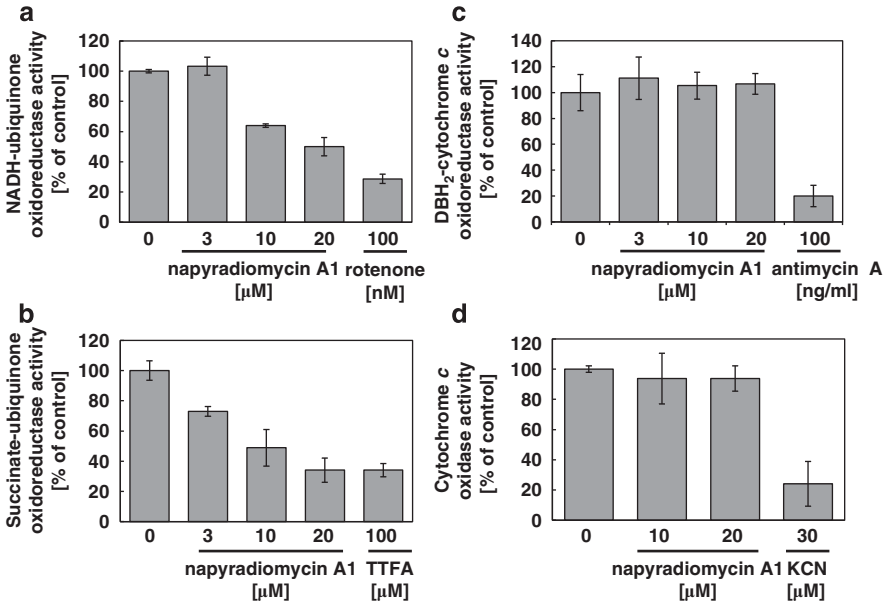


**Figure 2** Napyradiomycin A1 inhibited mitochondrial complexes I and II. A modification of previously reported methods[10,16–18] was used to measure complex I, complex II, complex III and complex IV activities. (**a**) Napyradiomycin A1 inhibited complex I activity. Mitochondrial complex I (NADH–ubiquinone oxidoreductase) activity was measured by monitoring the absorbance change of NADH at 340 nm using SMP. (**b**) Napyradiomycin A1 inhibited complex II activity. Mitochondrial complex II (Succinate–ubiquinone oxidoreductase) activity was measured by monitoring the absorbance change of 2,6-dichlorophenolindophenol at 600 nm using SMP. (**c**) Napyradiomycin A1 did not inhibit complex III activity. Mitochondrial complex III (decylubiquinol ($DBH_2$)–cytochrome *c* oxidoreductase) activity was measured by monitoring the absorbance change of cytochrome *c* at 550 nm using SMP. (**d**) Napyradiomycin A1 did not inhibit complex IV activity. Mitochondrial complex IV (cytochrome *c* oxidase) activity was measured by monitoring the absorbance change of the oxidation of reduced cytochrome *c* at 550 nm using SMP. SMP were obtained by standard different centrifugation.[4] All data are the mean ± s.d. of three independent experiments.

with or without napyradiomycin A1. Although KCN inhibited complex IV activity, 20 μM napyradiomycin A1 inhibited complex IV activity less than 10% (Figure 2d).

In conclusion, napyradiomycin A1 inhibited mitochondrial complexes I and II, but did not inhibit complex III or IV *in vitro*. Therefore, it was suggested that mitochondrial electron transport inhibition by napyradiomycin A1 was caused by the inhibition of mitochondrial complexes I and II.

It was reported that the isoprenyl unit of ubiquinone was important to bind to mitochondrial complex I.[11] It has been also reported that two isoprene units of ubiquinone could be modeled in the native structure of complex II by computational analysis using a protein-ligand docking program.[12] Napyradiomycin A1 is structurally similar to ubiquinone (Figure 1a); therefore, it is likely that napyradiomycin A1 binds with the ubiquinone-binding site of complexes I and II through its terpenoid residue. On the other hand, although mitochondrial complex III also includes a ubiquinone-binding site,[13] napyradiomycin A1 was unable to inhibit the activity of complex III up to 20 μM. Our result that napyradiomycin A1 inhibits mitochondrial complexes I and II but not complex III is interesting, because it has been reported that the structure of ubiquinone-binding sites in complex I and complex III may be similar, but they are different from a ubiquinone-binding site in complex II.[14] Further study is necessary to elucidate the mechanism by which napyradiomycin A1 inhibits mitochondrial complexes I and II but not complex III.

Isolation of napyradiomycins was first reported in 1986. Napyradiomycin group compounds were isolated from *Actinomycetes* and identified as antibacterial antibiotics.[3] At present, many napyradiomycins have been identified and reported to exhibit cytotoxicity against human cancer cell lines;[15] however, the molecular mechanism by which napyradiomycins show cytotoxicity has been unclear. Our results raise the possibility that an inhibitory effect of napyradiomycin A1 against the mitochondrial electron transport chain may explain the cytotoxicity of napyradiomycins against cancer cells.

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