International Journal of Pharmaceutics, 15 (1983) 61–71
Elsevier Biomedical Press

Development of lipophilic prodrugs of mitomycin C. II. Stability and bioactivation of 1a-N-substituted derivatives with aromatic pro-moiety

Hitoshi Sasaki, Eiji Mukai, Mitsuru Hashida, Toshikiro Kimura and Hitoshi Sezaki *

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606 (Japan)

(Received July 12th, 1982)
(Accepted November 1st, 1982)

Summary

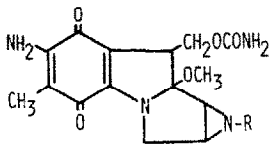
The decomposition and bioactivation characteristics of five 1a-N-substituted derivatives of mitomycin C possessing an aromatic pro-moiety with different linkage structures were studied to assess their suitability for prodrugs. Derivatives were stable in neutral aqueous media except for benzoyloxymethyl mitomycin C which decomposed with a half-life of 3.5 min at pH 7.4. Acyl derivatives such as benzoyl and benzylcarbonyl mitomycin C were stabler than mitomycin C under acidic conditions, but converted relatively rapidly to the parent drug in basic media. Derivatives having an ester bond in their linkages showed enzyme-mediated conversion to the parent drug in rat plasma and liver homogenate. Acyl derivatives were converted only by hepatic enzymes. No practical bioactivation was observed for benzyl mitomycin C. Species differences were observed in these bioactivation phenomena. These results suggested that 5 derivatives have intrinsic regeneration characteristics which might offer successful applications under various conditions of administration.

Introduction

A promising approach to improve drug delivery appears to be chemical transformation of the active drug substances into per se inactive derivatives (prodrugs)

* To whom correspondence should be addressed.

0378-5173/83/0000-0000/$03.00



I	R = H
II	R = CH_2-C$_6$H$_5$
III	R = CO-C$_6$H$_5$
IV	R = $COCH_2$-C$_6$H$_5$
V	R = $COOCH_2$-C$_6$H$_5$
VI	R = CH_2OCO-C$_6$H$_5$

Fig. 1. Structures of 1a-N-substituted mitomycin C derivatives.

which revert to the parent compounds by virtue of enzymatic or chemical lability, or both, within the body (Stella and Higuchi, 1975; Sinkula and Yalkowsky, 1975; Sinkula, 1977). Among the various physicochemical parameters, lipophilicity or lipid solubility occupies a dominant role in determining biopharmaceutical properties and, ultimately, therapeutic success of a prodrug (Yalkowsky, 1977). Combined application of lipophilic derivatives to lipoidal delivery systems would offer further utility of this approch.

In the previous paper(Sasaki et al., 1983), 5 lipophilic derivatives of mitomycin C (I), an antitumor antibiotic with severe myelotoxicities, were synthesized by substituting the 1a-position with benzyl (II), benzoyl (III), benzylcarbonyl (IV), benzyloxycarbonyl (V) and benzoyloxymethyl (VI) groups. As shown in Fig. 1, they possess a benzene ring, a model lipophilic moiety, through various linkage forms and exhibited increased lipophilicity and lipid solubility. Biological tests revealed that they exhibited characteristic antitumor activities after being regenerated to the parent drug. In the present paper, a chemical- and enzyme-mediated conversion of these derivatives were investigated.

Materials and Methods

Chemicals

Mitomycin C (I) was supplied by Kyowa Hakko Kogyo. The lipophilic derivatives of I, shown in Fig. 1, were prepared as reported previously (Sasaki et al., 1983). All other chemicals were of reagent grade and obtained commercially.

Stock solutions of all compounds were prepared in DMSO with a concentration of 5×10^{-2} M and an appropriate volume was mixed with the aqueous buffer or biological media for kinetic studies.

Stability measurements in aqueous solutions

All stability experiments were carried out in aqueous buffer solutions at 37 ± 0.2°C. The pH of the solution was maintained at the desired value by using appropriate buffer systems (citric acid–sodium citrate, NaH_2PO_4–Na_2HPO_4, and $NaHCO_3$–Na_2CO_3). The total buffer concentration was 0.05 M (citrate buffer) or 0.10 M (phosphate and carbonate buffer) except for experiments where buffer effects were studied specifically. The ionic strength of each buffer solution was adjusted to 0.3 with sodium chloride, if necessary. Degradation was initiated by the

addition of the stock solution to a preheated buffered solution to give a concetration of 1×10^{-4} M. Aliquots of the solution were withdrawn at suitable time intervals.

Stability measurements in biological media

Male Wistar rats weighing 200–220 g and male hybrid BDF_1 mice (C57Bl/6 × DBA/2) weighing 20–23 g were used to obtain plasma and liver homogenate. Human blood was obtained from healthy volunteers. The liver of rats and mice was homogenized at 0–5°C in a glass–teflon homogenizer, centrifuged at 4°C at 600 × *g* for 10 min, and the supernatant was used for the experiments. An isotonic phosphate buffer (pH 7.4) containing 0.25 M sucrose was used to prepare tissue homogenate and dilute plasma and tissue homogenate samples.

Bioactivation experiments were performed at 37 ± 0.2°C and initiated by adding the stock solution to give a final concentration of 1×10^{-4} M. At appropriate time intervals aliquots of the solution were withdrawn, acetonitrile added to precipitate the protein, and the supernatant was subjected to analysis.

Analysis

Degradation of the prodrugs and regeneration of I were monitored by a high-performance liquid chromatography (HPLC) system (TRIROTAR, Jasco) equipped with a variable wavelength UV-detector (UVIDEC 100-II, Jasco). The stationary phase used was Cosmosil $5C_{18}$ packed column (4.6 × 150 mm, Nakarai Chemicals) and a short column packed with Lichrosorb RP-2 (E. Merck) was used to guard the main column. Mixtures of methanol–water were used as the mobile phase with a flow rate of 0.8 ml/min. The standard solutions were chromatographed and calibration lines were constructed on the basis of peak-area measurements.

Results

Stability in aqueous buffer solutions

The kinetics of breakdown of 1a-N-substituted derivatives of I were studied in aqueous buffer solutions at 37°C over a wide range of pH. The degradation of derivatives and formation of I in pH 7.4 phosphate, pH 4.0 citrate, and pH 11.0 carbonate buffer solutions are shown in Figs. 2, 3 and 4, respectively. The degradation of derivatives at each pH followed apparent pseudo-first-order kinetics with respect to their concentrations, over more than 4 half-lives in the case of those with relatively rapid decomposition ($t_{1/2} < 5$ days).

As shown in Fig. 2, the derivatives were relatively stable in a neutral aqueous medium except for VI. VI reverted to I with a half-life of 3.5 min so that the time course of VI was almost equal to that of I soon after the initiation of experiment. The simultaneous disappearance of prodrugs and appearance of I was also observed in the case of IV, although the degradation rate was rather slow. At pH 4.0, I was unstable and hydrolyzed rapidly with a half-life of 30 min and II showed almost the same degradation rates with those of I (Fig. 3). Other compounds were relatively stable comparing with I and II and did not produce I within 60 min. In a basic

Fig. 2.

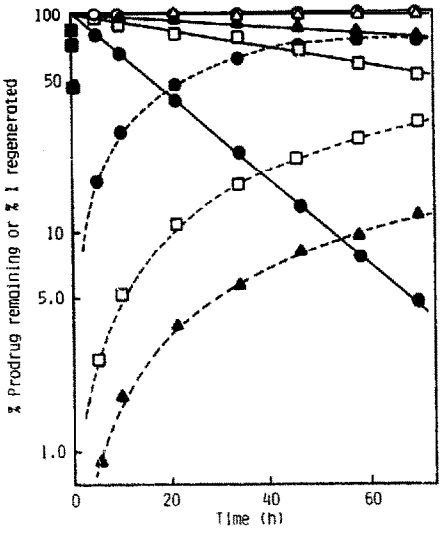


Fig. 3.

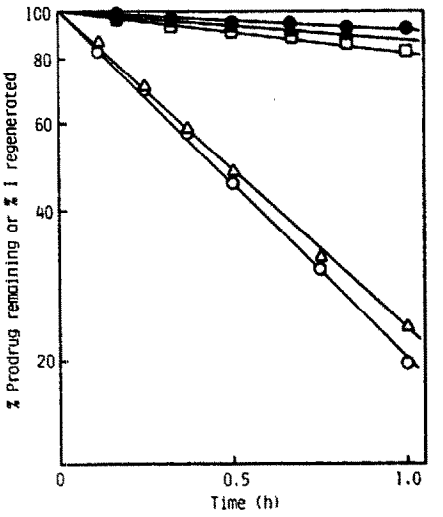


Fig. 2. Stability of 1a-N-substituted mitomycin C derivatives in a pH 7.4 buffer solution at 37°C. The solid lines represent time courses of degradation of prodrug and the dashed lines are for regeneration of I from prodrug, respectively. ○, I; △, II; □, III; ●, IV; ▲, V; ■, VI.

Fig. 3. Stability of 1a-N-substituted mitomycin C derivatives in a pH 4.0 buffer solution at 37°C. ○, I; △, II; □, III; ●, IV; ■, V.

Fig. 4.

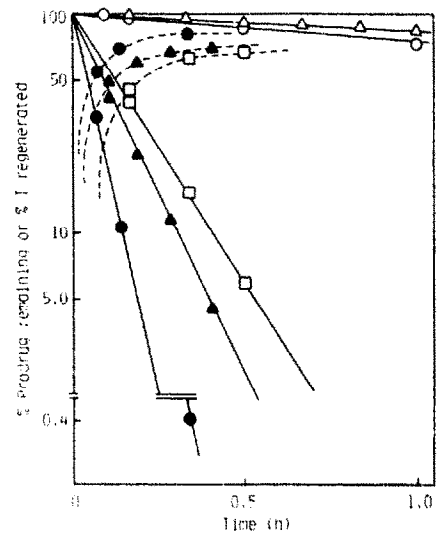


Fig. 5.

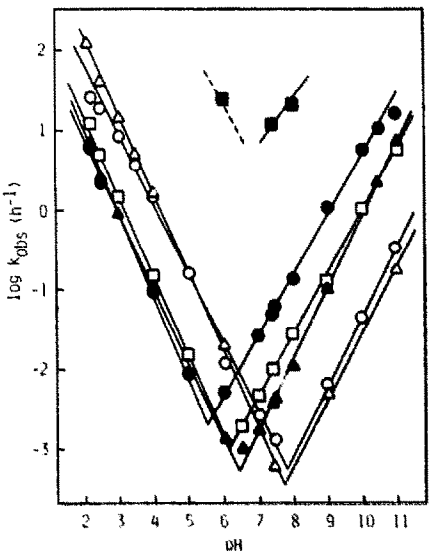


Fig. 4. Stability of 1a-N-substituted mitomycin C derivatives in a pH 11.0 buffer solution at 37°C. The solid lines represent time courses of degradation of prodrug and the dashed lines are for regeneration of I from prodrug, respectively. ○, I; △, II; □, III; ●, IV; ▲, V.

Fig. 5. Log pH–rate profiles for the degradation of 1a-N-substituted mitomycin C derivatives at 37°C and an ionic strength of 0.3. ○, I; △, II; □, III; ●, IV; ▲, V; ■, VI.

solution, IV and V decomposed to I relatively rapidly as shown in Fig. 4.

In addition to these pH conditions, the degradation reactions of these compounds were monitored widely in a pH range between 2.2 and 11.0. The observed pseudo-first-order rate constants for the overall degradation of these compounds (k_{obs}) were calculated by linear regression analysis of a plot of the logarithm of the concentration against time. The pH–rate profiles for the decomposition of compounds I–VI are shown in Fig. 5, where the logarithms of the observed apparent first-order rate constants are plotted against pH. The rate of decomposition was determined mostly in a single buffer concentration. Kinetic data determined in different buffer concentrations at several pH conditions revealed some buffer dependence but the effects were rather small and essentially produced no change to the pH-profiles.

As shown in Fig. 5, all compounds exhibited V-shape pH–rate profiles. Minimum degradation rates were shown at pH 7.5–8.0 for I and II, 5.5–6.5 for III and IV, and 6.0–6.5 for V, respectively. In an acidic medium, compounds III–V were about 10-fold stabler than I and II while these compounds reverted to I rather rapidly under the alkaline conditions. The decomposition rate constant of VI was much larger than those of other compounds.

Stability in biological media

The relative susceptibility of the various prodrugs to enzymatic hydrolysis was studied in vitro in plasma and in the supernatant fraction of liver homogenate at 37°C. The time courses of disappearance of prodrugs and appearance of I in 2% rat

Fig. 6.

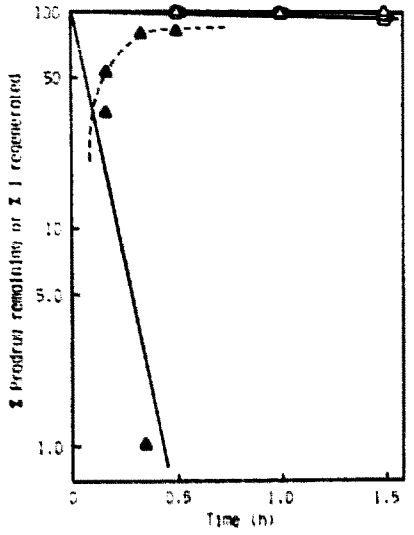


Fig. 7.

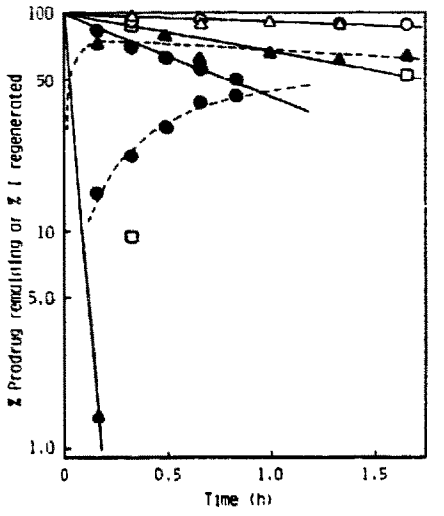


Fig. 6. Bioactivation of 1a-N-substituted mitomycin C derivatives in 2% rat plasma. The solid lines represent the time courses of degradation of prodrug and the dashed line is for regeneration of I from prodrug, respectively. ○, I; △, II; □, III; ●, IV; ▲, V.

Fig. 7. Bioactivation of 1a-N-substituted mitomycin C derivatives in 2% rat liver homogenate. The solid lines represent time courses of degradation of prodrug and the dashed lines are for regeneration of I from prodrug, respectively. ○, I; △, II; □, III; ●, IV; ▲, V.

TABLE 1

EFFECT OF TISSUE HOMOGENATE CONCENTRATION ON DEGRADATION RATES OF PRODRUGS IV AND V IN RAT PLASMA AND LIVER HOMOGENATE

Tissue concentration (%)	Degradation rate constant, $k_{obs}(h^{-1})$			
	IV		V	
	plasma	liver	plasma	liver
50	0.0921	–	> 15	–
10	–	1.82	> 15	> 30
2	0.0212	0.841	13.83	25.61
1	–	0.183	3.90	5.87
0.1	–	0.111	0.242	0.341
0.01	–	0.0745	0.0181	0.0582

plasma are shown in Fig. 6. Fig. 7 shows the degradation patterns of I–V in a 2% rat liver homogenate. As shown in these Figures, I was stable in plasma but degraded gradually in liver homogenate. V disappeared very rapidly in plasma and liver homogenate and production of I was observed simultaneously. The acyl derivatives of I, III and IV were relatively stable in plasma but rapidly reverted to I in a liver homogenate.

Table 1 summarizes the effect of plasma and liver homogenate concentrations on the degradation rates of IV and V. Their degradation rate constants showed a significant dependence on tissue concentration. The tissue concentration dependencies were also shown in the degradation of I, II and III in liver homogenate.

In order to examine species difference in the activity of each tissue sample for catalyzing the conversion of prodrugs II–V, disappearance rates were determined also in rat and mouse plasma and liver homogenate, and human plasma. Table 2

TABLE 2

DEGRADATION RATES OF 1a-N-SUBSTITUTED MITOMYCIN C DERIVATIVES IN PLASMA AND LIVER HOMOGENATE OF RAT, MOUSE AND HUMAN

Compound	Degradation rate constant, $k_{obs}(h^{-1})$				
	Rat		Mouse		Human
	2% plasma	2% liver	2% plasma	2% liver	50% plasma
I	0.0108	0.0461	0.00468	0.0238	0
II	0.0172	0.0876	0.0251	0.0413	0.0189
III	0.0781	0.412	0.0246	> 3.0	0.0125
IV	0.0212	0.841	0.0444	0.457	0.0380
V	13.83	25.61	0.184	3.889	0.0136

TABLE 3

ESTIMATED CONVERSION RATE CONSTANTS OF 1a-N-SUBSTITUTED MITOMYCIN C DERIVATIVES IN VARIOUS MEDIA [a]

Compound	pH 4.0	pH 7.4		pH 11.0		Rat 2% plasma		Rat 2% liver	
	$k_1 + k_2$	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2
I	1.628 [b]	0.00148 [b]		0.326 [b]		0.0108 [b]		0.0461 [b]	
II	1.492	0.00054 [c]	-	0.166 [c]		0.0172 [c]		0.0876 [c]	
III	0.144	0.00594	0.00277	4.458	1.216	0.0781 [c]		0.412 [c]	
IV	0.080	0.0373	0.00846	13.86	2.776	0.0212 [c]		0.781	0.060
V	0.113	0.00192	0.00170	6.162	1.580	9.945	3.882	18.43	7.185
VI	-	11.99 [c]		-		-		-	

[a] Calculated by simultaneous fitting of the results to Eqns. 1 and 2. See text.

[b] Values are equal to k_3.

[c] Values are obtained as the total degradation rate constants, k_{obs}, which can be expressed as $k_1 + k_2$.

summarizes the observed pseudo-first-order rate constants calculated from the slopes of semilogarithmic plots of drug concentration against time. The degradation of compounds I–V in mouse plasma and liver homogenate occurred like those in biological media obtained from rat, while these compounds were proved to be stable in human plasma.

Kinetics of degradation of prodrugs in various media

On the basis of the present results, overall decomposition reactions may be described roughly by the following scheme of parallel and consecutive reactions for all prodrugs:

$$\begin{array}{l} \text{prodrug} \xrightarrow{k_1} \text{mitomycin C (I)} \xrightarrow{k_3} \text{degradation products} \\ \quad\quad \downarrow k_2 \\ \text{degradation products} \end{array}$$

In this scheme k_1–k_3 are pseudo-first-order rate constants for the depicted chemical- or enzyme-mediated reactions (where $k_1 + k_2 = k_{obs}$). The corresponding rate equations to this model are integrated with Laplace transform to give following equations:

$$[\text{prodrug}] = [\text{prodrug}]_0 \times e^{-(k_1 + k_2)t} \tag{1}$$

$$[\text{I}] = \frac{k_1}{k_3 - (k_1 + k_2)} \times [\text{prodrug}]_0 \times (e^{-(k_1 + k_2)t} - e^{-k_3 t}) \tag{2}$$

where $[\text{prodrug}]_0$ represents the initial concentration of each prodrug, and [prodrug] and [I] are concentrations of prodrug and I at time t.

Based on these equations, curve-fitting and parameter estimation were done using a non-linear least-squares program MULTI (Yamaoka et al., 1979). Concentrations of prodrugs and I were fitted simultaneously. The degradation rate of I under each condition was used as k_3 for the corresponding experiment. The calculated rate constants are summarized in Table 3. The independent simulation of concentration of I employing Eqn. 2 with substitution of $k_1 + k_2 = k_{obs}$ values obtained from Fig. 5 gave parameters within 10% variation from those obtained by the simultaneous two-line fitting.

Discussion

In a prodrug design, not only a physicochemical property but also a lability of a leaving group is important to considerations bent on improving biopharmaceutical characteristics and therapeutic efficiencies of the drug (Roche, 1977). Thus, the lability of the disposable moiety under the condition available in vivo (pH, enzymes, etc.) is of great consequence and numerous reports have been focussed on problems such as a structure–chemical lability relationship, enzyme–substrate specificities,

and distribution of enzyme system (Sinkula and Yalkowsky, 1975; Charton, 1977; Amidon et al., 1977).

In designing 1a-N-substituted prodrugs of I in order to increase lipophilicity or lipid solubility, a benzene ring with various kinds of linkage moieties was preferred as model substituents in the previous investigation (Sasaki et al., 1983), and it was found that there were multiple differences in their biological activities regardless of their relatively similar lipophilicities.

Although I is a widely used antitumor antibiotic, little is known about the degradation kinetics except for few fragmentary reports (Schwartz et al., 1961; Edwards et al., 1979). As shown in the present results, I is fairly stable in a neutral solution but is subject to more rapid decomposition in an acidic and basic medium. The V-shaped pH–rate constant profile suggested that the degradation proceeded by general acid–base catalyzed reactions. But the reaction appeared to be somewhat complicated; e.g. acid-catalyzed hydrolysis was known to produce several mitosene compounds (Stevens et al., 1964) in the same manner as N-methyl mitomycin (porfiromycin) (Garrett, 1963; Garrett and Schroeder, 1964). The 1a-alkylated derivative, II, exhibited almost similar pH–rate constant profiles to that of I.

Other prodrugs except for VI also showed V-shaped profiles although the minimum rate constants were observed at lower pH. Substitution of 1a-position of I with acyl group led to a significant decrease of degradation rate in acidic solutions. Thus N-acylation of I appeared to stabilize I against acid-catalyzed hydrolysis. In a basic solution, these acylated mitomycins reverted to I relatively rapidly. As a result, these prodrugs are equally stable or stabler than the parent drug over the wide pH range in view of the residual quantity of the active drug. Prodrug VI, an acyloyloxymethyl derivative of I, was converted into I rapidly in aqueous solutions, while it was stable in organic solvents.

The present results revealed peculiar bioactivation profiles of these prodrugs under the physiological conditions. The rat plasma successfully catalyzed the hydrolysis of V which has a linkage consisting of carbamate. Non-specific esterase of carbamidase might be responsible for this raction (Sinkula and Yalkowsky, 1975). The acyl derivatives such as III and IV were relatively stable in plasma but were converted to I by liver homogenate. In the case of II, I was not produced in any tissue medium. Mouse tissue samples gave relatively similar results as those of rat in catalyzing hydrolysis of III, IV and V, but human plasma showed only slight activity.

The results shown in Table 3 suggest the predominant conversion of prodrug III and IV to I under most chemical and biological conditions. In the case of V, k_2 could not be neglected when comparing with k_1 regardless of the experimental conditions. The TLC analysis showed the production of an unknown decomposition intermediate of V in each medium which can be considered to be the cause for this peculiar behavior.

On the basis of the present results, it is quite evident that plasma and liver enzymes catalyze the hydrolysis of the prodrugs in vitro to a varying extent, and it can be anticipated that similar results would be obtained in vivo. As previous results had shown the fundamental activities of these prodrugs in a simple i.p.–i.p. system,

some relationships between their activities and lability characteristics were suggested in the present investigation. That is: prodrugs which are anticipated to revert to I in the body, i.e. V and VI, show high antitumor activities at low dose area. The acylated derivatives are considered to exhibit their activities after regeneration to I in the organ with high enzyme activity such as the liver. On the contrary, II can be considered to show its activity without any conversion, so that this compound is estimated to be an analogue of I.

Although the present report has preliminary characteristics in itself, the possible approach for controlling biopharmaceutical and chemotherapeutic properties of mitomycins by substituting the 1a-position with moieties of different labilities is suggested. An integrated design of linkage bond and leaving group would lead to a promising prodrug which could exhibit excellent activity by employing various routes of administration and/or in combination with various dosage forms particularly with lipoidal ones. In the series of this investigation, we compared percutaneous permeability of these compound and found that those with adequate lipophilicity and low melting point showed excellent absorbability. Liposomes and emulsions loaded with lipophilic prodrugs of I successfully showed specific lymphatic delivery of the parent drug following topical administration. Detailed results will be published in following papers.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Cancer Research (56-10) from the Ministry of Health and Welfare, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

Amidon, G.L., Pearlman, R.S. and Leesman G.D., Design of prodrugs through consideration of enzyme-substrate specificities. In Roche, E.B.(Ed.), Design of Biopharmaceutical Properties through Prodrugs and Analogs, American Pharmaceutical Association, Washington, DC, 1977, pp. 281–315.

Charton, M., The prediction of chemical lability through substituent effects. In Roche, E.B.(Ed.), Design of Biopharmaceutical Properties through Prodrugs and Analogs, American Pharmaceutical Association, Washington, DC, 1977, pp. 228–280.

Edwards, D., Selkirk, A.B. and Taylor, R.B., Determination of the stability of mitomycin C by high-performance liquid chromatography. Int. J. Pharm., 4(1979) 21–26.

Garrett, E.R., The physical–chemical characterisation of the products, equilibria, and kinetics of the complex transformations of the antibiotic porfiromycin. J. Med. Chem., 6(1963) 488–501.

Garrett, E.R. and Schroeder, W., Prediction of stability in pharmaceutical preparations. J. Pharm. Sci., 53(1964) 917–923.

Roche, E.B., Structural aspects of selective distribution. In Roche, E.B.(Ed.), Design of Biopharmaceutical Properties through Prodrugs and Analogs, American Pharmaceutical Association, Washington, DC, 1977, pp. 27–46.

Sasaki, H., Mukai, E., Hashida, M., Kimura, T. and Sezaki, H., Development of lipophilic prodrugs of mitomycin C. I. Synthesis and antitumor activity of 1a-N-substituted derivatives with aromatic pro-moiety. Int. J. Pharm., 15 (1983) 49–59.

Schwartz, H.S., Pharmacology of mitomycin C: III. In vitro metabolism by rat liver. J. Pharmacol. Exp. Ther., 136(1961) 250–258.

Sinkula, A.A. and Yalkowsky, S.H., Rationale for design of biologically reversible drug derivatives: prodrugs. J. Pharm. Sci., 64(1975) 181–210.

Sinkula, A.A., Perspective on prodrugs and analogs in drug design. In Roche, E.B.(Ed.), Design of Biopharmaceutical Properties through Prodrugs and Analogs, American Pharmaceutical Association, Washington, DC, 1977, pp. 1–17.

Stella, V. and Higuchi, T.(Eds.), Prodrugs as Novel Drug Delivery Systems, ACS, Washington, DC, 1975.

Stevens, C.L., Taylor, G., Munk, M.D., Marshall, W.S., Noll, K., Shah, G.D., Shah, L.G. and Uzu, K., Chemistry and structure of mitomycin C. J. med. Chem., 8(1964) 1–10.

Yalkowsky, S.H., Solubility and melting point considerations in drug design. In Roche, E.B.(Ed.), Design of Biopharmaceutical Properties through Prodrugs and Analogs, American Pharmaceutical Association, Washington, DC, 1977, pp. 392–408.

Yamaoka, K., Tanigawara, Y., Nakagawa, T. and Uno, T., A pharmacokinetic analysis program (MULTI) for microcomputer. J. Pharm. Dyn., 4(1981) 879–885.