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# Anticancer Activity for 4,4′-Dihydroxybenzophenone-2,4-dinitrophenylhydrazone (A-007) Analogues and Their Abilities to Interact with Lymphoendothelial Cell Surface Markers

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**Abstract**—The structure of the anticancer agent 4,4′-dihydroxybenzophenone-2,4-dinitrophenylhydrazone (**A-007**) has been modified through SAR and by incorporating barbituric acid, pyridine, quinoline, and alkylcarboxylic acids into **A-007**'s moieties. Analogue anticancer activity and interacting with CD surface markers on a T-cell leukemia cell line were evaluated and the correlation between SAR and biological properties are discussed.


4,4′-Dihydroxybenzophenone-2,4-dinitrophenylhydrazone (**A-007**, analogue **1** Fig. 1), continues to demonstrate significant anticancer activities in Phase I/II anticancer trials (IND 47,470).[1,2] To date, 33 people have been treated with topical **A-007** (as a 0.25% gel) and 31% objective remissions have been observed with two complete responses.[1,2] During the Phase I study, it became obvious that **A-007** was not acting via a cytotoxic mechanism, that is, no local or systemic toxicity was noted. However, histochemical review of biopsies of human skin topically treated with **A-007** revealed that increased infiltrates of T-lymphocytes—CD4+, CD3+, CD8+, and CD45+—had occurred after treatment.[2] Increased skin infiltrates of CD11c+ dendritic cells were also observed in treated areas.[3] Immunohistochemical (IHC) studies suggested that immune modulation had occurred in vitro and in vivo following exposure to **A-007**.[2,3]

X-ray crystallography data revealed that **A-007** (as monoclinic crystals) exists as two unique molecules, which differ only in the orientation within the bis-diphenylmethane group, where the rings are approximately perpendicular to each other (and rotated approximately 90° from the orientation of the rings in each rotamer) (Fig. 1).[4] Both rotamers showed strong intramolecular hydrogen bonds between the –NH of the –HN-N=C– moiety and an oxygen of the *o*-nitro group. Thus, there are at least three unique moieties present in **A-007** that may contribute to its overall biological activity—*a dihydroxy-bis-diphenylmethane, a hydrazone, and a dinitrophenyl moiety*. However, despite **A-007**'s high electrophilicity, it lacks chemical reactivity.[5] This paper presents support for **A-007** and its analogues as T-cell activators via CD45+ surface receptors on lymphoendothelial cells, in particular with dendritic cells.

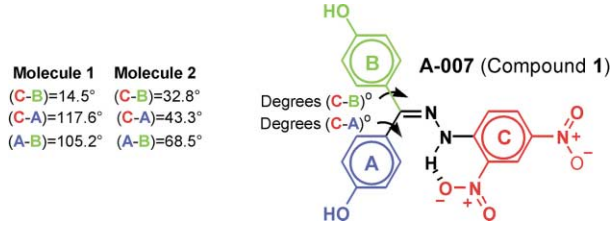


**Figure 1.** X-ray crystallography characteristics of **A-007**, **1**.[4]

Dendritic cells (DC) are antigen-presenting cells (APCs) involved in the initiation of the immune response.[6] Serving as immune system sentinels, DCs are responsible for antigen (An) acquisition and subsequent transport to T-lymphocyte rich areas. The former are present in

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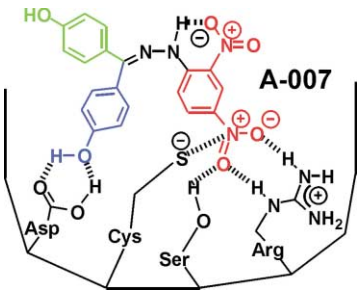



**Figure 2.** Proposed interactions of **A-007** with the CD45 receptor (modified from ref 9).

lymphatic tissues, such as peripheral cutaneous tissue, as well as in lymphoid organs. Once the DCs interact with antigens and have been activated, they become capable of specific immune responses. Secondary lymphoid organs, such as the skin, recruit both naïve T-lymphocytes and An-stimulated DCs into T-cell rich lymphoid zones/networks (nodes, etc). Co-localizing these early immune response constituents are cognitive of T-cell activation.[6]

Effective anti-tumor responses require both DC (APCs) and lymphocyte effectors.[7] Because tumor cells often have limited expression of microhistochemical (MHC) antigens and lack co-stimulatory molecules, they are not effective modulators of APCs.[8] **A-007** is a simple 'organic' molecule, that is sufficiently chemically endowed to act as a hapten or An (Fig. 1). This paper proposes that up-regulation of the CD45+ receptor is an initiation site for the **A-007**-induced immune modulations that are being observed in patients with cancer.[1,2]

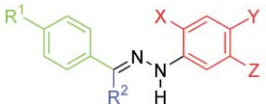
CD45+ is expressed on dendritic cells, lymphocytes, monocytes, and leukocytes, as well as some neoplastic cells, as a protein tyrosine phosphatase (PTP), which together with other members of the PTPs, are responsible for phosphorylating tyrosine residues.[9] Blockade of the CD45+ sites with anti-CD45 antibodies inhibited T-cell activation and prevented mitogen (lectin) activation of naïve T-cells.[10] CD45+ receptor surfaces contain arginine, serine/threonine, and cysteine moieties, which can bind to and/or transfer natural ligands to the surface of APCs, as well as hydrolyze tyrosyl phosphates.[11] **A-007** does not inhibit or block CD45+, but up-regulates lymphocytes and dendritic cells (to APCs) via electrostatic/non-covalent binding with Arg, Cys, Ser/Theo, etc (Fig. 2).[3] Furthermore, **A-007**-activated DCs are capable of initiating mitotic events with naïve human blood peripheral mononuclear cells (PBMC) and up-regulating both CD45+ and CD11c+ receptors in human peripheral dendritic cells.[12] Dendritic cells in cancer tissue are up-regulated from CD45RA+ to CD45RO+ following exposure to topical **A-007** during topical treatment of skin lesions.[12] Thus, **A-007** is not an inhibitor of CD45+, but an up-regulator or modulator of the molecular sites (Fig. 2). The influence that functional group substitutions may have on **A-007**'s intra-/inter-molecular hydrogen binding and electrostatic interactions is presented below.

In order to gather preliminary information on the above concept—that **A-007** may be binding to a CD45+ surface receptor and upgrading DCs and/or T–lymphocytes—analogues of **A-007** have been synthesized and tested in vitro to determine if they have the ability to bind to (or up-regulate) surface CD receptors—with cytotoxicity or apoptosis. Structural changes made to **A-007** include changes to the (a) bis-diphenyl methane and (b) the phenylhydrazone moieties.

## Chemistry

**A-007** analogues in Table 1 were prepared through the reactions of equivalent amounts of the corresponding hydrazine and aryl ketone in methanol or ethanol with sulfuric acid as a catalyst.[13] Resulting hydrazones were recrystallized from ethanol or glacial acetic acid in

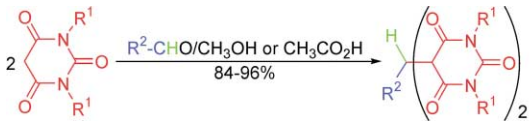
**Table 1.** Anticancer activities and up-regulation for substituted phenylhydrazones[a]




| | Analogues **1**–**11** | Cytotoxicity | Binding Intensity @ 5 μg/Ml | | |
|---|---|---|---|---|---|
| | | $IC_{50}$ (μg/mL) | CD4+ | CD11C+ | CD45+ |
| **1** | $R^1$=OH, $R^2$=4-PhOH, X=Y=$NO_2$, Z=H | 3.2 | NC[a] | ↑20% | ↑50% |
| **2** | $R^1$=OH, $R^2$=4-PhOH, X=$NO_2$, Y=H, Z=H | 2.7 | NC | NC | NC |
| **3** | $R^1$=OH, $R^2$=$CH_3$, X=H, Y=$NO_2$, Z=H | 4.1 | NA[b,c] | NA | NC |
| **4** | $R^1$=OH, $R^2$=H, X=H, Y=$NO_2$, Z=H | 5 | NA | NA | NC |
| **5** | $R^1$=OMe, $R^2$=4-PhOMe; X=Y=$NO_2$, Z=H | >10 | ↑25% | ↑25% | NC |
| **6** | $R^1$=H, $R^2$=Ph, X=Y=$NO_2$, Z=H | >10 | NC | NC | NC |
| **7** | $R^1$=OH, $R^2$=4-PhOH, X=Y=$NO_2$, Z=Cl | 1.5 | NC | NC | NC |
| **8** | $R^1$=$NH_2$, $R^2$=4-Ph$NH_2$, X=Y=$NO_2$, Z=H | 5 | NC | NC | ↑25% |
| **9** | $R^1$=$NHSO_2$Ph, $R^2$=4-Ph$NHSO_2$Ph, X=Y=$NO_2$, Z=H | 6 | NA | NA | NA |
| **10** | $R^1$=$OPO_3$, $R^2$=4-Ph$OPO_3$, X=Y=$NO_2$, Z=H | >10 | NC | NC | NC |
| **11** | $R^1$=$OSO_2$Me, $R^2$=4-Ph$OSO_2$Me, X=Y=$NO_2$, Z=H | >10 | NC | NC | NC |

[a] *Vs* HH leukemia cells.
[b] NC, no change in CD.
[c] NA, not available.

**Table 2.** Anticancer and up-regulation for quinoline and pyridine bis-barbituric acid analogues[a]

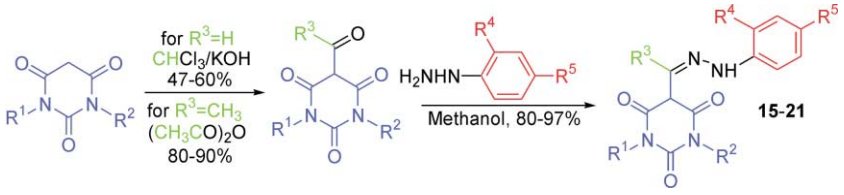



**12-14**

| | Analogues **12–14** | Cytotoxicity | Binding Intensity @ 5 μg/mL | | |
|---|---|---|---|---|---|
| | | $IC_{50}$ (μg/mL) | CD4+ | CD11C+ | CD45+ |
| **12** | $R^1$=H, $R^2$=4-quinoline | >10 | NC[b] | NC | ↑25% |
| **13** | $R^1$=$CH_3$, $R^2$=4-quinoline | >10 | NA[c] | NA | NA |
| **14** | $R^1$=H, $R^2$=3-pyridine | >10 | NA | NA | NA |

[a]*Vs* HH leukemia cells.
[b]NC, no change in CD.
[c]NA, not available.

**Table 3.** Anticancer and up-regulation for formyl and acetylbarbituric phenylhydrazone analogues[a]

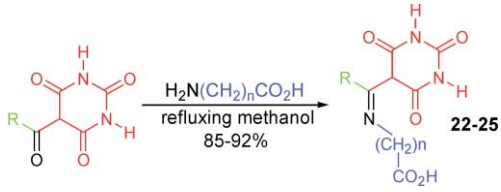



**15-21**

| | Analogues **15—21** | Cytotoxicity | Binding Intensity @ 5 μg/mL | | |
|---|---|---|---|---|---|
| | | $IC_{50}$ (μg/mL) | CD4+ | CD11C+ | CD45+ |
| **15** | $R^1$=$R^2$=$CH_3$; $R^3$=$CH_3$; $R^4$=$R^5$=$NO_2$ | 10 | NA[b] | NC | NA |
| **16** | $R^1$=$R^2$=$CH_3$; $R^3$=H; $R^4$=$R^5$=$NO_2$ | >8 | NA | NA | NA |
| **17** | $R^1$=$R^2$=H; $R^3$=$CH_3$; $R^4$=$R^5$=$NO_2$ | 3.5 | NC[c] | NC | NC |
| **18** | $R^1$=$R^2$=H; $R^3$=H; $R^4$=H; $R^5$=$NO_2$ | >6 | NC | NC | NC |
| **19** | R1=$R^2$=H; $R^3$=$CH_3$; $R^4$=H; $R^5$=$NO_2$ | >10 | NC | NC | NC |
| **20** | $R^1$=$R^2$=H; $R^3$=Ph; $R^4$=$R^5$=$NO_2$ | >10 | NC | NC | NC |
| **21** | $R^1$=$R^2$=H; $R^3$=4-PhOH; $R^4$=$R^5$=$NO_2$ | >10 | NC | NC | NC |

[a]*Vs* HH leukemia cells.
[b]NC, no change in CD.
[c]NA, not available.

**Table 4.** Anticancer and up-regulation for formylbarbituric acid Schiff base analogues[a]



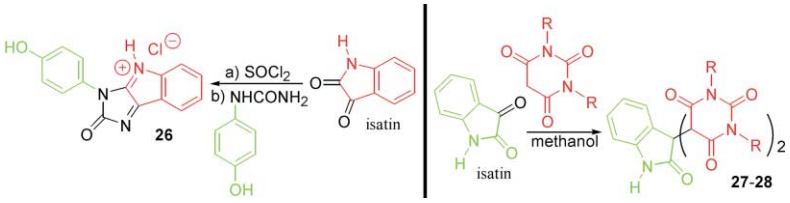

**22-25**

| | Analogues **22–25** | Cytotoxicity | Binding Intensity (@ 5 μg/mL) | | |
|---|---|---|---|---|---|
| | | $IC_{50}$ (μg/mL) | CD4+ | CD11C+ | CD45+ |
| **22** | n=2; R=H | >12 | NC[b] | NC | NC |
| **23** | n=3; R=H | 3.5 | NC | NC | ↑25% |
| **24** | n=5; R=H | >10 | NC | NC | NC |
| **25** | n=3; R=Ph | >10 | NC | NC | NC |

[a]*Vs* HH leukemia cells.
[b]NC, no change in CD.

70–90% yields. Barbituric acid analogues described in Tables 2–4 were prepared either by heterocyclic aldehyde condensation with barbituric acid or by formyl/acetyl barbituric acid condensation with phenylhydrazines/ω-aminoalkanoic acid in methanol as a solvent.[14,15] Isatin analogues in Table 5 were prepared and characterized according to Jursic and Stevens.[16] Analogue **7** was a gift from Dr. Johnny Easmon.[17]

**Table 5.** Anticancer and up-regulation for isatin analogues[a]




| | Analogues **26–28** | Cytotoxicity | Binding Intensity (@ 5 μg/mL) | | |
|---|---|---|---|---|---|
| | | $IC_{50}$(μg/mL) | CD4+ | CD11C+ | CD45+ |
| **26** | See above | >10 | NC[b] | NC | NC |
| **27** | R=H | >10 | NC | NC | NC |
| **28** | $R=CH_3$ | >10 | NC | NC | NC |

[a] *Vs* HH leukemia cells.
[b] NC, no change in CD.

## Biology

T-leukemia cell line—HH (CRL-2105) available from ATCC, Manassas, VA, that is, CD45+, CD3+, CD4+, and CD11C+ (dendritic cell surface CD)— was used to screen **A-007** (**1**) and analogues for up-regulation of CD surface receptor expression, loss of agglutination properties and cell death (Tables 1–5). Multicolor immunofluorescence staining and analysis were performed by standard procedures.[11] Primary and secondary antibodies were conjugated to biotin, fluorescin isothiocyanate (FITC), phyco-erythrin (PE), peridinin-chlorophyl protein or allo-phycocyanin. Antibodies and conjugates for CD3, CD4, CD8, CD11C, CD19, and CD30, were obtained from Becton-Dickinson; CD45 was obtained from PharMingen. Cells were analyzed using a FACScan flow cytometer (Becton-Dickinson). HH T-lymphocytic leukemia cells were cultured in RPMI 1640 media (BioCell) supplemented with activated 10% fetal calf serum, 10 mcg/mL streptomycin and 100 U/mL penicillin (Sigma) in a $CO_2$ incubator at 36 °C. All analogues were prepared in DMSO and stored in a 1:15 ratio of DMSO/RPMI1640 media. CD marker assays and standard cytotoxicity/apoptosis studies were conducted in Corning Cell Wells™. Assays involved $10^5$ HH-cells incubated with the analogues for 24 h, cells were removed, washed with RPMI media and analyzed by a BD fluorescent-activated cell sorter. Agglutination was documented using scanning density assays.[12] Cytotoxicity analysis was conducted using the MTT assay.[12] Apoptosis was followed with—Annexin V- FITC and fluorescein Fragel DNA kits (Oncogene, Inc, San Diego, CA) and DNA fragmentation/cell death analyzed with a FACScanner.

## Results and Discussion

Table 1 reviews toxicity and binding intensity values for a series of simple **A-007** analogues that vary functional groups existing in **A-007**. **A-007** (**1**) prevented HH cells from agglutination, resulting in well-differentiated cells with up-regulation of CD45+ and CD11C+ binding affinities. Apoptosis occurred 12–24 h post **A-007** exposure. Only analogues **1**, **2**, **17**, and **23** produced apoptotic changes, while analogue **7** induced immediate pyknotic nuclear changes and death. Of interest is that analogues **5** and **12** also up-regulated CD receptors. The up-regulation of CD receptors varied as indicated in the Tables.

Modification of the bis-diphenyl/phenylhydrazone moieties resulted in loss of activity as indicated in Table 2.

Replacement of **A-007**'s bis-diphenyl rings with a barbituric acid moiety resulted in some retention of activity (Table 3), but no surface CD up-regulation was noted.

Further modifications have eliminated the phenylhydrazone moiety (Table 4) and provided at least one analogue with equivalent properties to **A-007**. Table 4 reviews Schiff base analogues that have been prepared and screened. Analogue **23** is a very encouraging lead that also has slight (~25%) binding with CD45+. Similarly, effects on cell agglutination, but with less apoptosis (25% vs 100% for **A-007**) were noted.

To date, no analogue with improved immune modulating activity over **A-007** (apoptosis without direct cytotoxicity) has been identified. Analogue **7** is more cytotoxic but does not interact with the CD45+ receptor.[17] In designing cell surface modulators, care must be provided that the new products are not cytotoxic to the point where the therapeutic index has been surpassed.[17,18] The biological properties for some of the described simple analogues (**2**–**28**) appear to be associated with cell membrane modulation and resulting programmed death. This is in contrast to previously described analogues where rings A and B were fused—anthracenes, xanthenes, thioxanthenes, fluorenes, and phthalazenes—and only intracellular changes occurred with immediate cancer cell death, as well as toxicity to normal tissues.[3–17]

Fusion of **A-007**'s benzophenone moiety into heterocyclic rings did not improve the latter's ability to induce

cell death nor up-regulated CD receptor activities (analogues **26**–**28**). The diamino isostere, **8**, possessed some ability to up-regulate CD45+ binding. However, increasing the electronegativity of the DNP moiety (analogue **7**) significantly increased cytotoxicity (with cell death within 24 h) and no detectable up-regulation of CD markers. **A-007**'s-NH moiety is involved with intramolecular –H–O= binding and has little reactivity—Figure 1.[5] **A-007** may be initiating the CD4+/8+ T-cell cascade via up-regulation of the CD45+ receptor at the level of antigen presenting cells (APC).[1,2] The potential use of the immune modulating properties of these compounds in malignant, as well as infectious disease processes, is encouraging.

## Acknowledgements

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14. **Preparation of 4-[di(2,4,6-trioxohexahydropyrimidin-5-yl)] methylquinoline**. Quinoline-4-carbaldehyde (160 mg; 1 mmol) was added to a refluxing methanol (400 mL) containing barbituric acid (pyrimidine-2,4,6-trione) (256 mg; 2 mmol) refluxed for 2 h. Methanol was evaporated off until a slurry formed ~100 mL. The slurry was cooled to room temperature and an orange precipitate that formed was filtered, washed with ice-cold methanol (3×20 mL) and dried at 80 °C for 2 h. Mp 218.6 °C (decomposition); yield–91%; $^1$H NMR (DMSO-$d_6$) δ 10.324 (4H, s, NH), 9.096 (1H, d, $J$=6.0 Hz, quinoline 2-H), 8.483 (1H, d, $J$=9.0 Hz, quinoline 8-H), 8.155 (1H, d, $J$=9.0 Hz, quinoline 5-H), 8.031 (1H, t, $J$=6.0 Hz, quinoline 7-H), 8.401 (1H, t, $J$=6.0 Hz, quinoline 6-H), 7.830 (1H, d, $J$=6.0 Hz, quinoline 3-H), and 6.761 (1H, s, benzyl H). $^{13}$C NMR (DMSO-$d_6$) δ 161.139 and 161.016 (two different carbonyl carbons), 146.879, 139.916, 133.806, 130.040, 125.415, 123.624, 121.919, 117.936, 117.622 (nine carbon atoms in quinoline), 86.450 (benzyl carbon), and 27.479 (barbituric 5-C). MS–ES$^+$ $m/z$ 204 (100%), 223 (45%) 251 (40%), 268 (20%), 396 (M+1, 10%). Elemental analysis: calcd for ($C_{18}H_{13}N_5O_6$) C, 54.69; H, 3.31; N, 17.72. Found: C, 54.55; H, 3.40; N, 17.65.
**Preparation of 4-di[1,3-dmethyl-2,4,6-trioxohexahydropyrimidin-5-yl)methylquinoline**. A methanol (300 mL) solution of 1,3-dimethylbarbituric acid (1.56 g; 10 mmol) and quinoline-4-carbaldehyde (0.78 g; 5 mmol) was refluxed for 2 h. In the course of the reaction yellow precipitate was formed. From the still refluxing reaction suspension the solid was separated by filtration and washed with methanol (3×20 mL) and dried at on the open air to give 1.9 g (84%) of product. . Mp 203.3 °C (decomposition); $^1$H NMR (DMSO-$d_6$, 300 MHz) δ 9.071 (1H, d, $J$=6 Hz, quinoline 2-H), 8.388 (1H, d, $J$=6.0 Hz, quinoline 8-H), 8.157 (1H, d, $J$=9.0 Hz, quinoline 5-H), 8.029 (1H, t, $J$=6.0 Hz, quinoline C-7), 7.924 (1H, d, $J$=6.0 Hz, quinoline 3-H), 7.843 (1H, t, $J$=9.0 Hz, quinoline 6-H), 6.969 (1H, s, benzyl H), 3.687 (1H, s, barbituric 5-H), and 3.125 (12H, s, $CH_3$). $^{13}$C NMR (DMSO-$d_6$, 300 Mz) δ 160.528 and 159.151 (two different carbonyl carbons), 147.564, 139.865, 133.769, 130.026, 125.590, 123.551, 121.679, 117.980, and 117.936 (nine carbon atoms in quinoline), 85.853 (benzyl carbon), 30.319 (barbituric 5-C), and 24.536 (methyl carbons). Elemental analysis for $C_{22}H_{21}N_5O_6$: calcd C, 58.53, H, 4.69, N, 15.51. Found: 58.45, H 4.75, N 15.43.
**Preparation of 3-di(2,4,6-trioxohexahydropyrimidin-5-yl)-pyridine**. Into a hot (110 °C) acetic acid (200 mL) solution of barbituric acid (1.28 g; 10 mmol), pyridine-3-carbaldehyde (550 mg; 5 mmol) was added. A pink precipitate formed immediately; the suspension and solution was heated at 110 °C for additional 30 min and filtered hot. The product was washed with acetic acid (3×20 mL), acetone (3×20 mL), dried at 80 °C under vacuum. Mp 275.2 °C (decomposition); yield–96%; $^1$H NMR (DMSO-$d_6$) δ 10.214 (4H, s, NH), 8.638 (1H, d, $J$=5.7 Hz, pyridine 6-H), 8.427 (1H, s, pyridine 2-H), 8.191 (1H, d, $J$=5.7 Hz, pyridine 4-H), 7.892 (1H, d+d, $J_1$=5.7H, $J_2$=5.7 Hz, pyridine 5-H), and 6.128 (1H,s); $^{13}$C NMR (DMSO-$d_6$) δ 160.90 and 146.99 (two different carbonyls), 141.93, 140.81, 136.36, 135.16, and 122.73 (five pyridine carbons), 85.467 (benzyl carbon), and 25.89 (barbituric carbon); MS–ES$^+$ (in acetic acid) 140 (38%), 209 (43%), 223 (100%), 251 (84%), 283 (26%), and 346 (M+1, 10%). Elemental analysis for $C_{14}H_{11}N_5O_6$: calcd C 48.70%; H 3.21%; N 20.28%. Found: C 48.57%, H 3.35%, N 20.21%.
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