A Potent "Fat Base" Nucleotide Inhibitor of IMP Dehydrogenase†

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Received May 14, 1998; Revised Manuscript Received June 24, 1998

ABSTRACT: Inosine monophosphate dehydrogenase (IMPDH) is a target for anticancer, antiviral, immunosuppressive, and antimicrobial chemotherapy. Thus, IMPDH inhibitors have great potential as chemotherapeutic agents. Here we show that imidazo[4,5-*e*][1,4]diazapine nucleotide (**I**) is a potent inhibitor of both human type II and *Escherichia coli* IMPDH. **I** is a slow-binding inhibitor. The values of K_d are 1.4 nM and 53 nM for human and *E. coli* IMPDH, respectively. Inhibition is reversible, as demonstrated by the recovery of activity upon denaturation and renaturation of the enzyme·**I** complex. **I** is not a substrate for IMPDH. **I** may form a covalent adduct with the active-site Cys of IMPDH. Such an adduct would serve as an analogue for an intermediate in the IMPDH reaction.

Inosine 5′-monophosphate (IMP)[1] catalyzes the oxidation of IMP to xanthosine 5′-monophosphate (XMP) with the concomitant reduction of NAD^+ to NADH (Figure 1). This reaction is the first committed and rate-limiting step in guanine nucleotide biosynthesis (*1*). IMPDH inhibitors have antiproliferative activity and have been used as immunosuppressive, antiviral, and anticancer agents (*2*−*5*). In addition, IMPDH is believed to be a target useful in the development of antimicrobial chemotherapy (*6*−*8*).

The mechanism of the IMPDH reaction is shown in Figure 1. The active-site Cys attacks the 2-position of IMP, yielding E-IMP*. This intermediate collapses with expulsion of hydride to NAD^+, producing NADH and E-XMP*. E-XMP* hydrolyzes to produce XMP, probably via the tetrahedral intermediate E-XMP†. Although E-XMP* has been isolated and characterized (*9*−*11*), the intermediates E-IMP* and E-XMP† have not yet been identified and are most probably unstable outside the IMPDH active site.

Coformycin, a potent inhibitor of adenosine deaminase, acts as a transition-state analogue by mimicking the tetrahedral adduct formed during the adenosine deaminase reaction (Figure 2) (*12*). Similarly, we believe that imidazo-[4,5-*e*][1,4]diazapine nucleotide ("fat base" nucleotide **I**) and related compounds could be potent inhibitors of IMPDH. Carbonolamines such as **I** are in rapid equilibrium with the dehydro form; however, the equilibrium concentration of the dehydro form is quite small ($K_{eq} \approx 10^{-6}-10^{-8}$) (*13*−*16*). The dehydro form of **I** could react with IMPDH to form a tetrahedral adduct resembling E-IMP* and E-XMP† (Figure 2). Such an adduct would be stabilized by the same interactions that stabilize E-IMP* and E-XMP†, and would be a transition-state analogue of the IMPDH reaction. The following experiments demonstrate that **I** is a potent, time-dependent inhibitor of IMPDH.

MATERIALS AND METHODS

Materials. IMP, NAD^+, and XMP were purchased from Sigma Chemical Co. (St. Louis, MO). [8-^{14}C]IMP was purchased from Moravek Biochemicals (Brea, CA). Human IMPDH type II and *Escherichia coli* IMPDH were purified as previously described (*17*).

Synthesis of I. The nucleoside of **I** was synthesized according to published procedures (*18*). The N-1 position of inosine was alkylated with bromoacetaldehyde diethylacetal. Subsequent ring opening in NaOH, followed by acidification, yielded the nucleoside of **I**. The identity of the nucleoside was confirmed by NMR and mass spectrometry: calculated molecular weight = 300 g/mol; electrospray ionization (ESI)−positive ion, m/e = 301 g/mol; ESI− negative ion, m/e = 299 g/mol. The nucleoside was enzymatically phosphorylated by using *Serratia maracens* 5′-nucleoside phosphotransferase and *p*-nitrophenyl phosphate (*19*). The reaction mixture was extracted twice with ether and adjusted to pH 8 with 1 M NaOH. The mixture was centrifuged and the supernatant was applied to a preparative cellulose thin-layer chromatography plate. The plate was developed in *n*-propanol:NH_4OH:H_2O (6:3:1 by vol). **I** was recovered by removing the cellulose and washing with H_2O. The eluant was filtered and absorbed onto a Dionex column, and **I** was eluted from the column with a gradient of NH_4HCO_3 (50−1000 mM, pH 8.2). The NH_4HCO_3 was removed by lyophilization. The identity of **I** was confirmed by NMR spectroscopy: ^{1}H NMR (D_2O) 3.34 (dd, 1 H, C-6 H), 3.54 (dd, 1 H, C-6 H), 4.01 (m, 2 H, C-5′ H), 4.31 (dd, 1 H, C-4′ H), 4.42 (t, 1 H, C-3′ H), 4.65 (t, 1 H, C-2′ H), 5.52 and 5.62 (d, 1 H, C-5 H), 5.68 (d, 1 H, C-1′

† Supported by the Lucille P. Markey Charitable Trust to Brandeis University. L.H. is a Searle Scholar and a Beckman Young Investigator.

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[1] Abbreviations: IMPDH, inosine 5′-monophosphate dehydrogenase; IMP, inosine 5′-monophosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; XMP, xanthosine 5′-monophosphate; EICARMP, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide 5′-monophosphate; DTT, dithiothreitol; ESI, electrospray ionization.

S0006-2960(98)01132-5 CCC: $15.00
Published on Web 08/07/1998

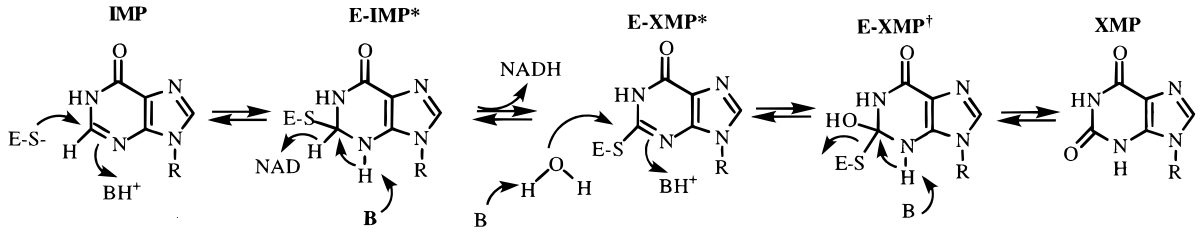

FIGURE 1: Mechanism of the IMPDH reaction.

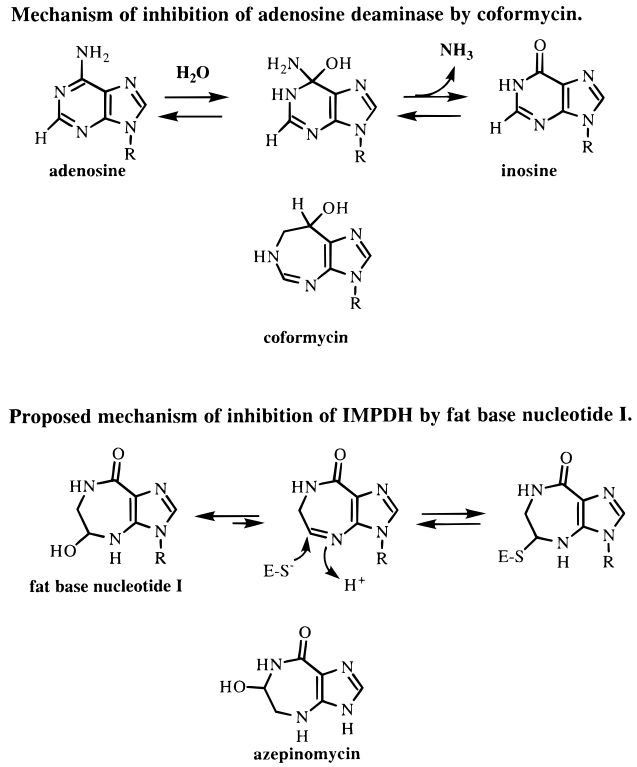

FIGURE 2: Structures and proposed mechanisms of inhibition of coformycin and **I** and structure of azepinomycin.

H), 7.51 and 7.50 (s, 1 H, C-1 H).

Enzyme Assays. The standard assay solution contains 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM dithiothreitol (DTT), and 3 mM EDTA. The following substrate concentrations were used: (*a*) for human IMPDH type II (henceforth human IMPDH) assay, 0.125 mM IMP and 0.100 mM NAD^+; (*b*) for *E. coli* IMPDH assay, 1.0 mM IMP and 2.5 mM NAD^+. The reaction was initiated by addition of enzyme, and the absorbance was recorded at 340 nm for 5 min at 25 °C. Protein concentration was determined by titration with 5-ethynyl-1-β-D-ribofuranosylimdiazole-4-carboxamide 5′-monophosphate (EICARMP) (*17*). A Hitachi U-2000 spectrophotometer was used for all spectrophotometric analysis.

*Kinetics of IMPDH Inhibition by **I***. IMPDH was incubated with various concentrations of **I**; substrates were added at appropriate times, and the enzyme activity was assayed. The loss of activity followed pseudo-first-order kinetics.

Inactivation, Denaturation, and Renaturation of E. coli IMPDH. *E. coli* IMPDH was incubated with **I**. After urea was added to a final concentration of 8 M, the solution was incubated for another hour at room temperature. The denatured enzyme was diluted six-fold with a solution containing 50 mM Tris-HCl, 1 mM DTT and 8 M urea, and concentrated to its original volume with a Centricon 30. This dilution−concentration process was repeated 4 times, and the final concentrate was dialyzed overnight against a buffer containing 50 mM Tris and 1 mM DTT. A control sample of *E. coli* IMPDH (not incubated with **I**) was treated by the same procedure. Enzymatic activity for both control and treated enzyme was monitored at each step.

RESULTS

*Inhibition of IMPDH by **I***. **I** was synthesized according to published procedures as described in *Materials and Methods*. Time-dependent inhibition is observed when both human and *E. coli* IMPDH are incubated with **I** (Figure 3). No activity is recovered upon dialysis, although activity is recovered when the E·**I** complex is denatured with urea and renatured. This observation indicates that inhibition is reversible. The presence of IMP protects the enzyme against inhibition, whereas the presence of NAD^+ has no effect (Figure 3a). High concentrations of IMP completely protect the enzyme from inhibition by **I** (Figure 3b), which indicates that IMP and **I** compete for the same binding site. **I** is not a substrate for IMPDH, however: No NAD^+ reduction was observed in the presence of 3.4 μM *E. coli* IMPDH, 34 μM **I**, and 1.0 mM NAD^+ by an experiment that could detect as little as 1 turnover of NAD^+.

*Determination of K_d for **I** Inhibition of IMPDH*. The values of K_d for **I** inhibition of both human and *E. coli* IMPDH were determined by incubating enzyme (6.2 and 5.0 μM of human and *E. coli* IMPDH, respectively) with IMP (500 μM) and **I** (5.4 and 11 μM for human and *E. coli* IMPDH, respectively). The presence of IMP served two purposes: (*a*) to stabilize the enzyme in the absence of inhibitor; e.g., no activity was lost in the control sample over the course of the experiment (unlike the experiments of Figures 3 and 4, this experiment requires reaction times of days); and (*b*), as shown above, to compete with **I**, which increases the observed K_d by the factor $(1 + [IMP]/K_{IMP})$, where K_{IMP} is the dissociation constant for the enzyme (E)·IMP complex. Only E·**I** and E·IMP complexes exist under the conditions of these experiments; no free enzyme will be present. The value of K_m for IMP was assigned to K_{IMP} for both enzymes. In the case of human IMPDH, the value of K_m for IMP is similar to the value of K_{IMP} determined by isothermal titration calorimetry [$K_m = 4$ μM versus a dissociation constant = 1 μM (*20*, *21*)], which justifies this assignment. However, we have not been able to measure K_{IMP} for *E. coli* IMPDH accurately because the enzyme tends to aggregate at high concentrations. Nevertheless, the results of equilibrium dialysis experiments with the *E. coli* enzyme are consistent with $K_{IMP} \approx 70$ μM, which justifies the assignment of $K_{IMP} = 61$ μM from the results of steady-state experiments (*22*). Activity was monitored over 3.5 days

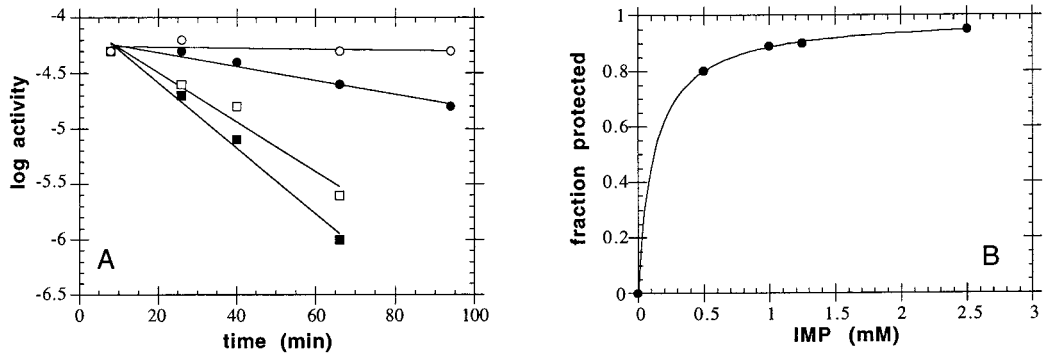

FIGURE 3: Inhibition of IMPDH by **I**. (A) Human IMPDH was incubated with **I** (17 μM) in the absence of substrates (open squares) or in the presence of 125 μM IMP (closed circles) or of 100 μM NAD^+ (closed squares) in 100 mM KCl, 1 mM DTT, and 50 mM Tris (pH 7.5, at 25 °C). The open circles denote the enzyme incubated in the absence of **I**. (B) *E. coli* IMPDH (0.3 μM) was incubated with **I** (50 μM) in the presence of various concentrations of IMP. The rate constants for inactivation are determined from plots as in (A). The fraction protected $= 1 - k/k_0$, where k is the rate constant for inactivation in the presence of IMP, and k_0 is the rate constant for inactivation in the absence of IMP. Extrapolation of these data to infinite concentrations of IMP by using the equation, fraction protected $= P_m[IMP]/(K + [IMP])$, where P_m is the maximal fraction of inactivation and K is the apparent binding constant of IMP, yields $P_m = 0.99$ (see solid line). Thus, high concentrations of IMP completely protect IMPDH from inhibition by **I**, which shows that **I** is a competitive inhibitor with respect to IMP.

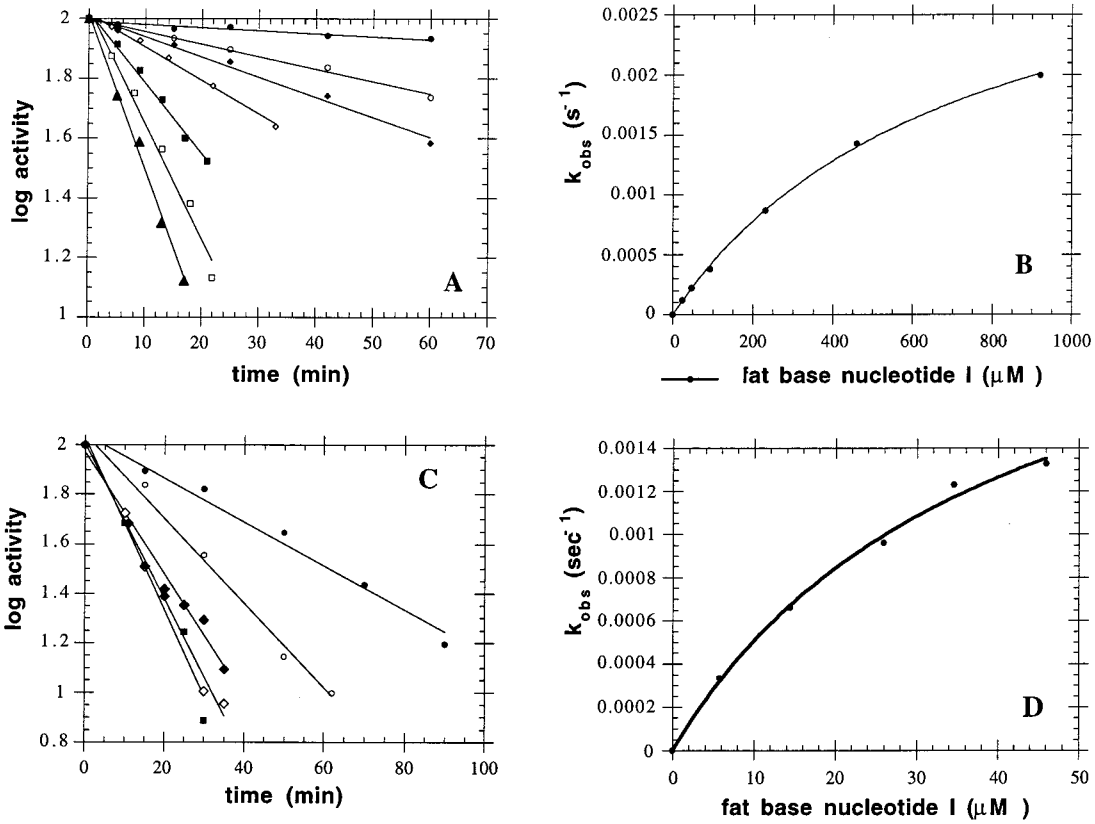

FIGURE 4: Kinetics of **I** inhibition of IMPDH. (A) Human IMPDH (1.6 μM) was incubated with various concentrations of **I**: closed circles, no **I**; open circles, 23 μM **I**; closed diamonds, 46 μM **I**; open diamonds, 92 μM **I**; closed squares, 230 μM **I**; open squares, 260 μM **I**; closed triangles, 920 μM **I**. (B) k_{obs} versus [**I**] plot for human IMPDH. (C) *E. coli* IMPDH (0.13 μM) was incubated with various concentrations of **I**: closed circles, 5.8 μM **I**; open circles, 14 μM **I**; closed diamonds, 26 μM **I**; open diamonds, 35 μM **I**; closed squares, 46 μM **I**. No activity is lost when *E. coli* IMPDH is incubated in the absence of inhibitor (data not shown). (D) k_{obs} versus [**I**] plot for *E. coli* IMPDH.

until the reactions reached equilibrium. The equilibrium activities of human and *E. coli* IMPDH were 24 and 6%, respectively, and the corresponding values of K_d were 1.4 and 53 nM (Table 1). Thus **I** is a more potent inhibitor of human IMPDH than of *E. coli* IMPDH; this difference in inhibitor affinity mirrors the difference in IMP affinity.

Kinetics of the Inhibition of IMPDH by I. Both human and *E. coli* IMPDH were incubated with various concentrations of **I**. The reactions were monitored by assaying activity at appropriate time intervals. Inhibition followed pseudo-first-order kinetics, as demonstrated by the exponential loss of activity (Figure 4). Replots of the apparent rate constants of inhibition versus concentration of **I** show biphasic behavior (Figure 4). This observation indicates that the inhibition of IMPDH is a two-step process described by:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}I^*$$

Table 1. Inhibition of IMPDH by I[a]

parameter	human	*E. coli*
K_d (nM)	1.4 ± 0.2	53 ± 6
initial K_i (μM)	650 ± 50	40 ± 8
k_2 (s^{-1})	0.0035 ± 0.0001	0.0025 ± 0.0002
k_{on} (M^{-1} s^{-1})	5.2 ± 0.2	63 ± 6
k_{off} (s^{-1})	$(7 \pm 1) \times 10^{-9}$	$(3.3 \pm 0.5) \times 10^{-6}$

[a] Enzyme and various concentrations of **I** were incubated in a solution of 50 mM Hepes, 100 mM KCl, and 1 mM DTT at 25 °C. Formation of E·**I** was monitored by loss of activity (Figure 4A and C). The initial K_i, k_2, and k_{on} ($= k_2/K_i$) were determined from the plots in Figure 4B and D. The final dissociation constant, K_d, was determined by incubating IMPDH (6.2 and 5.0 μM of human and *E. coli* IMPDH, respectively), **I** (5.4 and 11 μM for human and *E. coli* IMPDH, respectively), and 500 μM IMP. No free enzyme will be present under these conditions. The samples reached equilibrium in 3 days. No activity was lost in control samples containing only enzyme and IMP. The value of K_d for the E·**I** complex was calculated according to: $K_d = [E{\cdot}IMP][\mathbf{I}]_{free}/[E{\cdot}\mathbf{I}](1 + [IMP]/K_{IMP})$ where [E·IMP] and [E·**I**] were determined from the remaining activity and $[\mathbf{I}]_{free} = [\mathbf{I}]_0 - [E{\cdot}\mathbf{I}]$. The value of k_{off} was calculated from $K_d = k_{off}/k_{on}$. The values of K_{IMP} are 4 and 61 μM for human and *E. coli* IMPDH, respectively).

The data were fit to the following equation:

$$k_{obs} = k_2[\mathbf{I}]/(K_i + [\mathbf{I}])$$

Assuming that $k_{-1} \gg k_2$, which seems reasonable given the magnitude of k_2, then $K_i = k_{-1}/k_1$, $k_{on} = k_2/K_i$, and $k_{off} = k_{on}K_d$. Since k_{-1} is a fast step, k_{off} will equal k_{-2}. Table 1 summarizes the kinetic parameters for the inhibition of human and *E. coli* IMPDH by **I**. The value of k_{on} for **I** inhibition of *E. coli* IMPDH is 100-fold greater than for the human enzyme. More-rapid inhibition appears to be a general property of the *E. coli* enzyme: EICARMP also inactivates *E. coli* IMPDH 10-fold faster than it does the human enzyme (*17*).

DISCUSSION

Two unstable tetrahedral intermediates, E-IMP* and E-XMP†, are formed during the IMPDH reaction (Figure 1). Compounds that form enzyme adducts resembling these intermediates are expected to be very potent inhibitors of IMPDH. Although **I** is predominantly hydrated in solution, it is nevertheless in rapid equilibrium with a small concentration of the dehydro form (Figure 2). Precedents for this chemistry are well documented (*13*−*16*). The dehydro form reacts with IMPDH to form a tetrahedral adduct that resembles E-IMP* and E-XMP†. This adduct would be a transition-state analogue for IMPDH, and this reaction is analogous to the reaction of aldehyde transition state analogue inhibitors with serine and cysteine proteases.

The experiments described above demonstrate that **I** is a potent inhibitor of both human and *E. coli* IMPDH, as predicted. Although inhibition may result simply from the association of **I** with IMPDH, it seems reasonable to believe, based on chemical precedent, that a covalent adduct forms. The E·**I** complex is unstable when the enzyme is denatured, an observation generally taken as evidence that the complex is noncovalent; however, the enzyme-**I** adduct shown in Figure 2 would not be stable outside the active site of the enzyme (again, such behavior is observed in aldehyde inhibition of serine and cysteine proteases). Unfortunately, this observation precludes confirmation of adduct formation by mass spectroscopy, and the similarity of the hydrated **I** and the adduct, as well as the 220 kDa molecular mass of the IMPDH tetramer, suggests that NMR experiments using ^{13}C-labeled **I** would not be informative. Therefore, further identification of the adduct will require solution of the X-ray crystal structure of the E·**I** complex. The inhibition of IMPDH by **I** is time dependent; k_2 and k_{-2} may represent formation and decomposition of a covalent adduct (Figure 2) or may represent a conformational change. Of course, these alternatives are not mutually exclusive: The covalent intermediate could form and a kinetically significant conformational change could also occur.

Similar inhibitors of IMPDH may already exist in nature. The structure of azepinomycin, a natural product with antibiotic and antitumor activity (*23*), is very similar to that of the base of **I** (Figure 2). The activity of azepinomycin is believed to result from inhibition of guanase. Our work on **I** suggests that the nucleotide of azepinomycin will also be a potent inhibitor of IMPDH. Perhaps the antibiotic and antitumor properties of azepinomycin result from the inhibition of IMPDH.

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BI981132W