Notes

Immunologically Specific Activation of a Cephalosporin Derivative of Mitomycin C by Monoclonal Antibody β-Lactamase Conjugates

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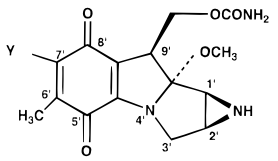
Received February 24, 1997[⊗]

The syntheses of two cephalosporin derivatives **2** and **3** of mitomycin C (**1**) containing 7-phenylacetamido and 7-δ-carboxybutanamido side chains, respectively, are described. These compounds were prepared for evaluation as cephalosporin prodrugs capable of being activated by mAb-β-lactamase conjugates. *In vitro* cytotoxicity assays performed on H2987 lung adenocarcinoma and clone 62 melanoma cell lines indicated that compound **2** was comparable in cytotoxicity to the parent drug. In an effort to improve upon the cytotoxic differential of **2**, an alternative prodrug **3** containing a polar carboxyl group in the side chain of the cephalosporin moiety was prepared. Compound **3** consistently behaved as a prodrug and was approximately 40- and 10-fold less toxic than **1** toward H2987 and clone 62, respectively. Determination of kinetic constants for hydrolysis by β-lactamase from *Enterobacter cloacae* P99 indicated k_{cat} values of 476 ± 170 and 248 ± 15.1 s^{-1} for **2** and **3**, respectively. The k_{cat}/K_m ratios for **2** and **3** were found to be approximately 9.7 and 2.1 μM/s, respectively. Comparison of these k_{cat}/K_m values with those obtained for similar cephalosporin derivatives of other antitumor agents demonstrated that compounds with δ-carboxybutanamido side chains generally have slightly diminished efficiency of enzymatic hydrolysis compared to the corresponding 7-phenylacetamido analog. It was also demonstrated that the less toxic prodrug **3** was activated in an immunologically specific manner by L6-F(ab′)-β-lactamase and 96.5-F(ab′)-β-lactamase conjugates, selective for H2987 and clone 62 cells, respectively.

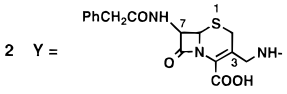
Introduction

Currently, several research groups are investigating the selective generation of cytotoxic compounds within tumor masses from anticancer prodrugs by targeted monoclonal antibody enzyme conjugates.[1] Research in our laboratory has been aimed at the development of anticancer prodrugs for activation by mAb-β-lactamase conjugates. To date, several prodrugs of cytotoxic agents have been developed for activation by bL. These include prodrugs of a number of mechanistically dissimilar agents such as doxorubicin,[2] paclitaxel,[3] nitrogen mustards,[4] vinca alkaloids,[5] and platinum derivatives.[6] Recently, we described the synthesis and immunologically specific activation of a cephalosporin prodrug (CCM[4c]) of the alkylating agent phenylenediamine mustard. This prodrug in combination with a mAb-β-lactamase conjugate effected complete regressions and cures of established human melanomas in nude mice.[7] This encouraged us to investigate prodrugs of the bioreductive alkylating agent mitomycin C (**1**). Clinically, **1** is used in combination chemotherapy against a variety of solid tumors.[8,9] A major limitation to the use of **1** is toxicity to the bone marrow.[10] Thus, selective generation of this reductively activated prodrug within the tumor may further improve its therapeutic index.

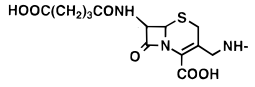
Derivatization at the 7′-position of mitosane had been used earlier by many groups to develop analogs of **1** that displayed variable cytotoxicity.[11] Some of these analogs, particularly those with polar side chains,[11b] were less toxic than the parent drug while others were more potent. We therefore decided to append the *exo*-methylene of cephem nucleus to the 7′-NH_2 position of mitosane. It was expected that the polar cephalosporin residue would diminish the cytotoxicity of mitosane to make the derivative a prodrug. Release of **1** after β-lactam hydrolysis was expected to be facile since the 7′-amino group of **1** is part of a vinylogous amide.



1 Y = NH_2

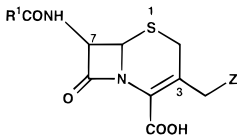


2 Y =



3 Y =

4 Y = OMe



5 R^1 = $PhCH_2$- Z = NH_2

6 R^1 = $HOOC(CH_2)_3$- Z = OAc

7 R^1 = $HOOC(CH_2)_3$- Z = N_3

8 R^1 = $HOOC(CH_2)_3$- Z = NH_2

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[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1997.

S0022-2623(97)00108-8 CCC: $14.00

Table 1. IC_{50} Values of Mitomycin C and Cephalosporin Derivatives of Mitomycin C

		mean IC_{50} (μM)	
compd	cell line	−bL	+bL
1	clone 62	1.5 ± 0.2	nd[b]
	H2987	1.6 ± 0.8	nd[b]
2	clone 62	3.5 ± 3.5	1.4 ± 0.5
	H2987	10.4 ± 10.2	2.8 ± 1.6
3	clone 62	30.1 ± 12.7	3.0 ± 1.2
	H2987	26.4+ 1.3	1.2 ± 0.6

[a] The values presented are the mean of five independent experiments for H2987 and clone 62 cells. [b] Not determined.

Here we describe the synthesis of cephalosporin prodrugs of **1**. Initially, cephalosporin derivative **2** with a phenylacetamido side chain was prepared and evaluated. In an effort to improve the drug to prodrug cytotoxicity differential, an alternative analog **3** with a polar 4-carboxybutanamido side chain was prepared. It was expected that the polar carboxyl group would impair the ability of compound **3** to traverse cell membranes, therefore rendering the molecule less toxic. The kinetics of enzymatic hydrolysis of **2** and **3** by bL are presented. In addition, **2** and **3** were evaluated for *in vitro* cytotoxic activity on melanoma and human lung adenocarcinoma cell lines.

Results and Discussion

Chemistry. Previous reports have made extensive use of the fact that the methoxy group in mitomycin A (**4**) is a good leaving group and can be displaced by amines under mildly basic conditions by nucleophilic addition elimination.[11] Suitably substitued 3-(aminomethyl)cephalosporins were therefore expected to react with **4** to yield cephalosporin derivatives of **1**. (Aminomethyl)cephalosporanic acid **5** was obtained as reported[12] by displacing the acetate in 7-phenylacetamidocephalosporanic acid with azide followed by reduction. Condensation of (aminomethyl)cephem **5** with **4** gave **2** in 37% yield. Similarly, acetoxymethyl derivative **6** was converted to azidomethyl derivative **7** which, after reduction to amine **8** followed by condensation with **4**, provided the (δ-carboxybutanamido)cephalosporin derivative **3** in 34% yield.

Cytotoxicity Assays. To address the question as to whether compounds **2** and **3** were prodrugs that could be activated *in vitro* by *E. cloaceae* bL, experiments were performed on the H2987 human lung adenocarcinoma cell line and on clone 62, a mouse melanoma cell line transfected with the human melanoma associated p97 antigen. Cellular incorporation of [^{3}H]thymidine was taken as a measure of cell viability after drug treatment, and the IC_{50} value for each compound was determined as the concentration that resulted in 50% incorporation of [^{3}H]thymidine in comparison to untreated control cells. Table 1 summarizes the IC_{50} values of compounds **1**−**3**. In a number of cytotoxicity assays, **2** compared to **1** displayed a highly variable cytotoxic differential. The mean IC_{50} ratio for **2**/**1** was 2.4 ± 2.4 for clone 62 cells and was 5.4 ± 3.5 for H2987 cells. These data suggest that **2** is as cytotoxic as mitomycin C and is therefore not a prodrug. In order to obtain a prodrug with a significantly reduced level of cytotoxicity, the phenylacetamido side chain of **2** was replaced with a 4-carboxybutanamido side chain containing a more polar carboxyl group. It was expected that the polar carboxyl group would impair the ability of compound **3** to traverse across cell membranes and therefore would make it a prodrug. Such a modification has been shown to improve the cytotoxic differential in the case of cephalosporin doxorubicin prodrugs.[2c] Compound **3** therefore was synthesized and evaluated. In contrast to the phenylacetamido derivative **2**, the 4-carboxybutanamido derivative **3** consistently behaved as a prodrug in that the compound was less toxic than **1** and full activation upon treatment with bL took place. The mean IC_{50} ratio for **3**/**1** was 13.06 ± 2.16 for the clone 62 and it was 37.8 ± 3.34 for H2987. The prodrug/drug cytotoxic differential observed with **3** is similar to that of other cephalosporin prodrugs of phenylenediamine mustard and doxorubicin that displayed *in vivo* antitumor activity in combination with mAb−bL conjugates.[7] Separate HPLC experiments demonstrated that **2** and **3** upon treatment with bL released parent drug **1** (data not shown).

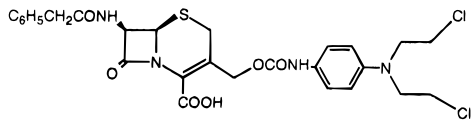
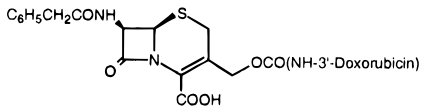
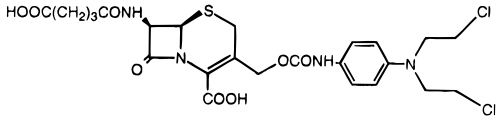
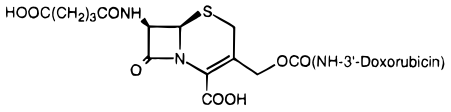
Enzyme Kinetics. Kinetic parameters for the bL-catalyzed hydrolysis of **2** and **3** were measured spectrophotometrically by measuring the loss of absorbance at 260 nm upon treatment with bL. Table 2 shows values of kinetic constants. Two striking features were the low K_m value seen with the phenylacetamido nitrogen mustard prodrug (entry 1) and the high k_{cat} value observed for compound **2**. These derivatives fall into two series bearing either a phenylacetamido group or a δ-carboxybutanamido group as side chain on position 7 of the cephalosporin and contain different linkages to the cephem *exo*-methylene. The k_{cat}/K_m ratio which is a measure of efficiency of catalysis was the highest for the nitrogen mustard prodrugs in both series. In the case of doxorubicin prodrugs, replacement of the phenylacetamido group by the δ-carboxybutanamido group resulted in a 2-fold diminishment of catalytic efficiency. A similar trend was observed for **2** and **3** resulting in approximately 4-fold diminishment of catalytic efficiency.

Immunologically Specific Prodrug Activation. The F(ab′) fragments of the mAbs 96.5, L6, and P 1.17 were conjugated via native reduced SH groups to bL that had been modified to contain maleimide groups as described previously.[13] The F(ab′)−bL conjugates thus obtained were biologically functional and retained their enzymatic and immunological activities (data not shown). *In vitro* cytotoxicity assays to demonstrate immunologically specific activation of mitomycin prodrug **3** were done with H2987 (L6 antigen positive and P1.17 antigen negative) and clone 62 (96.5 antigen positive and L6 antigen negative) cells. Cells were incubated with the mAb−bL conjugates, washed, and then treated with varying amounts of prodrug **3**. Comparison of cytotoxic effects depicted in Figure 1 indicated that compound **3** was less toxic than **1** and was activated by mAb−bL conjugates that could bind to surface antigens on the two cell lines. Prior incubation of cells with saturating amounts of unconjugated antigen binding antibody in the above experiment abrogated prodrug activation. Furthermore, the nonbinding mAb−bL conjugate did not potentiate the activity of **3**, demonstrating that the activation of the prodrug was immunologically specific.

Conclusion

We have demonstrated that it is possible to take advantage of the leaving group ability of a vinylogous amide to design cephalosporin prodrugs of the biore-

Table 2. Kinetic Constants for β-Lactamase Hydrolysis of Different Cephalosporin Derivatives

Entry	Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μMs^{-1})	Ref
1		7.0	119	17	4a
2		46 ± 11	280 ± 67	6.1	2c
3	**2**	48.9 ± 7.69	476 ± 170	9.7	
4		43.2	337	7.8	4c
5		83 ± 26	210 ± 39	2.5	2c
6	**3**	119 ± 20.6	248 ± 15.1	2.1	

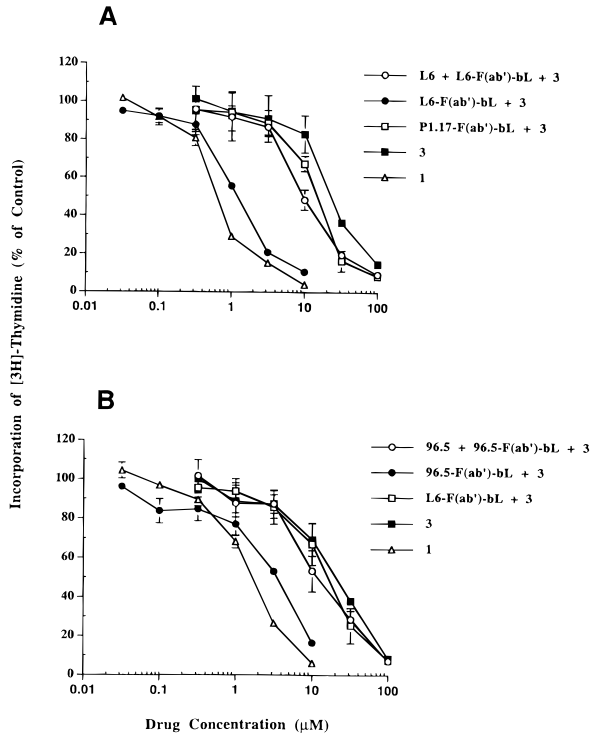


Figure 1. Immunologically specific activation of **3** on (A) H2987 (L6 antigen positive and P1.17 antigen negative) cells and (B) clone 62 (96.5 antigen positive and L6 antigen negative) cells. See Experimental Section for details.

ductive alkylating agent **1**. The synthetic methodology applied permits different side chain containing 3-(aminomethyl)cephalosporins to be condensed with mitomycin A as a common intermediate to obtain more polar mitomycin C analogs with varying degrees of polarity. Since modification at a site remote from β-lactam carbonyl is tolerated by bL, the cephalosporin side chain at position 7 can serve as a handle to design hydrophilic prodrugs of **1**.

Experimental Section

General. Crude *E. cloaceae* bL was obtained from Sigma Chemical Co. and purified according to published procedures.[4a,c] The human lung adenocarcinoma cell line H2987 (L6 antigen positive and P1.17 antigen negative) was established at Bristol-Myers Squibb, Seattle, WA. Clone 62 (96.5 antigen positive and L6 antigen negative) is a mouse melanoma cell line transfected with p97 antigen.[15] Description of analytical instruments used were as reported in previous publication.[2c] Chemical shifts are reported in ppm downfield from $(CH_3)_4Si$. HPLC analyses were performed on a spheri 5 RP18 reversed phase column (220 × 2.1 mm, Applied Biosystems) with the UV/vis detector set at 365 nm for mitomycin derivatives. Solvent systems used were as follows: system 1, 15−45% linear gradient of acetonitrile (MeCN) in 50 mM phosphate buffer pH = 7; system 2, solvent A = 50 mM KH_2PO_4 at pH 7 and B = MeCN, A used for 5 min, a linear gradient of 0−30% B in A for 10 min, 30% B in A for 5 min, linear gradient of 30−70% B in A for 10 min, and 70% B in A for 5 min. Phosphate-buffered saline, pH 7.2 (PBS), had the following composition: 0.14 M NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 8 mM Na_2HPO_4.

7′-[[*N*-(9′a-Methoxymitosanyl)]methyl]-7-phenylacetamido-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (2). A solution of mitomycin A[16] (147 mg, 0.42 mmol) in 5.6 mL of MeOH was combined with potassium salt of 7-phenylactamido-3-(aminomethyl)cephalosporanic acid[12] (3 equiv) and diisopropylethylamine (0.14 mL, 0.8 mmol) and stirred at room temperature for 3 h. MeOH was removed *in vacuo*, and the residue was purified by reversed phase chromatography on C18 silica gel (2.5 × 30 cm) by successive elution with H_2O (250 mL), MeCN:H_2O (1:9, 500 mL), and MeCN:H_2O (3:17, 500 mL). Fractions were analyzed by HPLC (system 1), and those containing the required compound were combined and lyophilized to give diisopropylethylammonium salt of **2** (131 mg, 37% yield) as a bluish green powder: HRMS [M + Na^+] = 687.1851 (found), 687.1842 (calcd); UV max (H_2O) 372 nm (log ϵ = 4.30); IR (KBr, cm^{-1}) 1772 (β-lactam CO), 1716 (mitosane O*CO*), 1668, 1604 (quinone *CO*); ^1H NMR (CD_3OD) δ 7.31−7.21 (m, 6H, ArH and unexchanged NH), 5.64 (d, 1H, H-7, $J_{6,7}$ = 4.7 Hz), 4.97 (d, 1H, H-6), 4.75 (d, 1H, 7′-N-CH_{2A}, J_{AB} = 15.0 Hz), 4.66 (dd, 1H, H-10′A, $J_{9',10'A}$ and $J_{9',10'B}$ = 4.3 and 10.5 Hz), 4.37 (d, 1H, 7′-N-CH_{2B}), 4.24 (t, 1H, H-10′B), 4.17 (d, 1H, H-3′A, J_{AB} = 13.1 Hz), 3.27 (s, 3H, OCH_3), 2.93

(d, 1H, H-1′, $J_{1',2'}$ = 4.4 Hz), 2.81 (m, 1H, H-2′), 1.96 (s, 3H, 6′-CH_3). Anal. ($C_{39}H_{51}N_7O_9S\cdot4.5H_2O$) C, H, N, S.

3-(Azidomethyl)-7-(4-carboxybutanamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (7). This compound was prepared by the same procedure described for the synthesis of the 7-phenylacetamido analog.[12] The mono potassium salt of the compound was isolated from an ethanol solution by precipitating it using 2 equiv of 0.1 M potassium 2-ethylhexanoate in ethanol: MS $[M - H]^-$ = 368 (found), 368 (calcd); IR (KBr) 1652 (NH*CO*-glutaryl), 1764 (β-lactam *CO*), 2108 cm^{-1} (N_3); ^1H NMR (DMSO-d_6) δ 8.80 (d, 1H, NH, $J_{NH,7}$ = 8.3 Hz), 5.48 (dd, 1H, H-7, $J_{6,7}$ = 4.7 Hz), 4.92 (d, 1H, H-6), 4.49 (d, 1H, CH_{2A}-N3, J_{AB} = 12.5 Hz), 3.78 (d, 1H, CH_{2B}-N3), 3.47 (d, 1H, H-2A, J_{AB} = 17.4 Hz), 3.22 (d, 1H, H-2B), 2.19 (t, 4H, CO$CH_2CH_2CH_2$CO, J_{vic} = 7.5 Hz), 1.70 (m, 2H, $COCH_2$-CH_2CH_2CO). Anal. ($C_{13}H_{14}N_5O_6SK\cdot0.2C_2H_5OH$) C, H, N.

7′-[[*N*-(9′a-Methoxymitosanyl)]methyl]-7-(δ-carboxybutanamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (3). (Aminomethyl)cephalosporin **8** was prepared by reducing the corresponding azide **7** (930 mg, 2.23 mmol) following the described procedure for the synthesis of the 7-phenylacetamido analog.[12] The compound was isolated as potassium salt from an ethanol solution by precipitation with 1 M potassium 2-ethylhexanoate (4.2 mL) in ethanol to give 665 mg (71% yield) of **8**: MS $[M - H]^-$ = 342 (found), 342 (calcd); IR (KBr) 1656 (NH*CO*-glutaryl), 1764 cm^{-1} (β-lactam *CO*); ^1H NMR (DMSO-d_6) δ 8.83 (d, 1H, NH, $J_{NH,7}$ = 8 0 Hz), 5.50 (dd, 1H, H-7, $J_{6,7}$ = 4.8 Hz), 4.88 (d, 1H, H-6), 3.57−3.39 (m, 5H, NH_2, NH_2CH_2, H-2′A), 3.10 (d, 1H, H-2B, J_{AB} = 12.8 Hz), 2.14 (m, 4H, CO$CH_2CH_2CH_2$CO), 1.69 (m, 2H, $COCH_2CH_2CH_2$CO). The coupling of **8** with mitomycin A was done exactly as in the preparation of **3** and on the same scale. Purification by C18 chromatography employing H_2O (250 mL) and MeCN:H_2O (1:9, 500 mL) as eluants and monitoring fractions by HPLC (system 2) led to recovery of diisopropylethylammonium salt of **4** as bluish green powder (118 mg, 34% yield): HRMS $[M + Na^+]$ = 683.1756 (found), 683.1740 (calcd); UV max (H_2O) 374 nm (log ε = 4.27); IR (KBr, cm^{-1}) 1660 (NH*CO* glutaryl), 1774 (β-lactam CO), 1722 (mitosane O*CO*), 1632, 1608 (quinone *CO*); ^1H NMR (D_2O) δ 5.53 (d, 1H, H-7, $J_{6,7}$ = 4.6 Hz), 4.99 (d, 1H, H-6), 4.64 (dd, 1H, 7′-N-CH_{2A}, J_{AB} = 15.8 Hz), 4.48 (dd, 1H, H-10′A, $J_{9',10'A}$ and $J_{9',10'B}$ = 4.6 and 10.9 Hz), 4.25 (dd, 1H, N-CH_{2B}), 4.14 (t, 1H, H-10′B), 4.07 (d, 1H, H-3′A, J_{AB} = 13.4 Hz), 3.18 (s, 3H, OCH_3), 2.96−2.93 (2 m, 2H, H-1′ and H-2′), 2.27 (t, 2H, glutaryl CH_2, J_{vic} = 7.4 Hz), 2.17 (t, 2H, glutaryl CH_2), 1.91 (s, 3H, 6′-CH_3) 1.80 (m, 2H, glutaryl CH_2). Anal. ($C_{36}H_{51}N_7O_{11}S\cdot2.5H_2O$) C, S; H: calcd, 6.76; found, 7.34, N: calcd, 11.74; found, 11.28.

Enzyme Kinetics. The kinetics of β-lactam hydrolysis of prodrugs **2** and **3** were determined spectrophotometrically by measuring the loss of absorbance at 260 nm using bL. Solutions of **2** and **3** in PBS containing 12.5 μg/mL of bovine serum albumin were incubated with 0.1 μg/mL of bL at 23 °C. Linear portions obtained from plots of absorbance versus time were used to calculate the initial velocities.

***In Vitro* Cytotoxicity Assays.** Cells were plated out at 4000 or 8000 cells/well in a 96-well microtiter plates in Iscove's modified Dulbecco's medium (IMDM) supplemented with fetal bovine serum (10% v/v), streptomycin (0.1 mg/mL), and penicillin-G (60 μg/mL) and were allowed to adhere for 18 h at 37 °C. The cells were then washed with Roswell Park Memorial Institute medium (RPMI) supplemented with fetal bovine serum (10%, v/v). After various concentrations of drugs or prodrugs were added, incubation with or without bL (10 μg/mL) was carried out for 1 h at 37 °C. Cells were then washed with IMDM and incubated for another 18 h at 37 °C. Pulsing with [^{3}H]thymidine at 1 μCi/well was carried out for 18 h in case of 4000 cells/well and for 6 h in case of 8000 cells/well. Following a PBS wash, the cells were detached with a solution of trypsin/EDTA, harvested onto filter mats with a TOMTEC Harvester 96, and counted with an LKB WALLAC 1205 liquid scintillation counter. The incorporation of [^{3}H]-thymidine was expressed as a percentage of untreated control cells. When mAb−bL conjugates were used to address immunologically specific activation of **2** and **3**, cells were treated with conjugates (10 nM mAb component) in 2 mL of RPMI for 30−45 min at 4 °C. The cells were then washed with 3 × 0.2 mL of RPMI and treated with prodrugs as described above.

Acknowledgment. The authors would like to thank Ian Frank for cytotoxicity and kinetic experiments, David Lowe, Steve Klohr, and John Emswiler for the analytical support and reagents, Phil Wallace for valuable discussions, and Karl Erik Hellström and Ingegerd Hellström for their support throughout the course of this work.

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JM970108Y