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Novel *N*-oxide of naphthalimides as prodrug leads against hypoxic solid tumor: Synthesis and biological evaluation

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Abstract—Novel tertiary amine *N*-oxides of naphthalimides were designed and synthesized as potential anticancer agents against hypoxic solid tumors. Although their ctDNA-binding affinities and cytotoxic activities against usual tumor cell lines were lower than those of corresponding amines, the *N*-oxides **A1** and **A4** showed hypoxia preference activities against A375 cells in vitro and might be used as interesting candidates of prodrug leads in hypoxic tumor cells.

The majority of clinically used anticancer drugs generally killed large numbers of tumor cells with constant proportion kinetics primarily by attacking their DNA at some level (synthesis, replication or processing).[1] However, these drugs were not truly selective for cancer cells. On one hand, normal cells such as those in the bone marrow and gut epithelia which divided rapidly were damaged;[2] on the other hand, in the treatment of solid tumors, where the majority of cells did not divide rapidly, their therapeutic efficacy was limited. It was, therefore, imperative that innovative approaches were employed to reduce the toxicity and improve the therapeutic index of the anticancer agents.

To fulfill this need, one strategy was the development of tumor-activated prodrugs (TAP), which were relatively non-toxic and can be selectively activated in tumor tissue.[3] We knew that, hypoxia was the common and unique property of cells in solid tumors,[4] which was an important potential mechanism for the tumor-specific activation of prodrugs, consequently hypoxia-dependent cytotoxicity arose such as Tirapazamine (TPZ), AQ4N, NLCQ-1, SN 23862,[5] and so on (see Fig. 1). Among them, TPZ discovered by Brown and Lee almost 20 years ago had been carried onto phase II clinical trials, and AQ4N also exhibited high selectivity against solid tumors, which encouraged us to develop novel prodrug leads for hypoxic tumor.

Naphthalimides with side chains as antitumor agents had been first discovered by Brana and co-workers.[6] Two famous compounds known as Amonafide and Mitonafide had been selected for phase II clinical trials, which inhibited the activity of topoisomerase through binding with DNA. Unfortunately, Mitonafide had inappropriate central nervous system (CNS) toxicity and Amonafide had myelosuppression, vomiting, and erythra side effects,[7] which hampered further studies. Therefore, in an attempt to find new agents with therapeutically advantageous profiles, we initiated a program to prepare novel tumor-activated prodrugs derived from naphthalimides such as Amonafide and Mitonafide (see Fig. 2).

The cationic tertiary amine side chains on these agents played an important role on electrostatic binding affinity with DNA, which also ensured good uptake into cells, then interfered with the topoisomerase function to inhibit the tumors.[8] In order to lower the toxicity and improve extravascular drug transport properties, the oxidized tertiary amine was introduced into the naphthalimide backbone to form a prodrug lead[9] instead of original amino side chain. When the compounds were induced into hypoxic cells, the tertiary amine *N*-oxides could be bioreduced to the corresponding tertiary amine and showed the high bioactivity of the amine. Of course, this process could be inhibited by oxygen.[10] Herein we designed, synthesized, and evaluated a series of novel

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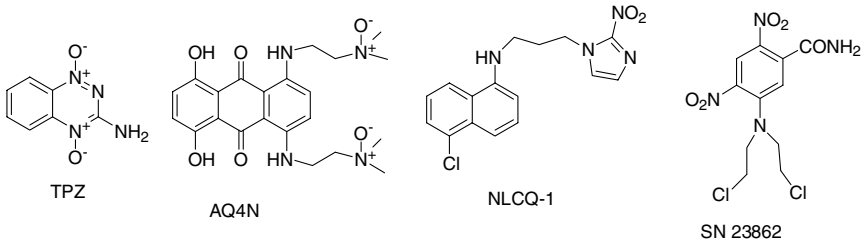

Figure 1. Structures of some reported hypoxia-selective prodrugs.

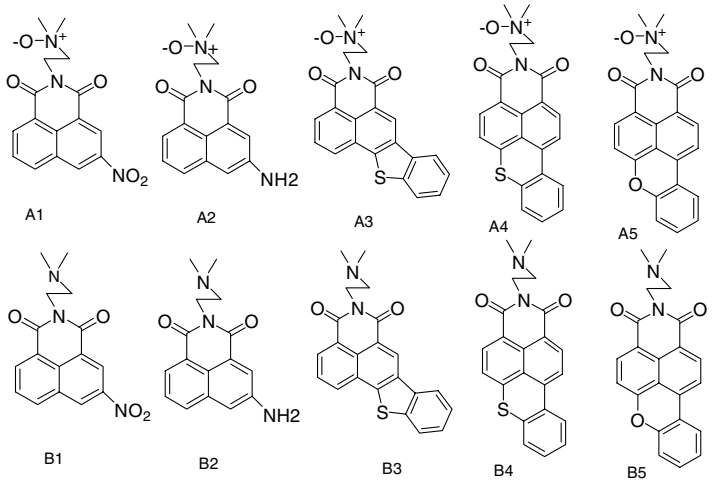

Figure 2. Novel compounds designed as potential hypoxia-selective antitumor agents (**A1**–**A5**) and some reported antitumor agents (**B1**–**B5**).

potential anticancer agents as prodrug leads against hypoxic solid tumor shown in Figure 2.

The compounds (**A1**–**A5**) were synthesized from 4-bromonaphthalic anhydride shown in Figure 3 (with compound **A5** as an example). 4-Bromonaphthalic anhydride and *o*-nitrophenol were dissolved in DMF and stirred for 1 h under reflux with NaOH and Cu as catalysts to give a yellow solid **1**. The solid **1** was treated with Fe powder in glacial acetic acid and refluxed for 1 h to afford khaki solid **2**. Then **2** was added into the hydrochloric acid and sodium nitrite at 0–5 °C for 1 h, followed by the addition of $CuSO_4$ solution, and refluxed for 0.5 h to give a yellow solid Benzo (*k*,*l*) xanthene-3,4-dicarboxylic anhydride **3**, which was mixed with *N*,*N*-dimethyl ethylenediamine in ethanol and refluxed for 3 h to give the intermediate product **4**. The important intermediates **B1**–**B4** were synthesized according to the reported methods.[11] Finally, the NO group was introduced by oxidation with H_2O_2 (30%) in CH_2Cl_2 or methanol under reflux for 1–3 h, and removal of the solvent gave the desired compounds **A1**–**A5** in high yields (Fig. 4). All of the structures were confirmed by IR, ^{1}H NMR, and HR-ESI.[12] A strong N–O stretching vibration appeared in the range of 2349–2338 cm^{-1} in their infrared spectra, which was characteristic of the *N*-oxides.

The UV–vis and fluorescence data of **A1**–**A5**, **B1**–**B5** were listed in Table 1. It was found that the quantum yields of the *N*-oxides were 0.13 and 0.123–0.99 (Φ), and the corresponding amines were 0.0046, 0.03–0.52 (Φ), which indicated that the fluorescence intensities have big changes between the *N*-oxides and their corresponding amines.

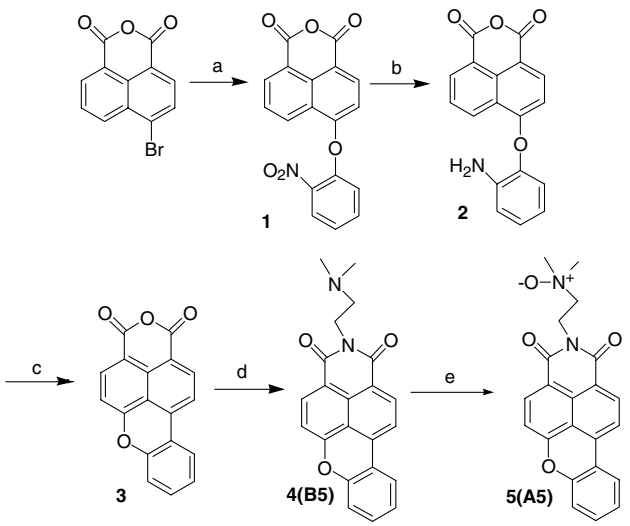

Figure 3. Reagents and conditions: (a) *o*-nitrophenol, NaOH, DMF, Cu, reflux 1 h; (b) Fe, acetic acid, reflux 1 h; (c) hydrochloric acid, acetic acid, $NaNO_2$, 0–5 °C, $CuSO_4$, HAc, H_2O; (d) *N*,*N*-dimethylethylenediamine, ethanol, reflux 3 h; (e) H_2O_2 30%, methanol, reflux, 3 h, 0.64 g (86%).

The binding properties between the compounds and ctDNA were evaluated (Fig. 5). The fluorescence data

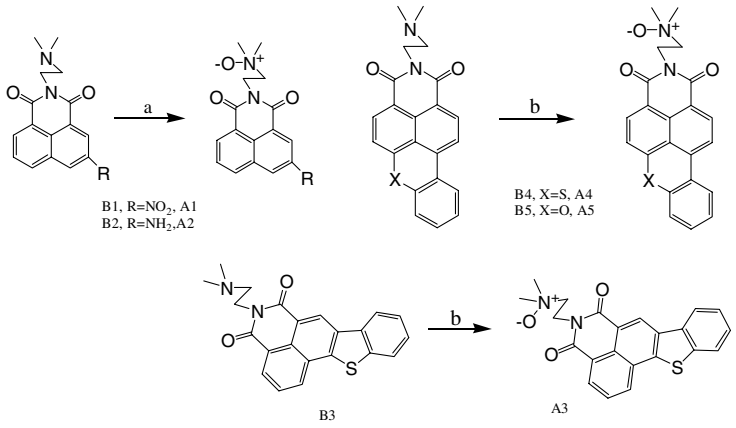

Figure 4. Synthesis of target compounds. Reagents and conditions: (a) H_2O_2 (30%), CH_2Cl_2, reflux 1 h; (b) H_2O_2 (30%), methanol, reflux 1–3 h.

Table 1. Spectral data of compounds **A1**–**A5**, **B1**–**B5**[a,b,c,d]

Compound	UV λ_{max} (log ε)	FL λ_{max} (Φ)
A1	331 (4.04)	368 (<0.001)
A2	427 (3.47)	549 (0.13)
A3	379 (4.14)	461 (0.123)
A4	461 (4.40)	522 (0.99)
A5	449 (4.32)	473 (0.92)
B1	316 (3.93)	368 (<0.001)
B2	431 (3.71)	547 (0.0046)
B3	378 (4.00)	456 (0.03)
B4	460 (4.34)	510 (0.52)
B5	447 (4.53)	472 (0.19)

[a] In absolute ethanol.
[b] With quinine sulfate in sulfuric acid as quantum yield standard ($\phi = 0.55$).
[c] The fluorescence of the compounds with strong intensity was measured at low voltage.
[d] As the spectra change for **A2** and **B2** was sharp, the medium voltage data of quinine sulfate were used for **A2** and **B2** to value their relative quantum yield, and the low voltage data for the other compounds.

were analyzed to give the binding constants of **A4** and its corresponding amine **B4** at approximately 6.62 and 2.9×10^5 M^{-1}, respectively.[13] It was about 15-fold lower for the *N*-oxide than that of the corresponding amine, which might be caused by the disappearance of protonated amine for electrostatic binding with DNA. It implied that weaker ctDNA binding probably endowed the *N*-oxides with lower toxicity and extravascular drug transport properties.[9]

The *N*-oxides and their corresponding amines were initially screened for their cytotoxicities in vitro against usual A549 and P388 cell lines, respectively, as shown in Table 2. It was found that for oxic cell lines, the bioactivities of **A2**, **A3**, **A4**, and **A5** were decreased by about 5-, 36-, 44-, 675-fold against A549, respectively, compared to the corresponding amines **B2**, **B3**, **B4**, **B5**, and **A3**, **A4**, **A5** decreased by about 11-, 8-, 165-fold against P388, respectively. Especially, the cytotoxicity of **A2** against P388 could not be evaluated. The results proved that the *N*-oxides might be used as potential prodrugs.

The antiproliferative activities for the compounds **A1**–**A5**, **B1**–**B5** were then measured in vitro against A375 and V79 solid tumors which were maintained as exponentially growing suspension cultures in Eagle's minimal essential medium with Earle's salts, modified for suspension cultures with 7.5% fetal calf serum. The compounds were added to cell suspensions at indicated concentrations. Then the suspensions were incubated

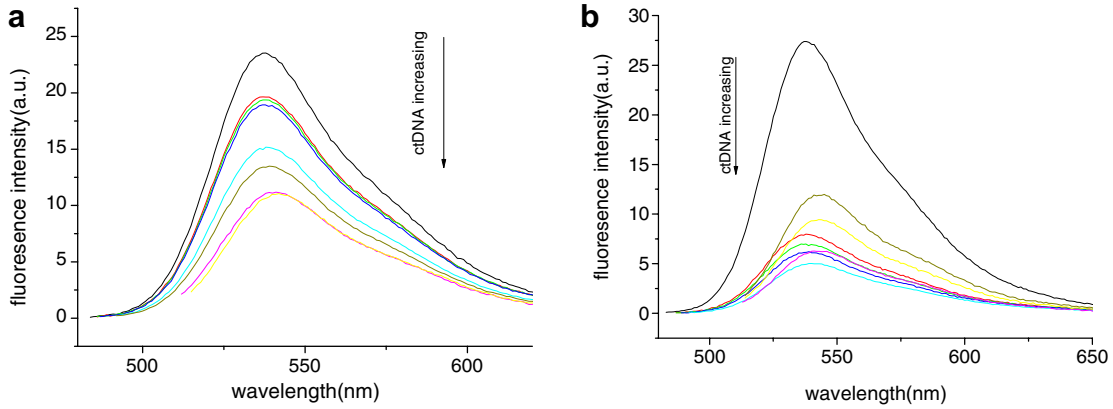

Figure 5. Fluorescence spectra of compounds **A4** (a) and **B4** (10×10^{-6} M) (b) with addition of ctDNA in increasing concentration ($0–400 \times 10^{-6}$) in 20 mM tris–HCl (pH7.5) at room temperature with excitation at 460 nm.

Table 2. Cytotoxicity of **A1**–**A5**, **B1**–**B5** against cell lines A549 and p388

Compound	Cytotoxicity (IC_{50}, μM)	
	A549[a]	P388[b]
A1	3.31	31.6
A2	5.62	>1000
A3	0.251	2.88
A4	6.3	12.6
A5	26.9	34.7
B1	NR	NR
B2	1.1	0.2
B3	0.007	0.263
B4	0.144	1.583
B5	0.0398	0.21

NR, not reported.

[a] Cytotoxicity (CTX) against human lung cancer cell (A549) was measured by sulforhodamine B dye-staining method.[6].

[b] CTX against murine leukemia cells (P388) was measured by microculture tetrazolium–formazan method.[7]

Table 3. Cytotoxicity of **A1**–**A5** and **B1**–**B5** against cell lines A375 and V79[a,b] in hypoxic–oxic cell cultures

Compound	A375			V79		
	IC_{50} (μM)		HCR	IC_{50} (μM)		HCR
	O_2	N_2	O_2/N_2	O_2	N_2	O_2/N_2
A1	168	90	1.9	313	184	1.7
A2	310	253	1.2	184	286	0.64
A3	30.1	19.3	1.6	15	76.8	0.19
A4	29	11.9	2.4	21	85	0.25
A5	13.3	12.3	1.1	17.8	12.1	1.47
B1	5.3	NR	NR	41.6	NR	NR
B2	14.7	NR	NR	70.3	NR	NR
B3	18.4	NR	NR	62.7	NR	NR
B4	2	NR	NR	14	NR	NR
B5	>200	NR	NR	4.1	NR	NR

[a] The most effective compounds, in terms of both hypoxic potency and hypoxia selectivity in vitro as established by hypoxic:oxic cytotoxicity ratio commonly referred to as hypoxic cytotoxicity ratio (HCR).[15]

[b] IC_{50} value was determined using an exposure time of 24 h.

in special gases (air + 5% CO_2, nitrogen + 5% CO_2) at 37 °C, following 24 h continuous drugs, exposure.[14] The concentration required for 50% cell growth inhibition (IC_{50}) was determined by the MTT colorimetric assay and the hypoxia selectivity for the prodrugs as antitumor agents was evaluated as shown in Table 3.

Compared with the corresponding naphthalimides, the *N*-oxides showed less cytotoxicity in oxic A375 cell cultures and more cytotoxicity in hypoxic cultures (Table 3). The results implied that the N–O group in hypoxic cell cultures could be bioreduced to the corresponding amine and led to the localization of bioreductive metabolites (active cytotoxins) in hypoxic cells and should further interfere with topoisomerase function.[8] The hypoxic cytotoxicity ratios (HCR) for **A1**–**A5** were 1.9, 1.2, 1.6, 2.4, and 1.1-fold, respectively. However, the HCR was very low, which might be caused by their insufficient bioreduction revealed by the fluorescence changes. On the other hand, the prodrugs did not show hypoxia selectivity against V79 cells except for **A1** and **A5** with HCR of 1.7 and 1.47, respectively, which indicated that this series of *N*-oxides showed hypoxia-selective antitumor activity for A375 solid tumor cells, but not for V79.

In order to illustrate the bioreduction efficiency, the fluorescent intensities of *N*-oxides in hypoxic and oxic A375 cell cultures in indicated concentrations were measured using fluorescence scan ascent. The fluorescent intensities of the *N*-oxides and their corresponding amines in water were measured as well. The relative changes of the fluorescent intensity to the *N*-oxides are listed in Table 4.

Table 4 shows that the intensities of the *N*-oxides in hypoxic cell cultures increased by 17.7%, 9.0%, 3.4%, 5.0%, and 8.0%, respectively, compared with those in oxic cell, in which the *N*-oxides could not be bioreduced; however, the fluorescent intensities of the pure amines increased by 53.0%, 33.0%, 40.0%, 14.4%, and 24.0% in water, respectively, which indicated that bioreductive efficiency of the *N*-oxides to naphthalimides was low in cell, and probably resulted in lower activity against hypoxia antitumor. Meanwhile, although the aqueous solubility of the compounds was improved by introducing the NO group, the activity of the *N*-oxide naphthalimides in the hypoxia cell cultures was still limited by the solubility, in comparison with the case of the corresponding amine. Considering the complexity of the interaction between the drugs and the solid tumor cells, the reasons for low bioreductive efficiency were being further studied. But, this approach at least showed the *N*-oxide strategy was useful for hypoxic tumor cells, which could have more bioactivities than that for oxic tumor cells.

Table 4. The changes of the fluorescent intensity[a,b]

	A1	**A2**	**A3**	**A4**	**A5**
$(F_{hy}\text{-}F_{ox})/F_{ox}$ in cell cultures (%)	17.7	9.0	3.4	5.0	8.0
$(F_B–F_A)/F_A$ in water (%)	53.0	33.0	40.0	14.4	24.0

[a] F_{ox} and F_{hy} indicate the fluorescent intensity of the *N*-oxides (10^{-4} M) in oxic and hypoxic A375 cell cultures.

[b] F_A and F_B indicate the fluorescent intensity of the *N*-oxides and their corresponding pure amines (10^{-4} M) in water (1% DMSO, V/V).

In summary, we described the synthesis, DNA-binding affinities, and hypoxic evaluation of the novel aliphatic *N*-oxide of naphthalimides. All of the *N*-oxides showed more potential against hypoxic A375 cells, which revealed that they could be used as potential candidate of prodrug leads with hypoxic tumor preference.

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12. **A-1** ^{1}H NMR (CD_3OD-d4) δ (ppm): 3.33 (s, 6H, $NOCH_3$), 3.70 (t, J_1 = 7.00 Hz, J_2 = 7.09 Hz, 2H, $NOCH_2$), 4.69 (t, J_1 = 6.85 Hz, J_2 = 7.00 Hz, 2H, $CONCH_2$), 8.00 (t, J_1 = 7.65 Hz, J_2 = 7.87 Hz, 1H, 2-H), 8.63 (d, J = 8.26 Hz, 1H, 1-H), 8.77 (d, J = 7.24 Hz, 1H, 3-H), 9.19 (d, J = 2.14 Hz, 1H, 9-H), 9.33 (d, J = 2.10 Hz, 1H, 7-H). Mp 184-185 °C. IR (KBr): 3069, 2353, 2338, 1664, 1341, 800 cm^{-1}; HR-MS: $C_{21}H_{16}N_3O_5$ $(M + H)^+$ calcd 330.1130. found: 330.1083. **A-2** ^{1}H NMR (CD_3OD-d4) δ (ppm): 3.30 (s, 6H, $NOCH_3$), 3.64 (t, J_1 = 7.13 Hz, J_2 = 6.92 Hz, 2H, $NOCH_2$), 4.63 (t, J_1 = 6.96 Hz, J_2 = 7.08 Hz, 2H, $CONCH_2$), 7.35 (d, J = 2.38 Hz, 1H, 9-H), 7.57–7.61 (m, 1 H, 1-H), 7.98 (t, J_1 = 4.51 Hz, J_2 = 3.12 Hz, 1H, 2-H), 8.03–8.04 (m, 1H, 7-H), 8.18 ~ 8.20 (m, 1H, 3-H). Mp: 160–161 °C. IR (KBr): 3416, 3338, 2353, 2326, 1649, 1622, 789 cm^{-1} HR-MS: $C_{16}H_{18}N_3O_3$ $(M + H)^+$ calcd 300.1348; found: 300.1333. **A-3** ^{1}H NMR (CD_3OD-d4) δ (ppm): 3.35 (s, 6 H, $NOCH_3$), 3.68 (t, J_1 = 7.27 Hz, J_2 = 7.13 Hz, 2 H, $NOCH_2$), 4.58 (t, J_1 = 7.04 Hz, J_2 = 7.33 Hz, 2 H, $CONCH_2$), 7.50 ~ 7.54 (m, 2H, 9-H, 10-H), 7.66 (t, J_1 = 7.74 Hz, J_2 = 7.71 Hz, 1H, 2-H), 7.87 ~ 7.89 (m, 1H, 8-H), 8.10 ~ 8.12 (m, 1H, 1-H), 8.21 (d, J = 7.42 Hz, 1H, 11-H) 8.35 (d, J = 7.31 Hz, 1H, 3-H) 8.76 (s, 1H, 7-H). Mp: 152-153 °C. IR (KBr): 3326, 2365, 2338, 1696, 1660, 1334, 734 cm^{-1}. HR-MS: $C_{22}H_{18}N_2O_3$: $(M + H)^+$ calcd 391.1116; found: 391.1119. **A-4** ^{1}H NMR (CD_3OD-d4) δ(ppm): 3.32 (s, 6H, $NOCH_3$), 3.66 (t, 2H, $NOCH_2$), 4.65 (t, 2H, $CONCH_2$), 7.45 (s, 3H, 9-H, 10-H, 11-H), 7.58 (d, J = 8.03 Hz, 1H, 8-H) 8.34 (d, J = 8.08 Hz, 3H, 1-H, 2-H, 7-H), 8.54 (d, J = 8.20 Hz, 1H, 6-H). Mp: 159–160 °C. IR (KBr): 3350, 2361, 2338, 1688, 1649, 1583, 1369, 758.1 cm^{-1}. HR-MS: $C_{22}H_{18}N_2O_3$ $(M + H)^+$ calcd 391.1116; found: 391.1114. **A-5** ^{1}H NMR (CD_3OD-d4) δ (ppm): 3.35 (s, 6H, $NOCH_3$), 3.69 (t, J_1 = 7.07 Hz, J_2 = 7.17 Hz, 2H, $NOCH_2$), 4.63 (t, J_1 = 6.97 Hz, J_2 = 7.21 Hz, 2H, $CONCH_2$), 7.23 (d, J = 8.33 Hz, 1H, 7H), 7.32 (d, J = 8.25 Hz, 1H, 8-H), 7.35 (t, J_1 = 8.19 Hz, J_2 = 8.11 Hz, 1H, 9-H), 7.55 (t, J_1 = 8.49 Hz, J_2 = 8.51 Hz, 1H, 10-H), 7.97 (d, J = 7.95 Hz, 1H, 11-H), 8.12 (d, J = 8.02 Hz, 1H, 1-H), 8.43 ~ 8.46 (m, 2H, 2-H, 6-H). Mp: 169-274 °C, IR (KBr): 3536, 2349, 2338, 1645, 1590, 1380, 777 cm^{-1}. HR-MS: $C_{22}H_{18}N_2O_4$ $(M + H)^+$ calcd 391.1345; found: 375.1347.
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