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9-DEAZAADENOSINE—A NEW POTENT ANTITUMOR AGENT*

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Abstract—9-Deazaadenosine (9-DAA), a novel purine analog, was found to be a potent inhibitor of the growth of nine different human solid tumor cell lines *in vitro* and of pancreatic carcinoma (DAN) in antithymocyte serum (ATS)-immunosuppressed mice. In culture, IC_{50} values ranged from 1.1 to 8.5×10^{-8} M. Ovarian carcinoma (MR) was the only cell line in which the activity of 9-DAA was potentiated (about 10-fold) by pretreatment with the adenosine deaminase inhibitor 2′-deoxycoformycin (dCF). After incubation of cultured pancreatic DAN cells with 9-DAA (10^{-5} M) for 2 hr, a peak appeared in the triphosphate region of HPLC nucleotide profiles that was identified tentatively as 9-deazaATP. Under the same incubation conditions, the incorporation of [^{3}H]uridine into RNA and of [^{3}H]thymidine into DNA was inhibited by 34 and 80% respectively. *In vivo* studies using ATS-immunosuppressed mice showed that 9-DAA at 0.4 mg/kg/day for 3 consecutive days reduced pancreatic carcinoma (DAN) tumor weights to approximately 50% of untreated controls. The nucleoside transport inhibitor *p*-nitrobenzyl-6-thioinosine (NBMPR) was shown to selectively protect host tissues from 9-DAA toxicity and, thereby, potentiated the antitumor activity of 9-DAA *in vivo* at optimal dosages.

The discovery of tubercidin (7-deazaadenosine), a powerful antibiotic, has led to the synthesis of several structurally related deazapurine nucleosides. One of these, the C-nucleoside 9-deazaadenosine (9-DAA),** has been shown to have pronounced growth inhibition against several murine and human leukemia cell line *in vitro* (IC_{50} values ranged from 2.3 to 6.6×10^{-9} M) [1].

It has been demonstrated that the nucleoside transport inhibitor *p*-nitrobenzyl-6-thioinosine (NBMPR) significantly increases the long-term survival of tumor-bearing mice treated with potentially lethal doses of cytotoxic nucleosides such as nebularine, tubercidin and toyocamycin [2]. This is apparently accomplished by differential inhibition of the transport of these nucleosides into normal and tumor cells in the mouse [2–6].

In this paper, we report the results of studies designed to determine: (1) the growth-inhibitory effects of 9-DAA on various types of human solid tumor cell lines *in vitro* and on pancreatic carcinoma (DAN) in ATS-immunosuppressed mice; (2) the degree of potentiation of the *in vivo* antitumor activity of 9-DAA by NBMPR; (3) the extent of incorporation of 9-DAA into the nucleotide pools of treated cells; and (4) the effects of 9-DAA treatment on the uptake of [^{3}H]uridine and [^{3}H]thymidine into RNA and DNA respectively.

MATERIALS AND METHODS

Cell cultures. Cell lines were grown in plastic tissue culture flasks (Falcon 3012) in growth medium consisting of RPMI-1640 with 10% fetal bovine serum (FBS) and 0.02 M Hepes buffer. Cells were recovered from culture flasks by using a solution of trypsin (0.5 g/l) and EDTA (0.2 mg/l) diluted 10-fold with Hanks' Balanced Salt Solution (calcium, magnesium and bicarbonate free). All media, FBS, Hepes, trypsin-EDTA and Hanks' solutions were obtained from the Grand Island Biological Co., Grand Island, NY. Tightly capped flasks were incubated at 37° in a non-humidified incubator with air as the gaseous phase. Human solid tumor cell lines used for this study included EP and MW breast, pancreas (DAN), lung (LX-1), lung mesothelioma (VAMT-1), and ovary (MR), all of which were established in our laboratory [7–10]. Other lines used were Tompkins' HCT-8

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** Abbreviations: 9-DAA, 9-deazaadenosine; NBMPR, *p*-nitrobenzyl-6-thioinosine; HPLC, high performance liquid chromatography; ATS, antithymocyte serum; dCF, 2′-deoxycoformycin; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; PBSG, phosphate-buffered saline and glucose; 9-deazaATP, 9-deazaadenosine triphosphate; ADA, adenosine deaminase; 8-azaATP, 8-azaadenosine triphosphate; HK, hexokinase glucose; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

and HRT-18 [11] and a melanoma (Tang)* line established at the Roger Williams Cancer Center. Samples of all cell lines are maintained at −120° in growth medium with 10% dimethyl sulfoxide (DMSO) at early passages (10-20). All cell lines were analyzed routinely and found to be free of mycoplasma contamination [12].

Drugs. Drugs were used as follows: 9-DAA was prepared by a previously published procedure [1]; NBMPR was purchased from the Aldrich Chemical Co. (Milwaukee, WI); and dCF was obtained from the National Cancer Institute (Bethesda, MD). [^{3}H] Uridine and [^{3}H]thymidine were purchased from the New England Nuclear Corp. (Boston, MA).

9-DAA and dCF were dissolved in water, while NBMPR was first dissolved in 100% DMSO and diluted with four parts of water. All drugs were sterilized by filtration (0.45 μm) and serially diluted for *in vitro* studies.

In vitro *cytotoxicity studies.* Screw-capped plastic tissue culture tubes (Corning 25200) were seeded with identical numbers of cells (approximately 2×10^4 cells/ml) in 4.8 ml of growth medium and incubated at 37° overnight on their sides in racks (A. H. Thomas, No. 9272-C30, Philadelphia, PA). The next day, the starting cell number in duplicate tubes was determined as follows: the medium was decanted and replaced with 4.0 ml of the trypsin-EDTA solution (see above). The tubes were again incubated at 37° for 5–7 min. The monolayer cells were shaken loose, and 1.0 ml of growth medium was added to inactivate the trypsin. Single cell suspensions were obtained by forceful ejection (3–4 times) with a 10-ml glass pipette. Cell viability was determined by trypan blue dye exclusion, and the total number of cells per tube was recorded. The drugs were then added in 0.1-ml volumes to duplicate tubes. DCF (1 μg/ml) or NBMPR (2.5×10^{-5} M, 1.0×10^{-6} M) was added 15 min prior to 9-DAA (10^{-4} to 10^{-9} M). Sterile saline (0.2 ml) was added to the controls. Tubes were gently mixed and incubated at 37° for 72 hr. At the end of the incubation, cells were counted and the number of doublings was calculated. The number of doublings at 72 hr for each drug concentration was plotted on semi-log paper, and the concentration of drug that inhibited the number of doublings by 50% was determined from the graph [13].

In vivo *toxicity studies.* Groups of six normal female (non-ATS, non-tumor-bearing) $B_6D_2F_1$ mice (Simonsen Laboratories, Gilroy, CA) were given varying dosages of 9-DAA (0.1 to 0.5 mg/kg/day) as single daily i.p. injections for 3 consecutive days. Mice were observed daily for deaths and weight loss over a 12-day period.

In vivo *immunosuppression, tumor cell injection, and chemotherapy studies.* Female $B_6D_2F_1$ mice (18–20 g) were immunosuppressed by a schedule modified from Stanbridge *et al.* [14]. Subcutaneous injections (0.25 ml) of ATS (M. A. Bioproducts, Bethesda, MD) were given into the right inguinal region on days −1, 0, 1, 3 and 6. On day 0, each animal received a subcutaneous injection of 1–2×10^6 tumor cells into an area over the sternum. Suspensions of cells were obtained from tissue culture flasks by trypsinization, centrifugation and resuspension in a phosphate-buffered saline plus glucose solution (PBSG, Dulbecco's phosphate-buffered saline and 1% D-glucose, GIBCO No. 300-4080). Immediately prior to injection, syringes were "twirled" to resuspend cells [13].

On days 1, 2 and 3, drugs were given intraperitoneally in 0.2-ml volumes. Mice treated with the combination of NBMPR and 9-DAA received 9-DAA immediately after NBMPR. Mice were killed by cervical dislocation, and tumors were removed and quickly weighed to the nearest 0.01 mg on day 10. Six to eight mice were used for each drug-treated and control group. The mean tumor weight of each treated group was divided by the mean weight of the control tumors to obtain percent of control tumor weight. Student's T-2 test was used for statistical analysis.

Inhibition of RNA and DNA synthesis, incorporation into nucleotide pools. Exponentially growing pancreatic carcinoma (DAN) cells (7.8×10^6/20 ml, 5.0 ml/tube) were pretreated for 2 hr with 9-DAA (10^{-5} M). Following the addition of [^{3}H]uridine (5×10^{-5} M, 20 μCi/μmole) or [^{3}H]thymidine (5×10^{-5} M, 20 μCi/μmole), 9-DAA-treated and control tubes were incubated at 37° for 30 min. The cells were then washed three times with ice-cold PBSG and fractionated for cold acid-soluble, RNA and DNA fractions [15, 16]. The cold acid-soluble extract, treated or non-treated with hexokinase-glucose [17], was fractionated by anion-exchange high performance liquid chromatography (HPLC) [18].

The material eluted from the HPLC columns was collected in 1-ml fractions and the radioactivity in each fraction was determined with a Packard Tri-Carb Liquid Scintillometer at an efficiency of approximately 45%. Quenching was not detected in any of the samples tested.

RESULTS

In vitro *cytotoxicity.* 9-DAA exhibited a wide range of inhibitory activity against nine human solid tumor cell lines in culture, as evidenced by IC_{50} values of 1.1 to 8.5×10^{-8} M (Table 1). In eight of the cell lines studied, a 15-min pretreatment of dCF failed to enhance 9-DAA cytotoxicity. However, with the ovarian tumor cell line, dCF increased 9-DAA inhibitory activity by approximately 10-fold. DCF (1 μg/ml) alone produced no inhibition in any of the lines.

In protecting cells from the cytotoxicity of 9-DAA, NBMPR showed differential effects on human pancreatic carcinoma (DAN), lung (LX-1) and melanoma (Tang). For pancreatic carcinoma (DAN) and lung (LX-1), NBMPR (2.5×10^{-5} M) reduced the inhibitory activity of 9-DAA (10^{-7} M) by 43 and 20% respectively. At a higher concentration of 9-DAA (10^{-6} M), these two cell lines were not protected by NBMPR (2.5×10^{-5} M). In comparison, the activity of 9-DAA (10^{-7} M) against melanoma (Tang) was reversed completely by NBMPR (10^{-6} M). In this cell line, equimolar concentrations (10^{-6} M) of

* S. Sato and M. Y. Chu, Abstract, Meeting of the New England Pharmacologists, January 1982.

Table 1. Effect of 9-deazaadenosine in combination with 2′-deoxycoformycin against human carcinomas in culture*

Cell line	IC_{50} (10^{-8} M) −DCF	IC_{50} (10^{-8} M) +DCF
HRT-18	1.1	1.1
Mesothelioma (VAMT-1)	1.3	1.3
EP breast	1.8	1.8
HCT-8	2.1	2.1
Melanoma (Tang)	2.4	2.4
MW breast	2.5	2.5
Pancreas (DAN)	2.8	2.8
Lung (LX-1)	3.5	3.5
Ovarian (MR)	8.5	0.76

* Human carcinoma cells in the exponential phase of growth were incubated with 9-DAA (10^{-4} to 10^{-9} M) in the presence and absence of dCF (1 μg/ml) at 37° for 72 hr. Molar concentrations causing 50% inhibition were determined as described in Materials and Methods.

NBMPR and 9-DAA reduced growth inhibition by 45% (Table 2).

Under the conditions studied, hypoxanthine, adenosine, guanosine, 2′-deoxyadenosine, 2′-deoxyguanosine and 2′-deoxycytidine, alone and in various combinations, failed to protect pancreatic carcinoma (DAN), lung carcinoma (LX-1) and melanoma (Tang) cells from 9-DAA cytotoxicity (data not shown).

In vivo *sensitivity*. The host toxicity of 9-DAA was determined on the basis of weight loss using non-ATS-treated, non-tumor-bearing mice. Dosages ranged from 0.1 to 0.5 mg/kg/day for 3 consecutive days and were based on the LD_{50} (0.5 mg/kg/day for 5 consecutive days) (J.S. Burchenal, personal communication). Although all animals survived, the most toxic dose examined, 0.5 mg/kg/day (×3), elicited an average weight loss of 2.2 g per mouse over a 10-day period.

To obtain a wide range of responses, three dosages (0.3, 0.4 and 0.5 mg/kg/day) were studied against

Table 2. Protection of human pancreatic carcinoma (DAN), lung carcinoma (LX-1) and melanoma (Tang) by NBMPR against the cytotoxicity of 9-DAA *in vitro**

9-Deazaadenosine	% Growth inhibition −NBMPR	% Growth inhibition +NBMPR		
		Pancreatic carcinoma† (DAN)	Lung carcinoma† (LX-1)	Melanoma‡ (Tang)
1×10^{-6} M	100	100	100	56
3×10^{-7} M	100	72	83	32
1×10^{-7} M	100	57	80	0

* Human pancreatic carcinoma (DAN), lung carcinoma (LX-1) or melanoma (Tang) cells were pretreated with NBMPR for 15 min followed by various concentrations of 9-DAA. At the end of 72 hr, cells were counted and the number of doublings was determined to obtain percent inhibition.

† NBMPR = 2.5×10^{-5} M.

‡ NBMPR = 1.0×10^{-6} M.

Table 3. Antitumor activity of 9-deazaadenosine in combination with NBMPR against human pancreatic carcinoma (DAN) in ATS mice*

Group	Daily dosage [mg/kg/day (×3)]	% of control tumor weight ± S.E.	Mean weight change (g/mouse)	P value
Control	0	100	+2.4	
9-DAA	0.4	51.4 ± 7.7	+1.8	<0.02
NBMPR	2.5	63.2 ± 13.1	+2.3	<0.01
NBMPR → 9-DAA	2.5 0.4	9.9 ± 5.8	+2.1	<0.001

* Mice immunosuppressed with ATS were inoculated s.c. with 1.2×10^6 pancreatic carcinoma (DAN) cells in an area on top of the sternum. Twenty-four hours later, therapy was initiated and continued once daily i.p. for 3 days. On day 10 tumors were removed and weighed. The percent of control tumor weight was determined by dividing the mean tumor weight of treated groups by the mean tumor weight of the controls. The mean change in body weight from onset to termination of drug therapy was used as a measure of toxicity.

Table 4. Effects of 9-deazaadenosine on [^{3}H]uridine and [^{3}H]thymidine incorporation in human pancreatic carcinoma (DAN) in culture*

Precursor	Treatment	Cold acid-soluble (cpm/10^6 cells × 10^3)	RNA (cpm/mg × 10^3)	DNA (cpm/mg × 10^3)
[^{3}H]Uridine	Control	23.0	80.3	4.6
	9-DAA	30.0	53.1	1.6
[^{3}H]Thymidine	Control	3.8		29.0
	9-DAA	3.0		6.0

* Human pancreatic carcinoma (DAN) cells (7.8×10^6/20 ml) in the exponential phase of growth were incubated at 37° for 2 hr with 9-DAA (10^{-5} M) followed by a 30-min exposure to [^{3}H]uridine (5×10^{-5} M, 20 μCi/μmole) or to [^{3}H]thymidine (5×10^{-5} M, 20 μCi/μmole). Cells were rinsed with ice-cold PBSG and fractionated for cold acid-soluble, RNA and DNA fractions.

pancreatic DAN tumors in ATS-immunosuppressed mice. At a dose of 0.5 mg/kg/day (×3), 9-DAA produced 85% inhibition of tumor growth with 75% mortality after 10 days. The remaining survivors had an average weight loss of 1.2 g. Therapy with 0.4 mg/kg/day (×3) and 0.3 mg/kg/day (×3) reduced tumor weights to 50 and 55% of untreated controls respectively. All animals in both groups survived and gained weight. Consequently, for further *in vivo* evaluation, 9-DAA at a dosage of 0.4 mg/kg/day (×3) was combined with various dosages of NBMPR (1.25, 2.5 and 5.0 mg/kg/day for 3 consecutive days, data not shown). The optimal dose ratio for the NBMPR–9-DAA combination was found to be 6:1 (2.5:0.4 mg/kg/day × 3). Higher doses of NBMPR caused non-selective protection of both normal and tumor cells and therefore reduced 9-DAA antitumor efficacy.

Further *in vivo* antitumor studies (Table 3) were carried out using ATS-immunosuppressed mice bearing pancreatic carcinoma (DAN, 1.2×10^6 cells/mouse). At a dosage of 0.4 mg/kg/day (×3), 9-DAA reduced tumor weights to 52% of untreated controls ($P < 0.02$).

Host toxicity was not observed as mice gained an average of 1.8 g over a 10-day period. When NBMPR at 2.5 mg/kg/day (×3) was coadministered with 9-DAA (0.4 mg/kg/day × 3), tumor weights were reduced significantly to 10% of untreated controls ($P < 0.001$) with no sign of host toxicity (animals gained an average of 2.1 g). NBMPR alone produced 37% ($P < 0.01$) inhibition with a gain in body weight (Table 3).

Inhibition of [^{3}H]*uridine and* [^{3}H]*thymidine uptake.* In pulse-labeling studies using pancreatic carcinoma (DAN), as shown in Table 4, incubation of 9-DAA (10^{-5} M) for 2 hr caused 34 and 66% inhibition of [^{3}H]uridine incorporation into RNA and DNA respectively. Under similar conditions, the incorporation of [^{3}H]thymidine into DNA was inhibited by 80% (Table 4). The incorporation of [^{3}H]uridine into acid-soluble uridine nucleotides was not decreased by 9-DAA treatment (data not shown).

Incorporation of 9-DAA into cellular nucleotide pools. Analysis of the cold acid-soluble fraction of 9-DAA-treated and control cells by anion-exchange HPLC, with monitoring of column effluents at 254 and 276 nm, revealed a large peak in the triphosphate region of the nucleotide profile (Fig. 1). This peak, which eluted on the leading edge of the UTP peak, was identified tentatively on the basis of the 276/254 absorbance ratio, as 9-deazaATP. When a hexokinase-glucose peak shift procedure [17] was performed on an aliquot of the same cold acid-soluble fraction, a shift of the new peak from the triphosphate to the di- and monophosphate regions of the profile occurred (data not shown).

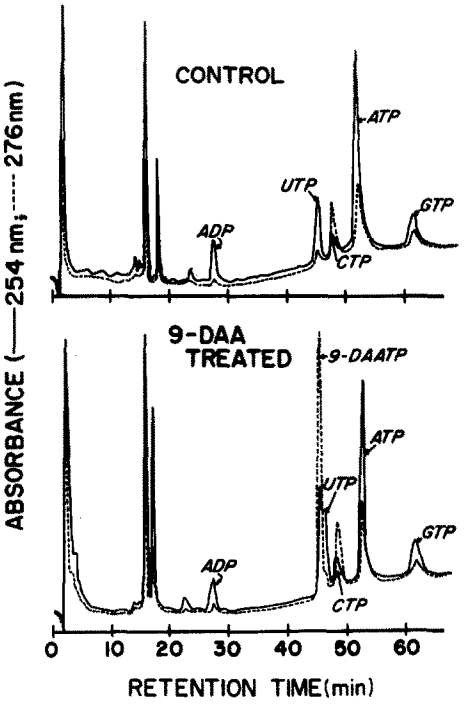

Fig. 1. Incorporation of 9-DAA into analog nucleotide pools of human pancreatic carcinoma (DAN). Anion-exchange high performance liquid chromatography was carried out under the following conditions: HPLC Varian 5000 Liquid Chromatograph equipped with a Schoeffel SF770 Spectroflow Monitor; buffers: low concentrate 0.001 M potassium phosphate (pH 4.5), high concentrate 0.5 M potassium phosphate (pH 4.5); gradient: linear increase in high concentrate at 2.5%/min for 40 min, followed by a 10-min elution at 100% high concentrate and then a 10-min decrease in high concentrate at a rate of 10%/min; column: Whatman Partisil PXS 10/25 SAX; flow rate: 1.2 ml/min.

DISCUSSION

The adenosine analog 9-DAA is a potent inhibitor of the growth of nine human solid tumor cell lines *in vitro* and of pancreatic carcinoma (DAN) *in vivo*. In the past, the antineoplastic efficacy of adenosine analogs has been limited by the deaminating effects of adenosine deaminase (ADA) [19–24]. Thus, in an effort to potentiate the inhibitory activity of 9-DAA, cells were pretreated with dCF, a tight-binding inhibitor of this enzyme. However, with the exception of a 10-fold increase in efficacy against the ovarian cell line, dCF pretreatment failed to enhance 9-DAA activity.

This lack of potentiation by dCF can be explained in two ways. In eight of the nine cell lines studied, the insensitivity to dCF might reflect uniformly low ADA levels. Therefore, the relative responsiveness of the ovarian cell line would indicate that this line possesses greater ADA activity. Alternatively, of these nine cell lines, MR ovarian may possess a form of ADA with a high substrate affinity for 9-DAA.

It is unlikely that the insensitivity to dCF pretreatment in eight of the nine cell lines is due to a low ADA content. Our previous studies indicate that HRT-18, HCT-15, LX-1 and pancreatic DAN all possess significant activities of this enzyme. For example, *in vitro* and *in vivo*, dCF effectively potentiated the inhibitory activity of 8-azaadenosine (a good substrate for ADA) against these four cell lines. Moreover, in LX-1 cells treated with 8-azaadenosine, dCF pretreatment increased 8-azaATP levels by approximately 6-fold [25]. Thus, it appears that 9-DAA may be a poor substrate for ADA and hence is not subject to rapid inactivation by this enzyme.

In *in vitro* studies of 9-DAA cytotoxicity, three human carcinomas responded differently to the protective effects of NBMPR. In order of responsiveness, they were: melanoma (Tang) > pancreatic carcinoma (DAN) > lung carcinoma (LX-1). When used in combination with NBMPR, higher concentrations of 9-DAA were required to elicit a comparable 50% inhibitory level in these three cell lines (Tables 1 and 2). Therefore, pretreatment with this nucleoside transport inhibitor afforded protection against higher 9-DAA concentrations *in vitro*.

When NBMPR was used in combination with 9-DAA *in vivo*, protection of host tissues against 9-DAA toxicity was demonstrated. An 82% increase in overall weight gain concomitant with a 40% increase in the antitumor activity of 9-DAA was attained. The fact that NBMPR caused 37% inhibition as a single agent indicates an interference with the transport of both normal nucleosides and their analogs. The usefulness of NBMPR as an adjuvant chemotherapeutic agent lies in its ability (1) to protect normal host tissues from 9-DAA toxicity at optimal doses, and (2) to potentiate the antineoplastic effects of 9-DAA by broadening the useful range of otherwise lethal doses.

These studies demonstrate that 9-DAA is a potent antineoplastic agent against a variety of human tumor cell lines *in vitro*. The host toxicity of 9-DAA can be selectively prevented by coadministration with the nucleoside transport inhibitor NBMPR, thus increasing the therapeutic index of 9-DAA in ATS-mice bearing human pancreatic carcinoma (DAN) xenograft tumors. In addition, 9-DAA can be incorporated into the nucleotide pools of treated cells and may exert its effects, in part, by inhibiting not only the interconversion of purine nucleotides, but also RNA and DNA synthesis.

Clearly, the significance of this work rests with the clinical potential of 9-DAA in combination with NBMPR. Most importantly, in studies carried out in culture and in ATS mouse xenografts, 9-DAA has demonstrated a potent antineoplastic efficacy against a wide variety of human solid tumors and leukemias. Secondly, because of the apparent biological stability of 9-DAA relative to other adenosine analogs, the use of toxic ADA inhibitors (e.g. dCF) may be circumvented. Finally, our *in vivo* results suggest that NBMPR should be most effective in protecting normal tissues from 9-DAA toxicity at clinical levels.

Future studies will focus on: (1) the relationship between 9-DAA cytotoxicity and inhibition of the incorporation of [^{3}H]uridine into RNA and DNA, (2) the effects of 9-DAA on DNA polymerase, (3) [^{14}C]leucine incorporation into protein fractions of 9-DAA-treated cells, (4) correlation of cell survival with [^{3}H]-9-DAA incorporation into RNA and DNA fractions, and (5) the combination of 9-DAA with NBMPR-phosphate (a more soluble form of NBMPR) in ATS-immunosuppressed mice with the hope of increasing the chemotherapeutic efficacy of 9-DAA.

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