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Tetrasubstituted naphthalene diimide ligands with selectivity for telomeric G-quadruplexes and cancer cells

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ABSTRACT

A series of tetrasubstituted naphthalene diimide compounds with *N*-methylpiperazine end groups has been synthesized and evaluated as G-quadruplex ligands. They have high affinity and selectivity for telomeric G-quadruplex DNA over duplex DNA. CD studies show that they induce formation of a parallel G-quadruplex topology. They inhibit the binding of hPOT1 and topoisomerase IIIα to telomeric DNA and inhibit telomerase activity in MCF7 cells. The compounds have potent activity in a panel of cancer cell lines, with typical IC_{50} values of ~0.1 μM, and up to 100-fold lower toxicity in a normal human fibroblast cell line.

The immortality of cancer cells is at least in part dependent on the maintenance of telomeres, the nucleoprotein complexes at the ends of chromosomes.[1] Telomeres are shortened in somatic cells, leading to their limited life span, whereas they are stabilized in cancer cells. The function of telomerase, which is over-expressed in >80% of cancer cells, is to both physically protect and maintain telomere length.[2] As telomeric DNA comprises repeated short G-tracts, it is able to fold into G-quadruplex structures, in which three planes of hydrogen-bonded G-quartets are held together by π–π interactions. Depending on the coordinating ion, the presence of small-molecule ligands, the DNA sequence and other factors such as molecular crowding, telomeric DNA G-quadruplexes can have a diversity of topologies.[3]

Quadruplex formation can affect a wide range of cellular processes. The stabilization of G-quadruplex structures in the single-stranded 3′ telomeric DNA overhang by small-molecule ligands has been shown to indirectly inhibit telomerase and telomere maintenance in cancer cells.[4] Quadruplex formation along the overhang can also displace the single-stranded binding protein hPOT1.[5] The function of telomere-associated Holliday junction decatenases such as topoisomerase IIIα (Topo IIIα), (essential for the maintenance of telomeres in the ALT (alternative lengthening of telomeres) pathway,[6] can be affected by quadruplex-binding small molecules.[21c] Promoter regions of oncogenes such as *c-myc* and *c-kit* contain sequences that may fold into G-quadruplexes,

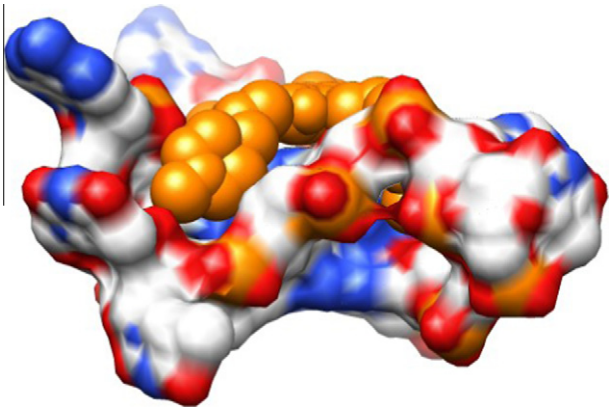

Figure 1. Modeled structure of compound **1** (orange) docked on the 3′ face of the parallel human telomeric G-quadruplex 22-mer DNA (side view) using the crystal structure of a known ND complex as a starting-point.[11] Positive and negative charge regions on the solvent-accessible surface of the DNA quadruplex are marked in blue and red, respectively.

and their stabilization can down-regulate oncogene expression.[7] The multiple pathways caused by the stabilization of telomeric G-quadruplexes can lead to uncapping and end-to-end fusions of chromosomes, followed by cell cycle arrest, senescence and growth inhibition, as well as DNA damage response and apoptosis in cancer cells.[5b,c]

A large number of G-quadruplex binding small molecules have been reported.[8] Tetrasubstituted naphthalene diimides (NDs) are

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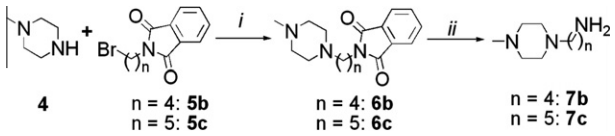
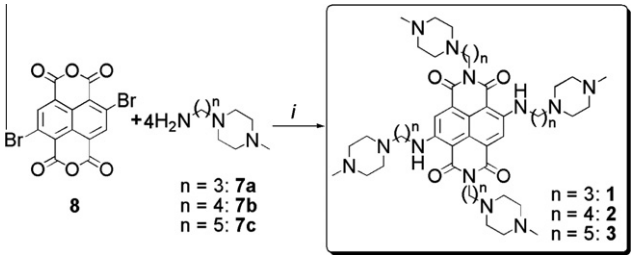


Scheme 1. Synthesis of the side chain amines **7b** and **7c**. Reagents and conditions: (i) Na_2CO_3, toluene, reflux, 16–18 h, 58–85%. (ii) (1) N_2H_4, EtOH, reflux, 3 h; (2) HCl, reflux, 30 min, 46–61%. The amine **7a** ($n = 3$) was commercially available.



Scheme 2. Synthesis of the NDs **1**–**3**. Reagents and conditions: (i) microwave, 150 °C, 20 min–2 h, 11–19%. The starting material **8** was synthesized according to the established procedure.[14]

Table 1
G-quadruplex stabilization of compounds **1**–**3** in the FRET melting temperature assay. Esds in ΔT_m are ±0.1 K.

DNA type	FRET ΔT_m [K], $c = 0.5$ μM		
	1	2	3
F21T G4	28.3	24.7	23.8
c-kit1 G4	1.8	4.9	1.5
c-kit2 G4	15.2	16.7	7.7
T-loop	1.3	0.1	0.2

very potent G-quadruplex ligands with high cellular toxicity.[9] NDs have been of broader interest because of their tunable electro- and photo-chemical properties and their applications in supramolecular chemistry.[10] They have a delocalized electron system, which is able to effectively stabilize the terminal G-quartets of a G-quadruplex by stacking interactions. Four substituents with amino end groups can fit into the four grooves at the sides of the G-quadruplex and interact electrostatically, as indicated by molecular modeling (Fig. 1), and demonstrated by X-ray crystallography.[11] One compound from these series, which has high affinity for the G-quadruplexes found in the promoter region of the *c-kit* gene, has been studied for its effects on gastrointestinal stromal tumor cell lines.[12] We report here on a novel subset of NDs with *N*-methylpiperazine end groups, which have enhanced telomeric quadruplex and cellular selectivity.

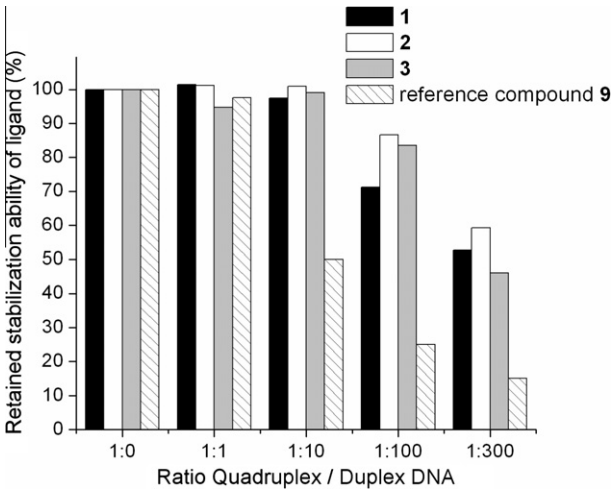


Figure 2. Competition FRET experiment showing the selectivity of **1**–**3** for the F21T G-quadruplex DNA sequence over duplex DNA. Different ratios of excess duplex DNA were added (concentrations of G-quartets vs nucleotide phosphates of duplex DNA). The reference compound **9** is a derivative of compound **1** with dimethylamine end groups (see Supplementary data).

Initially, novel ND derivatives with enhanced selectivity for telomeric G-quadruplexes were conceived by qualitative molecular modeling using a parallel G-quadruplex from a ND co-crystal structure.[11] Side-chains of 3–5 carbon atoms with bulky methylpiperazine end groups gave optimal groove interactions. Overlay of **1** onto the known position of the related ND compound (Fig. 1) indicated that the *N*-methylpiperazine groups, which are bulky and can be protonated easily, reach deep into the grooves at the sides of the G-quadruplex and interact strongly with phosphate moieties.

The amines **7b** and **7c**, which were used as side-chains, were obtained via Gabriel synthesis (Scheme 1).[13] For this, *N*-methylpiperazine was added to the bromoalkylphthalimides **5b** or **5c** in

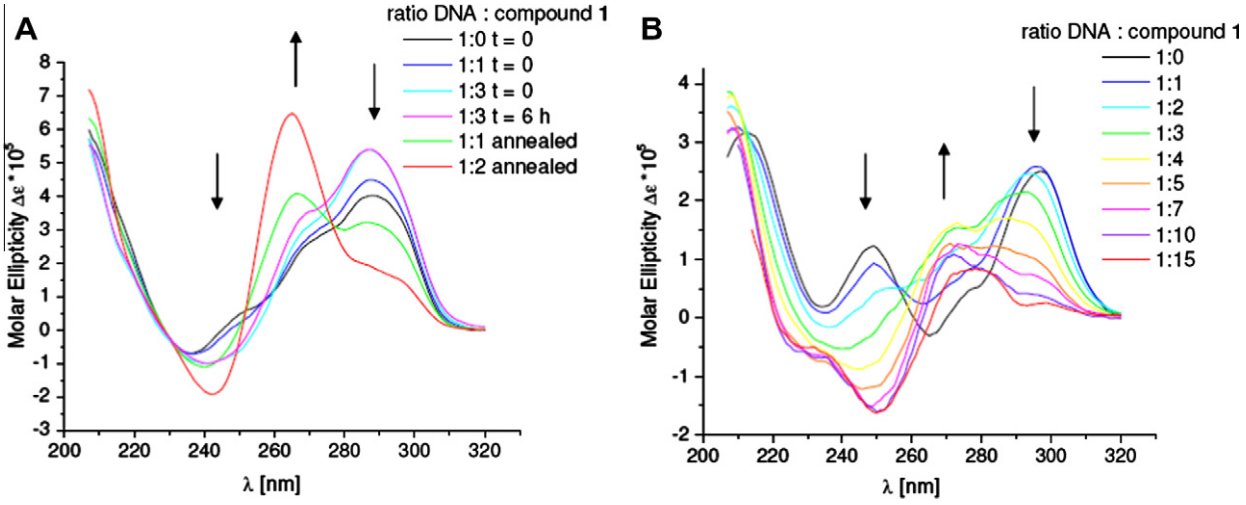


Figure 3. Circular dichroism (CD) spectra of a human telomeric 23-mer DNA with various ratios of compound **1**, at pH 7.4. (A) CD spectrum, 100 mM K^+. (B) CD spectrum, 100 mM Na^+. Compounds **2** and **3** gave similar results (see Supplementary data).

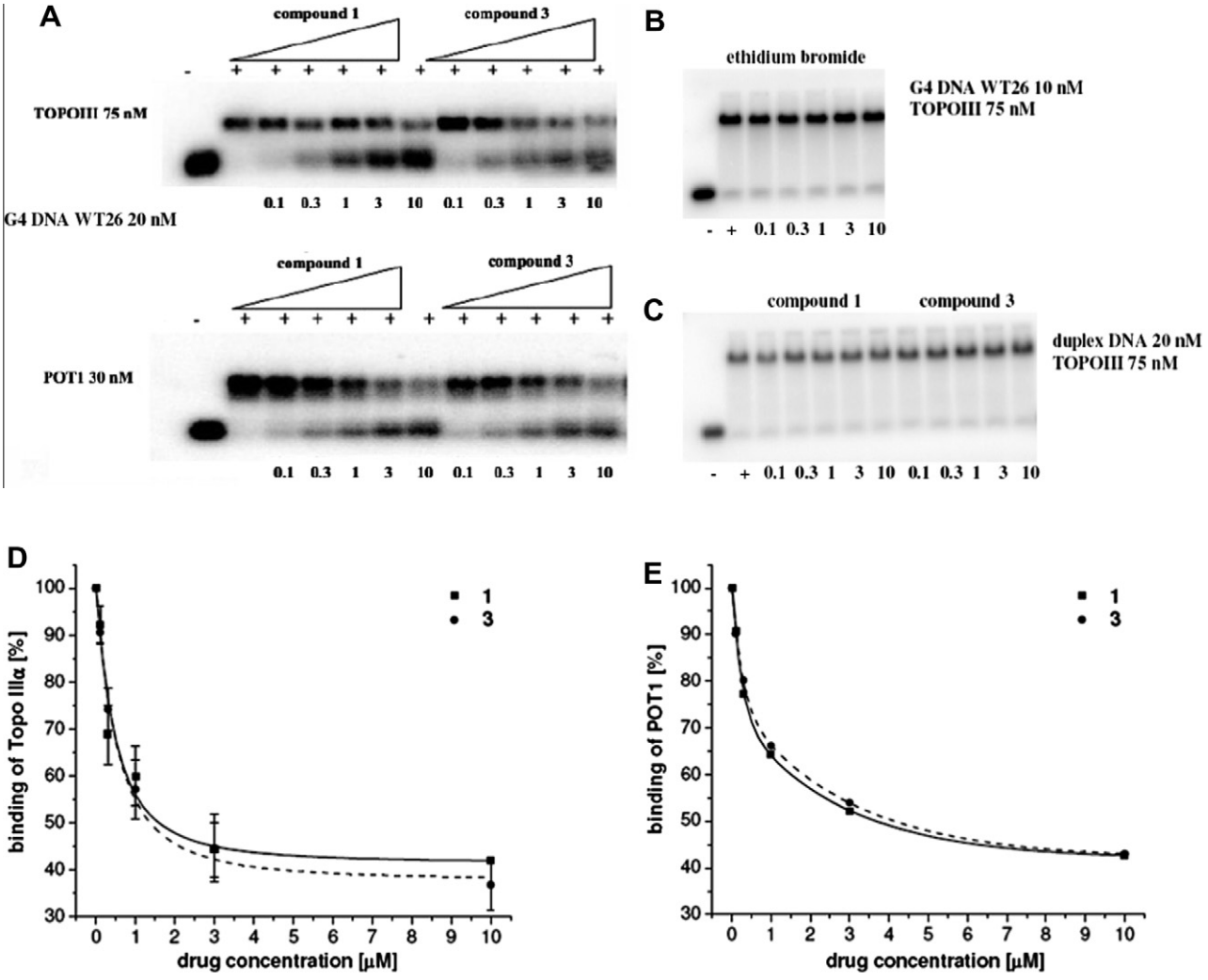


Figure 4. (A, D, E) Inhibition of POT1 (30 nM) and Topo IIIα (75 nM) binding to the telomeric G-quadruplex DNA sequence WT26 (20 nM) by **1** and **3** in an in vitro EMSA assay.[21] (B) The duplex DNA binding molecule does not inhibit the binding of Topo IIIα to WT26. (C) Compounds **1** and **3** do not inhibit the binding of Topo IIIα to duplex DNA.

toluene to give the imides **6b** and **6c**, respectively. Those were cleaved with hydrazine in ethanol to give the amines **7b** and **7c**. The propylamine analog **7a** was commercially available. The tetrasubstituted NDs **1**–**3** were synthesized from the dibromodianhydride **8** with the respective aminoalkyl-methylpiperazine **7** as the solvent in the microwave (Scheme 2) as variants of the established method.[14] The tetrasubstituted species was isolated from the side products, containing a trisubstituted species, by HPLC with yields of 11–19%. In contrast to previously reported NDs, compounds **1**–**3** are highly water-soluble, which simplified their evaluation.

The ability of compounds **1**–**3** to stabilize G-quadruplex DNAs was evaluated in a FRET (Fluorescence Resonance Energy Transfer) melting temperature assay.[15] Melting temperature shifts in Table 1 were compared at a drug concentration of 0.5 μM, as some of the melting curves were already saturated at the standard comparison concentration of 1 μM. All three compounds show very high affinity for the human telomeric G-quadruplex sequence F21T, leading to increases in melting temperature of 24–28 K. Moderate affinity for the c-kit2 sequence was observed, whereas there was only low affinity for the c-kit1 sequence and the duplex DNA T-loop sequence.

The selectivity of **1**–**3** for G-quadruplex DNA was evaluated in a competition FRET experiment,[16] in which different ratios of duplex DNA from calf thymus were added to the classic FRET experiment with the telomeric sequence F21T (Fig. 2). This showed that a 10:1 duplex:quadruplex ratio does not interfere with the stabilization of the G-quadruplex by **1**–**3**, and even at a 300-fold excess of duplex DNA, G-quadruplex stabilization is reduced by ca. 50%. The methylpiperazine compounds have higher selectivity for G-quadruplex DNA over duplex DNA than earlier NDs[9] such as the reference compound **9**, which contains dimethylamine end groups.

The effect of the ligands on the topology of a telomeric G-quadruplex 23-mer DNA was investigated by circular dichroism (CD).[17]

Table 2
Short term cytotoxicity of compounds **1**–**3** in a panel of cancer cell lines (WI38 is a normal fibroblast line), determined by 96 h SRB assays.

Cell line	SRB IC_{50} (nM)		
	1	2	3
MCF7	167 ± 30	100 ± 20	196 ± 8
A549	108 ± 17	69 ± 28	258 ± 42
MIA-Pa-Ca-2	111 ± 24	73 ± 11	121 ± 18
PANC-1	148 ± 45	106 ± 33	193 ± 46
HPAC	186 ± 17	117 ± 28	205 ± 30
BxPc-3	1520 ± 133	1020 ± 120	759 ± 134
WI38	9042 ± 3197	5500 ± 1234	8430 ± 2904

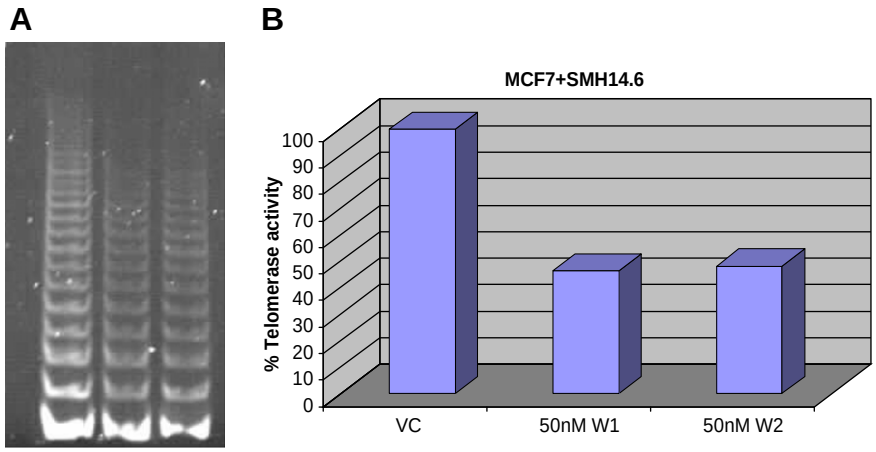


Figure 5. Effects of compound **1** on telomerase activity in MCF7 cells. (A) Denaturing gel from the TRAP–LIG assay[22] with compound **1** at a concentration of 50 nM. The left-hand lane shows a positive control with telomerase and no ligand. The middle and right-hand lanes are for **1** and **2** weeks exposure, respectively. (B) The quantitation of the gel.[23]

The 23-mer DNA with K^+ as the coordinating ion formed a G-quadruplex in solution with a mixed/hybrid type topology,[3b] indicated by a negative peak at 235 nm, a shoulder peak at 270 nm and a positive peak at 290 nm (Fig. 3A, black graph).[18] Similar sequences form a parallel quadruplex in the crystalline state[19] and in crowded solution.[3c] Addition of 1–3 equiv of compound **1** did not lead to immediate changes in the spectrum, but after 6 h of incubation, the equilibrium shifted in the direction of a parallel-type topology, which was found in DNA annealed in the presence of **1**, indicated by a negative peak at 240 nm and a positive peak at 260 nm (Fig. 3A, red graph). The Na^+ coordinated 23-mer DNA formed a G-quadruplex with an anti-parallel topology, with a positive peak at 245 nm, a negative peak at 265 nm, and a positive peak at 290 nm (Fig. 3B, black graph). Upon addition of compound **1**, the ratio of the parallel arrangement increased instantly (Fig. 3B, red graph). The equilibration towards the parallel topology was more rapid than for the more stable K^+ complex. Compounds **2** and **3** gave very similar results, but with the absence of the shoulder at 290 nm in the CD spectrum of **1** in the presence of sodium, suggesting a more complete shift of the equilibrium towards the parallel structure (see Supplementary data). A number of G-quadruplex ligands have been reported, which stabilize either the parallel or the anti-parallel form of G-quadruplex DNA.[17,19,20] The potent ligands telomestatin and RHPS4 for instance stabilize the anti-parallel form.[19,20] The crystal structure of a complex of a telomeric G-quadruplex 23-mer DNA and an ND has a parallel topology, which is in agreement with the results of the CD study.[11]

The inhibition of hPOT1 and Topo IIIα binding to a telomeric DNA sequence WT26 by the ligands **1** and **3** was examined in vitro using EMSA assays.[21] Both compounds inhibit the binding of hPOT1 and Topo IIIα at low μM concentrations, probably due to G-quadruplex stabilization (Fig. 4A, D, E), by analogy with earlier compounds examined in this assay.[21c] In vivo, interference with these targets may lead to telomere uncapping and end-to-end fusions of chromosomes. This will hinder maintenance of telomeres both in telomerase-positive and in ALT cells[18]; these effects have yet to be examined for **1** and **3**. Compounds **1** and **3** do not inhibit the binding of Topo IIIα to duplex DNA (Fig. 4C). effects have yet to be examined for **1** and **3**. The inhibition of Topo IIIα binding to the G-quadruplex DNA sequence WT26 is specific, as the duplex binding molecule ethidium bromide does not inhibit the binding of Topo IIIα to WT26 (Fig. 4B), and compounds **1** and **3** do not inhibit the binding of Topo IIIα to duplex DNA (Fig. 4C).

The toxicity of **1**–**3** on a panel of cancer cell lines and the normal human fibroblast cell line WI38 was evaluated with 96 h sulforhodamine B (SRB) assays (Table 2).[12] The toxicity of the drug to most tested cancer cell lines, including the pancreatic cancer cell lines MIA-Pa-Ca-2, PANC-1 and HPAC, is high with IC_{50} values of 0.1–0.2 μM. The pancreatic cancer cell line BxPc-3 is ~10-fold less sensitive than the other pancreatic lines. The compounds are significantly less toxic to the normal human fibroblast cell line WI38 (which does not express telomerase) than to the panel of cancer cell lines examined here.

The effects of compound **1** on telomerase activity in the MCF7 cell line were also examined (Fig. 5). Incubation with a 50 nM concentration of **1** (<50% of the IC_{50} value) resulted in a 50% reduction in telomerase activity after 1 week, using a modified TRAP (Telomerase Repeat Amplification Protocol) assay,[22] which was maintained after a further week. Since cell populations tend to have telomere length heterogeneity,[24] the high potency of this compound may be attributable to its effect on a sub-population of susceptible cells with short telomeres.

The compounds reported in this paper show high selectivity and affinity for telomeric G-quadruplex DNA, together with selective toxicity in a panel of cancer cell lines. They inhibit the binding of hPOT1 and Topo IIIα to telomeric G-quadruplex DNA, and stabilize G-quadruplexes with a parallel-type topology. The observation of inhibition of cellular telomerase activity is consistent with the hPOT1 displacement, in particular in accord with the hypothesis that these compounds act by displacing hPOT (and telomerase) from the telomeric DNA overhang and stabilizing quadruplex structures along its length or during telomere replication,[25] which act as signals of DNA damage.[26] The compounds are notably potent against the pancreatic cancer cell lines used here, and they are currently being evaluated in a xenograft model for pancreatic cancers, which are especially challenging to treat.[27]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.066.

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