As stated in the introduction, we have compared the conformations of the TRH analogues with those observed in two crystal structure determinations[18,19] for Leu[5]-enkephalin. Literature reports[6,24] indicate that TRH, opiates (such as morphine), and opiate antagonists (such as naloxone) interact but apparently not by binding to the same receptors. We are unaware of any direct comparisons of TRH and enkephalin receptor binding but felt that since both are CNS active and interact with some of the same drugs, a comparison of conformational properties for the two peptide systems could be of interest.

We have detected a surprising degree of similarity between the conformations of the TRH analogues and those of the last three residues of the enkephalin. There are eight independent observations for the latter system (four in XI and four in XII). We have compared the conformations directly,[26] by least-squares superposition methods, with the conformation of I. Figure 7 displays the best and worst fit conformations from each crystal structure determination plotted, together with representative examples of the TRH analogues. The similarities are sufficient to suggest that TRH might bind to some enkephalin receptors or vice versa. If TRH does bind to enkephalin receptors, the smaller molecule will very likely only interact with part of the binding site, which implies that different physiological effects might result. We hope that these observations will provide incentive for suitable biological studies for the determination of the presence or absence of TRH–enkephalin receptor interactions.

Acknowledgment. This work was supported in part by the Deutsche Forschungsgemeinschaft (Grant No. Ste 230/5-1) and by the Verband der Chemischen Industrie e.V. We are particularly grateful to L. Flohé and his colleagues at Grünenthal GmbH for samples of several R[1]-TRH analogues and to W. Voelter of the Universität Tübingen for the Phe[2]-TRH analogue.

Supplementary Material Available: Tables of atomic coordinates, bond distances, bond angles, torsion angles, temperature factors, and observed and calculated structure factors for compounds I–VII (169 pages). Ordering information is given on any current masthead page.

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Notes

The Covalent Linking of Two Nucleotide Analogues to Antibodies

Esther Hurwitz,* Rina Kashi, Ruth Arnon, Meir Wilchek, and Michael Sela

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel. *Received March 26, 1984*

Two anticancer drugs, antagonists of nucleic acids, were covalently linked to antibodies specifically reactive with B leukemia cells and thus with a potential possibility of drug targeting to the tumor site. The drugs cytosine 1-β-D-arabinoside and 5-fluorouridine, competitive inhibitors of enzymes involved in DNA synthesis, were linked to antibodies via a dextran bridge. Cytosine 1-β-D-arabinoside was linked to periodate-oxidized dextran and fluorouridine to dextran hydrazide. The dextran derivatives were in turn linked to antibodies that recognized a specific membrane IgM on B leukemia cells. The drug–antibody conjugates maintained most of the original antigen-binding capacity of the antibody and the full pharmacological activity of the drugs.

The major drawback of cancer chemotherapy is its toxicity to normal cells. One possible way to increase the effectiveness of antitumor drugs would be to find methods of altering their distribution in the body so as to increase their local concentration at the tumor site while maintaining lower systemic concentrations. This could be achieved by chemical coupling of drugs to carriers such as antibodies with preferential affinity toward tumor cells. The linking of antineoplastic agents such as drugs or toxins to antibodies specifically or preferentially reactive with tumor cells has gained wide interest in recent years. Antibodies with the capacity to specifically recognizing tumor cells and tissues have by now been developed and some of them were shown to be able to concentrate the tumors sites in vivo.[1] We have previously bound the antineoplastic-reactive antibiotics daunomycin and adriamycin to antibodies and used these conjugates as therapeutic agents in vitro and in vivo in several tumor systems.[2] Although these two drugs are effective against many types of neoplasia, some tumors are almost unresponsive to them yet are sensitive to other drugs such as 5-fluorouracil. A malignant growth is usually made up of more than one type of cell.[3] The development of this variability is a continuous process often furthered by drug treatments that can lead to the development of resistant cell clones. For that reason chemotherapeutic treatment regimes often involve mixtures of drugs with different mechanisms of action. We have, therefore, extended our studies to the linking of two additional drugs of a different nature, antimetabolites of nucleic acids, to antibodies. Both these drugs inhibit competitively enzymes involved in DNA synthesis. 5-Fluorouridine (FU) inhibites thymidylate synthetase, while cytosine arabinoside (ARA-c) inhibits DNA polymerase.[4]

We describe the binding procedures of these drugs to antibodies through backbones of dextran derivatives and their inhibitory activity on culture tumor cell lines.

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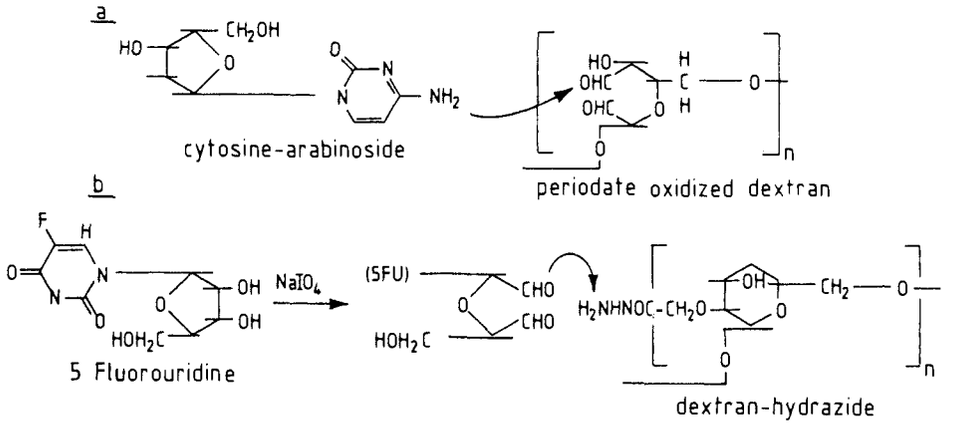
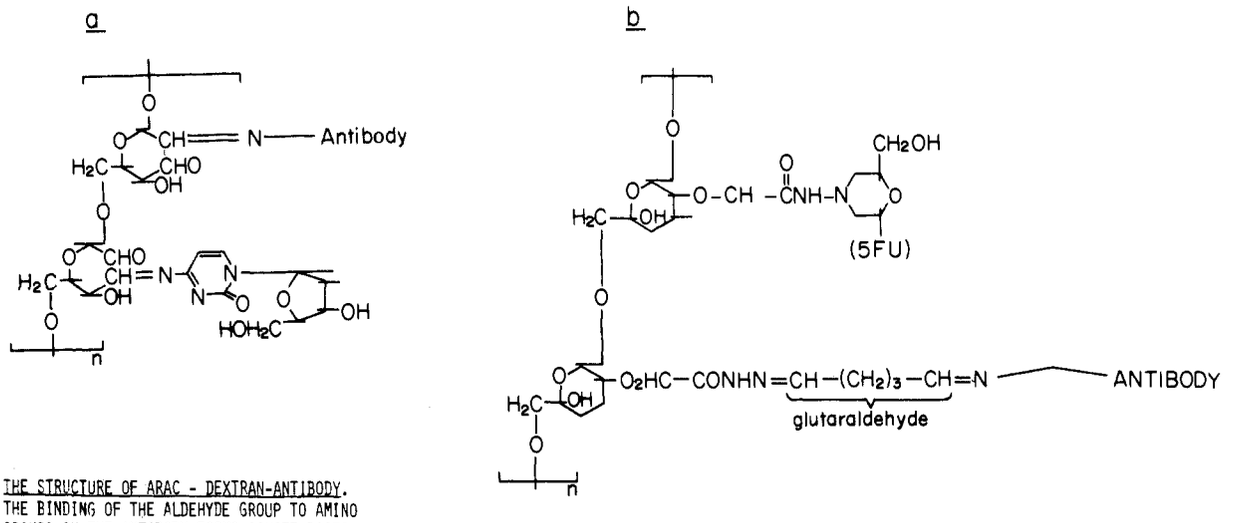
Scheme I



Scheme II



THE STRUCTURE OF ARAC - DEXTRAN-ANTIBODY.
THE BINDING OF THE ALDEHYDE GROUP TO AMINO GROUPS ON THE ANTIBODY FORMS SCHIFF BASES OR 7-MEMBERED RINGS ENCLOSING THE M.

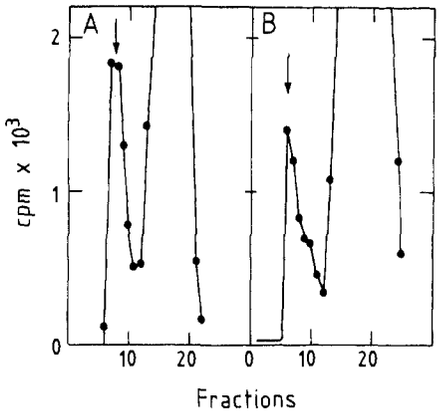


Figure 1. The separation of free from bound drugs on Bio-gel P60 columns (1 × 10 cm). The bound drugs were determined by radioactivity. (A) 5-Fluorouridine, (B) cytosine arabinoside. The antibody in the preparations was determined by absorption at 280 nm. The absorption of each drug at that wavelength is substracted from the total OD.

THE STRUCTURE OF 5 FUR-DEXTRAN ANTIBODY.

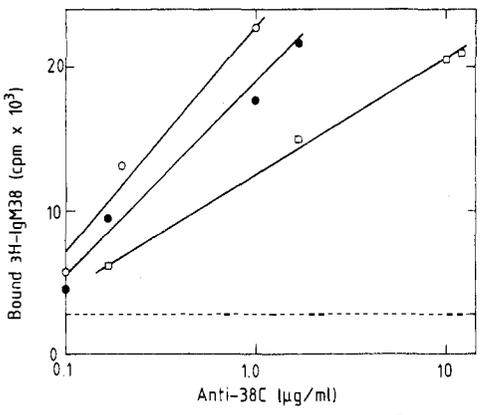


Figure 2. The antibody activity of the drug–antibody conjugates. The anti-38 IgM antibody activity was measured by its binding to ^{3}H-38c IgM antigen. Goat anti-38c (●), ARA-c–dextran–anti-38c (○), FUR–dextran–anti-38c (□).

Experimental Section

Cytosine Arabinoside–Dextran–Antibody Conjugate (1). The binding of cytosine 1-β-D-arabinofuranoside (Sigma, St. Louis, MO) was similar to that previously described for linking daunomycin, using a periodate-oxidized dextran derivative as a backbone.[5] ARA-C, 30 mg in 0.15 mL of phosphate-buffered saline (PBS) was mixed with 45 mg of oxidized dextran in 0.2 mL of PBS and left for 20 h at room temperature. Then 5 mg of antibody in 1 mL was added and the mixture was allowed to stand at room temperature for another 20 h. The drug and the antibody bind to the aldehyde groups on the periodate-oxidized dextran to form Schiff bases, which in turn are reduced by addition of sodium cyanoborohydride (Sigma, St. Louis, MO) in equimolar amounts to the amount of periodate used for oxidation of the dextran (Schemes Ia and IIa). The reduction was performed for 1 h at 37 °C. The reduction step was essential for the stability of the product and did not affect the drug's activity. Free drug and drug dextran were separated from the conjugate by gel fil-

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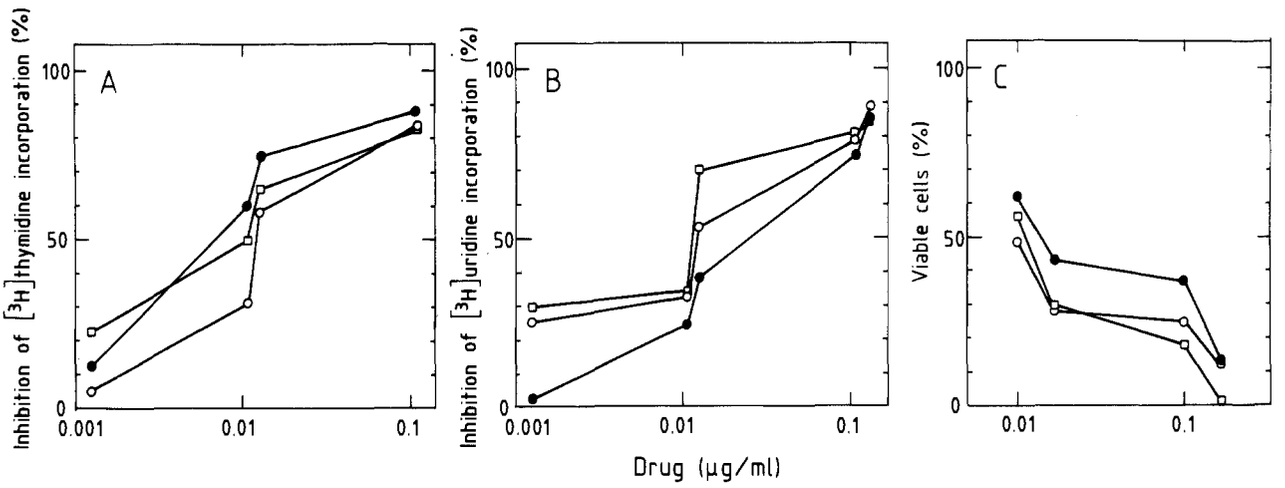

Figure 3. The pharmacological activity of the ARA-c–dextran–antibody derivative on 38c B leukemia cells. (A) Inhibition of [*methyl*-^{3}H]thymidine incorporation. The cells (10^5/mL in 100 L of medium) were incubated in the presence of 50 L of inhibition solution. After 2 h [*methyl*-^{3}H]thymidine was added and incubation continued for another 2 h. The experiment was terminated, harvested, and counted. (B) Inhibition of [^{3}H]uridine incorporation. Same as in A except the incubation was 20 h with the inhibitors and with [^{3}H]uridine 5 h. (C) Cytotoxicity. The cytotoxicity was evaluated by incubating the drug and its derivatives with 10^5 cells in 1 mL for 24 h. The number of viable cells was determined by trypan blue exclusion. The degree of substitution varied from 25 to 60 mol/mol and did not have a marked effect on the results. ARA-c (●), ARA-c–dextran (○), ARA-c–dextran–anti-38c (□).

tration on Bio-Gel P-GO (100–200-mesh Bio-Rad, Richmond, CA). The stability of the compounds was tested by repeated fractionations on the same column. In order to determine the amount of drug bound to antibody ^{3}H-labeled cytosine arabinoside (Radiochemical Centre, Amersham, England) was used. It was mixed with the cold ARA-c and the binding was performed as described above (Figure 1). The extent of substitution was 25–60 mol of ARA-c/mol of antibody in different preparations. The amount of antibody in the conjugate was determined by the absorption at 280 nm after substraction of the drug's optical density at this wavelength. The yield of bound ARA-c varied in different preparations from 2% to 7%.

5-Fluorouridine–Dextran–Antibody (2). 5-Fluorouridine (FUR) (Calbiochem-Bearing, La Jolla, CA) (15 mg in 0.1 mL of PBS) was oxidized by $NaIO_4$ (0.15 mL of 0.3 M) for 30 min at room temperature. The oxidation was stopped by the addition of excess ethylene glycol (150 μL of a 0.8 M solution). After 30 additional min, oxidative byproducts were removed by several extractions with 1 mL of ethyl acetate, which was carefully aspirated (some residual IO_3^- was still present after the extraction). The oxidized fluorouridine was mixed with dextran hydrazide[6] (20 mg in 0.15 mL of 0.1 M sodium acetate buffer, pH 5.6) and the mixture was kept at room temperature for 5–7 h. The attachment of the FUR–dextran hydrazone to the antibody was performed by cross-linking with glutaraldehyde.[7] The drug–polymer was mixed with 7 mg of antibody in a final concentration of 0.08% glutaraldehyde (Ladd, Jerusalem, Israel) in PBS for 20 min at room temperature (Schemes Ib and IIb). The separation from unbound drug was obtained by fractionation on Bio-Gel-P-60 (Figure 1) or by dialysis. The amount of drug bound to the antibody was determined by mixing [^{3}H]uridine (Radiochemical Centre, Amersham, England) (10 μCi) to the 5-fluorouridine during its oxidation and binding it along with the latter to the antibody. The extent of substitution was calculated from the radioactivity of the product. The results indicated that in various preparations 7–24 mol of FUR were bound per mole of antibody.

Biological Test Methods. The drug activity of the conjugates was tested by its effects on a B leukemia 38c which has a membrane-bound IgM (IgM 38c). Inhibition of the incorporation of either [*methyl*-^{3}H]thymidine (6.25 Ci/mmol, Nuclear Research, Beer-Sheva, Israel) or [^{3}H]uridine (29 Ci/mmol, Radiochemical Centre, Amersham, England) or both were performed by using a similar procedure to that previously described.[8] The tumor cells, 1 × 10^5/mL, were suspended in medium (RPMI 1640, containing 10% fetal calf serum, Biolab, Jerusalem, and 5 × 10^{-5} M 2-mercaptoethanol) and dispensed in 100-μL aliquots into the wells of microtiter plates (Micro test II 3040, Falcon Plastics, Oxford, CA). The drug or the drug conjugates were added to the cells in 50-μL amounts. The plates were incubated for 2 or 24 h at 37 °C (in 5% CO_2 in air). At that time the radioactive nucleotides were added, and the plate was further incubated for 2–4 h. The reaction was terminated by harvesting on a Flow harvester (Flow Laboratories, Ayrshire Scotland), and the filters were counted in a liquid scintillation counter. The cytotoxic effect of the drug derivatives on the tumor cells was determined by incubating 10^5 38c cells/mL for 24 h and counting the number of viable cells by trypan blue exclusion.[8]

The antibodies used for linking the drugs were specifically reactive with the tumor cells by recognition of the IgM expressed on 38c leukemia cells. Antiserum against the 38c IgM was passed through an affinity column of sepharose–mouse immunoglobulins. Subsequently, the effluent was absorbed on and eluted from a second affinity column of 38c type IgG-sepharose. The eluate consisted of purified idiotype specific antibodies.[9] The antigen-binding capacity of the anti-38c antibody before and after its conjugation with drugs was tested by using ^{3}H-labeled 38c IgM.[10]

Biological Evaluation

Antibody Activity of the Drug–Anti-38c Conjugates. The antigen-binding capacity of ARA-c–dextran–anti-38c and of FUR–dextran–anti-38c and its comparison to the binding of the original antibody is shown in Figure 2. Most of the original antigen-binding capacity is maintained by both derivatives.

The antibody activity of the ARA-c–dextran–anti-38 was somewhat higher than that of the FUR derivative possibly due to the use of a cross-linking agent in the latter's preparation.

Pharmacological Activity of ARA-c–Dextran–Anti-38. The inhibition of the incorporation of both thymidine and uridine by the ARA-c derivative is depicted in Figure 3A and 3B. Bound ARA-c maintained its full

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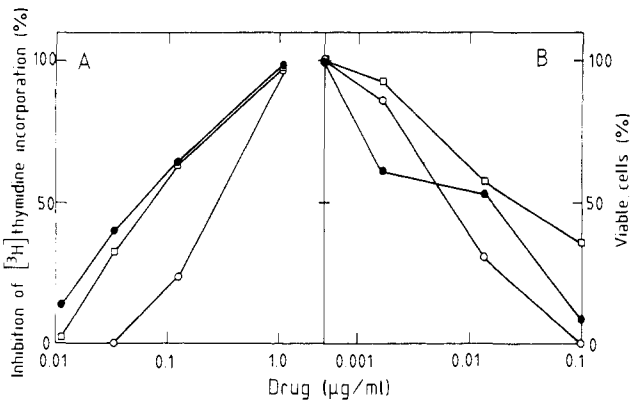

Figure 4. The pharmacological activity of the 5-fluorouridine–dextran–antibody derivative. (A) Inhibition of [*methyl*-^{3}H]thymidine incorporation as in Figure 2A except incubation with the drug and its derivatives was for 20 h. (B) Cytotoxicity as in Figure 2. (C) FU (●), FUR–dextran (○), FUR–dextran–anti-38c (□).

original activity as tested by the inhibition of DNA and RNA synthesis as well as its cytotoxic activity on the tumor cells (Figure 3C). ARA-C–dextran–anti-38 was slightly less effective on a control cell line, Daudi (human lymphoma), than a specific antibody–drug conjugate.

Pharmacological Activity of FUR–Dextran–Anti-38. The inhibition of the incorporation of [*methyl*-^{3}H]-thymidine exerted by the FUR–antibody derivative was similar to that obtained by FUR (Figure 4A) and is slightly better than that obtained by FUR–dextran without the antibody. Similar results were observed when the cytotoxic effects on the tumor cells were tested (Figure 4B). The FUR–dextran–anti-38c had a similar killing effect as FU. It should be noted that the inhibitory activity in vitro of FUR, the drug originally used for the conjugation, was much more potent than that of FU. Nevertheless, we though it right to compare the activity to FU, since we have opened the sugar ring by the periodate oxidation and used the resultant aldehyde groups for the binding. Both FUR and FU are thought to be metabolized to the same products: 5-fluorouridine monophosphate (FUMP) and 5-fluorodeoxyuridine monophosphate (FUdMp). These products exhibit the inhibitory action: FUMP by incorporation into RNA while FUdMP inhibits thymidylate synthetase. In spite of the metabolism through the same pathways, differences in their activities were noted. In vivo FUR was not active while FU was effective against the murine B leukemia 38 by prolonging survival (unpublished data). As can be seen from the data, both antibody–drug derivatives, ARA-c–dextran–anti-38 and FUR–dextran–anti-38 maintained their drug activity. ARA-c and FUR are converted biochemically in the cells to other antinucleotides which are actually the active analogues.[4] The active nucleotide of ARA-c is arabinoside cytidine triphosphate, which functions as the antagonist of the physiologic substrate deoxycitidine triphosphate and competitively inhibites DNA polymerase. The active analogues of FU are FUMP and FUdMP as mentioned above. If we assume that a similar mechanism prevails here also, thus, the drugs attached to the antibody not only have to be taken up by the cells but also have to be converted to the active analogues in situ. The penetration of drug–antibody into cells and nuclei was previously demonstrated with other anticancer agents of the tetracycline group daunomycin[11] and adriamycin.[7] The fact that the conjugates are biologically active indicates that some of the necessary biochemical conversions of antinucleotide metrabolites can be performed rapidly even on the immobilized drug. Other possibilities are that the drug conjugates operate via different mechanisms or that the drug is released slowly due to the nature of the bond. A slow release mechanism may perhaps overcome the development of resistance to antimetabolic drugs by shunt mechanisms, which is often the case with this type of drugs, particularly ARA-c. It is also assumed that a selective delivery to the site of action may result in more beneficiary chemotherapy.

Acknowledgment. The antibodies were kindly provided by Prof. J. Haimovich of the Tel Aviv University, Tel Aviv, Israel. The research was supported by a grant from Summa Corp., Albuquerque, NM.

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Substrate Specificity of Pyroglutamylaminopeptidase[1]

John T. Capecchi and Gordon Marc Loudon*

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907. *Received March 20, 1984*

Three synthetic peptides (compounds 4–6) were tested as substrates of pyroglutamylaminopeptidase. In addition, inhibition of the hydrolysis of these substrates by compounds 8 and 9 was also examined. The enzyme does not appreciably catalyze the hydrolysis of peptides with six-membered ureido rings at the amino terminus, but it tolerates well a five-membered ureido ring at this position.

Recently in our laboratory we observed[2] that one of the products resulting from the Lossen rearrangement of α-glutamyl peptides (eq 1) is a hexahydro-2-oxo-pyrimidinyl-4-carbonyl peptide, **1**. A similar five-membered derivative **2** has been observed in the Hofmann degradation of asparaginyl peptides.[3-5] The overall sequence in eq 1 is potentially valuable as a specific cleavage at glutamic acid residues, as noted by Spande et al.,[6] provided that the ureido group can be removed from the amino terminus of

(1) Abbreviations used in this paper are as follows: Pep^N, Pep^C, amino- and carboxyl-terminal portions, respectively, of a general peptide; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; DMF, *N,N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid.

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