Reactions of 4-bis(2-chloroethyl)aminophenylacetic acid (phenylacetic acid mustard) in physiological solutions

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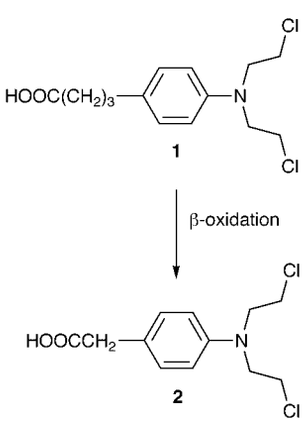
4-Bis(2-chloroethyl)aminophenylacetic acid (phenylacetic acid mustard, **2**) is the major metabolite of the cancer chemotherapeutic agent chlorambucil (**1**). Although its high antitumor activity and high toxicity to normal tissues have been known for a long time, no detailed chemical data on its reactions in aqueous media have been available. According to the present results **2** is decomposed in aqueous solutions by the same mechanism as other aromatic and aliphatic nitrogen mustards: an intramolecular, rate determining attack of the unprotonated nitrogen to form an aziridinium ion intermediate is followed by attack of an external nucleophile. Species **2** is considerably more stable in whole plasma than in plasma ultrafiltrate ($t_{1/2}$ at 37 °C 10 h and 37 min, respectively). Also the product distribution is completely different: while in protein-depleted plasma the only reaction is hydrolysis, in plasma the predominant reactions are non-covalent and covalent binding of **2** to albumin. The present information is important when clinical evaluation of chlorambucil, or *in vitro* evaluation of phenylacetic acid mustard, is performed in order to determine efficacy and bioavailability of these compounds.

Introduction

4-[4-Bis(2-chloroethyl)aminophenyl]butyric acid (chlorambucil, CLB, **1**) is an alkylating drug routinely used in the chemotherapy of chronic lymphocytic leukemia.[1] Other indications include Hodgkin's lymphoma, non-Hodgkin's lymphoma, Waldenström's macroglobulinaemia, ovarian and breast cancer, some other tumors and certain autoimmune diseases. CLB is an orally administrated drug that is readily absorbed from the gastrointestinal tract giving peak plasma concentrations 1 h after ingestion.[2] CLB is metabolized predominantly by β-oxidation of the butyric acid side chain,[3] giving an intermediate (*E*)-4-[4-bis(2-chloroethyl)aminophenyl]but-3-enoic acid, that is further converted into the final metabolite 4-bis(2-chloroethyl)aminophenylacetic acid (phenylacetic acid mustard, PAM, **2**; Scheme 1). Despite its known high anti-tumor activity and broad area under curve (AUC),[4–6] no detailed chemical data on the reactions of **2** in human fluid matrixes have been available. In order to understand the therapeutic index of CLB it is essential to know its metabolism and protein binding in detail, especially since PAM is known to be considerably more toxic to normal tissues than the parent drug.[3] The task of the present work was to verify the kinetics and mechanism of PAM decomposition in aqueous solutions, and clarify its reactions in human plasma.



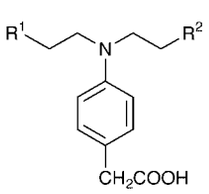
Scheme 1

Results

It is widely accepted that aromatic and aliphatic nitrogen mustards decompose in aqueous media by a mechanism involving an intramolecular, rate determining attack of the unprotonated nitrogen to form an aziridinium ion intermediate followed by an attack of an external nucleophile.[7–11] To verify if this is true also in the present case, PAM was allowed to react under various strictly controlled conditions, and the reactions were followed *via* HPLC techniques at 267 nm.

pH–rate profile

The effect of pH on the rate of PAM decomposition was investigated at 37 °C at constant ionic strength (1 M, adjusted with $NaClO_4$) in the absence of chloride ion (Fig. 1). PAM disintegration followed cleanly first order kinetics over the whole pH range studied. In the absence of nucleophiles (*i.e.* in cacodylic acid buffers and in solutions of perchloric acid) the only significant reaction observed was PAM hydrolysis yielding 4-(*N*-chloroethyl-*N*-hydroxyethylamino)phenylacetic acid (**3**) as the reaction intermediate and 4-bis(2-hydroxyethyl)aminophenylacetic acid (**4**) as the stable end product. When the reactions were performed in formic acid and acetic acid buffers,



2 $R^1 = R^2 = Cl$
3 $R^1 = Cl$; $R^2 = OH$
4 $R^1 = R^2 = OH$
5 $R^1 = Cl$; $R^2 = SCN$
6 $R^1 = R^2 = SCN$
7 $R^1 = SCN$; $R^2 = OH$
8 $R^1 = GS$; $R^2 = Cl$
9 $R^1 = R^2 = GS$
10 $R^1 = GS$; $R^2 = OH$

Table 1 HPLC/MS analyses of the reactions of PAM in the presence of 5 mM nucleophiles analyzed at $t = 2\ t_{1/2}$ [$T = 37$ °C, pH = 6.8, $I = 1$ M ($NaClO_4$)]. For chromatographic conditions, see Experimental

Nucleophile	t_R/min[a]	[M − 1]	Compound
SCN^-	18.7	238	**4**
	22.8	279	**7**
	23.6	256, 258	**3**
	28.4	320	**6**
	30.1	297, 299	**5**
	32.0	274, 276	**2**
GSH	18.3	527	**9**
	18.7	238	**4**
	21.2	545, 547	**8**
	23.6	256, 258	**3**
	32.0	274, 276	**2**

[a] Refers to retention time on mass detector.

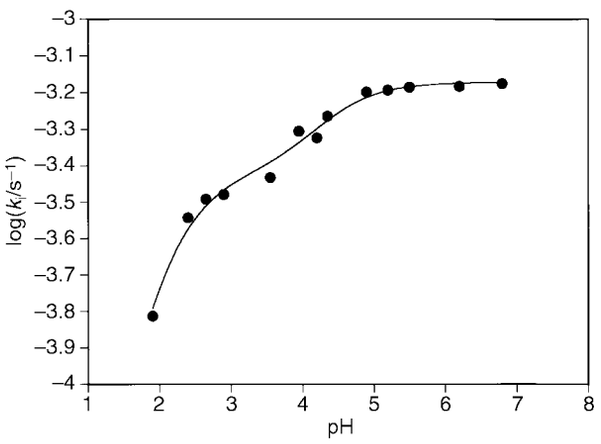


Fig. 1 pH–rate profile of PAM decomposition at 37 °C and 1.0 M ionic strength (adjusted with $NaClO_4$). The solid line is the theoretical curve derived from the rate law [eqn. (4)] developed in the Experimental section.

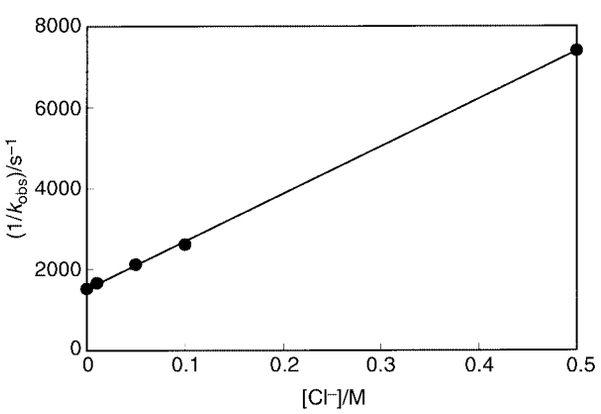


Fig. 2 The effect of chloride ion on the rate of PAM decomposition [$T = 37$ °C, pH = 6.8, $I = 1.0$ M ($NaClO_4$)].

additional reaction intermediates and stable end products were formed, which were tentatively assigned as resulting from the reactions of acetate and formate ions with the aziridinium ions derived from **2**. No catalysis by acetate or formate ions was observed.

The chloride effect

Addition of chloride ion is known to retard the rate of decomposition of aromatic nitrogen mustards significantly.[7–11] Also in the present case the rate of PAM decomposition was considerably slower in the presence of Cl^-. The rate retardation was dependent on chloride ion concentration in the solution (Fig. 2).

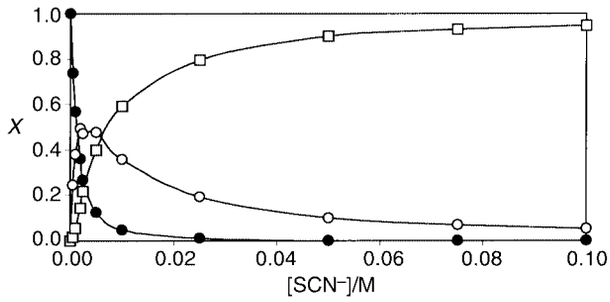


Fig. 3 End product distribution of PAM decomposition as a function of thiocyanate ion concentration [$T = 37$ °C, pH = 6.8, $I = 1.0$ M ($NaClO_4$)]. Compound **4** (filled circles), compound **6** (open squares), compound **7** (open circles).

The effect of glutathione and thiocyanate ion

The effect of two biologically significant nucleophiles, glutathione (GSH) and thiocyanate ion, on the rate and product distribution of PAM decomposition was investigated (pH 6.8, $I = 1.0$ M). In the presence of 5 mM SCN^-, PAM was converted into two reaction intermediates (**3**,**5**) and three stable end products (**4**,**6**,**7**). The identity of **2**–**7** was easily confirmed on HPLC/MS analysis (Table 1). By contrast, HPLC/MS analysis of the reactions of PAM with GSH was more complicated. The products of PAM hydrolysis (**3**,**4**) could be characterized as above. Analogously, the molecular ions of the reaction intermediate **8** and the stable glutathione monoadduct **10** could be analyzed, although their molecular ions were considerably weaker than those of the corresponding thiocyanate derivatives (**5**,**7**). By contrast, the expected molecular ion of the glutathione bis-adduct, **9**, could not be detected. This is not surprising, since the loss of glutamic acid residues of glutathionyl (GS) derivatives of chlorambucil on thermospray LC/MS analysis is well documented.[12] In all likelihood, a similar problem is also present in electron spray MS analysis.

As expected[11] SCN^- concentration had no effect on the rate of PAM decomposition: no rate enhancement was observed when [SCN^-] was increased from 0 to 0.1 M, which is considerably larger than the biologically relevant thiocyanate concentration.[13] By contrast, the effect of SCN^- on the product distribution was significant: when SCN^- was over 0.02 M, practically no PAM hydrolysis could be detected (Fig. 3). In contrast to SCN^-, GSH retarded slightly the rate of PAM decomposition ($t_{1/2}$ was 7.5 and 30 min in the presence of 0 and 0.1 M GSH, respectively). This is due to the decrease of the pH of the reaction medium caused by the acidity of GSH; the buffer capacity of the reaction mixture was not high enough to keep the pH constant at high [GSH].

Reactions of PAM in physiological media

The reactions of PAM in whole human plasma and its ultrafiltrate were investigated at 37 °C. In protein-depleted plasma, $t_{1/2}$ of PAM decomposition was 37 min, and the only reaction detected was hydrolysis, yielding **3** as the reaction intermediate, and **4** as the stable end product (Fig. 4A). When the same reaction was performed in whole plasma, the rate of PAM decomposition was considerably reduced ($t_{1/2}$ was 10 h). Furthermore, there was practically no **3** and **4** in the reaction mixtures when analyzed *via* HPLC techniques after protein precipitation (Fig. 4B). Similar rate retardation and product distribution was detected when the reactions of PAM were followed in a cacodylic acid buffer in the presence of purified HSA (Table 2).

Discussion

The proposed mechanism of PAM decomposition in aqueous solutions is shown in Scheme 2. As in the case of other nitrogen mustards, the mechanism is supported by the following

Table 2 The effect of HSA and Cl^- on the rate of PAM disintegration at 37 °C

Matrix	pH	Albumin/ g dm^{-3}	$[Cl^-]$/ mM	$k_{obs}/10^{-5}$ s^{-1}	Reaction observed
Protein-depleted plasma	7.5	—	114	31.5 ± 0.4	hydrolysis
Cacodylic acid buffer	7.0	—	100	38.4 ± 0.2	hydrolysis
Plasma	7.9	48.3	105	1.94 ± 0.05	binding to proteins
HSA in cacodylic acid buffer	7.3	55.1	—	0.69 ± 0.03	binding to HSA

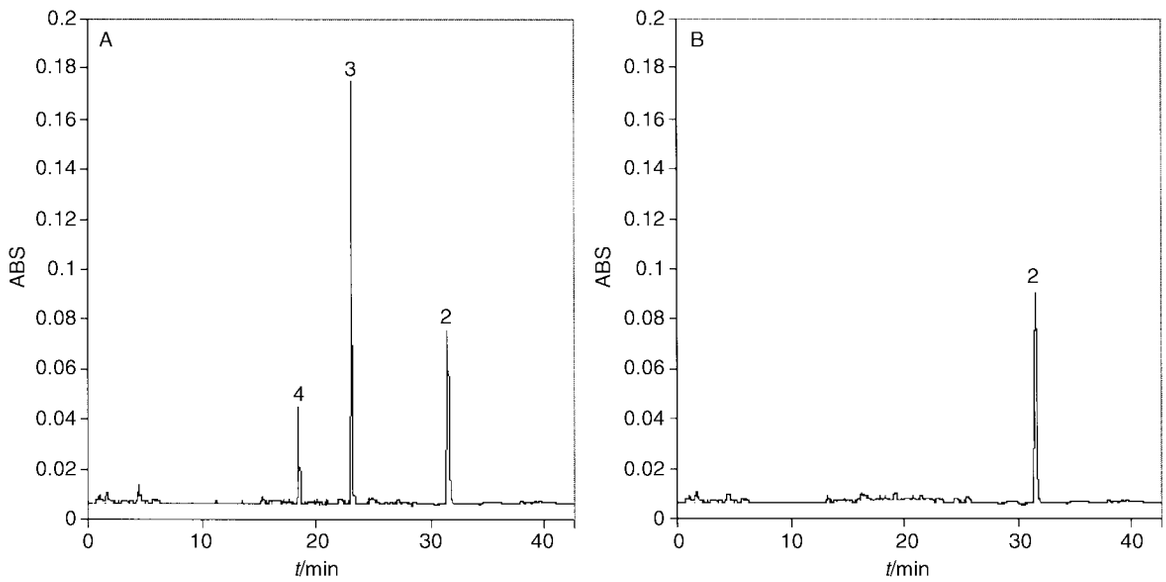
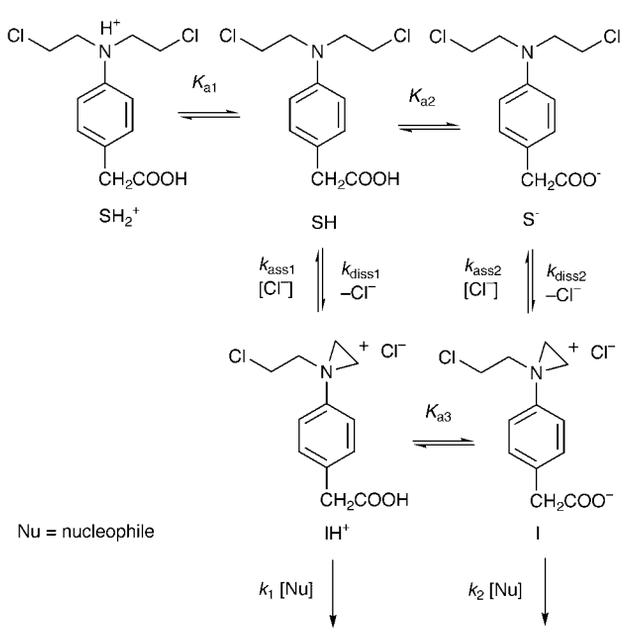


Fig. 4 Reversed phase HPLC traces of the reaction of PAM in human plasma ultrafiltrate (A) and human plasma (B) analyzed at $t \approx 2\ t_{1/2}$. For chromatographic conditions, see Experimental.



Scheme 2

observations. (i) The rate of decomposition is proportional to the mole fraction of unprotonated PAM in solution. Since only deprotonated nitrogen can form the aziridinium ion intermediate, the reactivity of the protonated PAM is insignificant: the rate of decomposition drops sharply below the pK_a of the nitrogen atom (Fig. 1, for development of the rate law, see Experimental). Furthermore, the rate levels off when the mole fraction of the deprotonated PAM reaches 1. The additional inflection at *ca.* pH 4 can be shown to result from the protolysis of the carboxylate ion of the acetic acid side chain: protonation of the acetate ion decreases inductively the electron density at the anilinic nitrogen and diminishes its capacity to act as a nucleophile. Furthermore, the electrostatic stabilization of the positively charged transition state by the carboxylate ion is lost. Hence the formation of the aziridinium ion is less favorable and a rate retardation is observed. A similar effect has been reported also for CLB[8] but the effect is naturally less significant due to the longer distance of the carboxylic acid group from the reaction center. The solid line in Fig. 1 is the best theoretical fit of eqn. (4), which gives the dissociation constants $K_{a1} = (9.46 \pm 1.67) \times 10^{-3}$ M (*i.e.* pK_{a1} 2.02), and $K_{a2} = (4.90 \pm 1.78) \times 10^{-5}$ M (*i.e.* pK_{a2} 4.31), and the rate constants $k_{diss1} = (3.73 \pm 0.24) \times 10^{-4}$ s^{-1} and $k_{diss2} = (6.73 \pm 0.21) \times 10^{-4}$ s^{-1}. (ii) Chloride ion retards the rate of decomposition by attacking the aziridinium ion derived from **2** and reforming the unchanged PAM. As shown in Fig. 2 and eqn. (5) the rate retardation is inversely proportional to chloride ion concentration. (iii) The rate of decomposition is of zero order with respect to external nucleophiles.

It is of common knowledge that approximately 98–99% of CLB is bound to plasma proteins.[14] We have shown that the non-covalent binding is practically immediate, and albumin is the main protein responsible for the binding in human blood.[15] The binding to albumin also has a stabilizing effect on CLB. Indeed, PAM was also found to be considerably more stable in human plasma and in the presence of purified human serum albumin (HSA) than in the plasma ultrafiltrate.

Albumin is the most abundant plasma protein; its concentration is normally 0.6 mM.[16] It its known to provide a very high drug binding capacity. It has different binding sites for cationic and anionic drugs. Also van der Waals interactions between albumin and hydrophobic drugs are important: the albumin binding correlates with the drug hydrophobicity. Hence, the large rate retardation of the PAM disintegration in plasma is

easily explained by its non-covalent binding to the hydrophobic pockets of albumin: in the absence of bulk water the hydrolysis is naturally less prominent. Furthermore, the hydrophobic environment makes the formation of the aziridinium ion intermediates less favorable. Similar effects, though less significant, have been reported for CLB stabilization in the presence of cyclodextrins[17] and micelles.[10]

Drug binding to albumin is normally instantaneous and reversible. In accordance with our previous findings with CLB,[15] covalent drug binding is also observed. These interactions between PAM and albumin may explain the inter- and intra-individual differences in the pharmacokinetics and bioavailability of orally administered CLB, as shown previously.[18] Molecular details of these interactions are currently being explored in our laboratories.

Experimental

General

4-Bis(2-chloroethyl)aminophenylacetic acid (**2**) was synthesized as described previously.[19] All inorganic reagents were of analytical grade. GSH was purchased from Fluka and human serum albumin (99%; globulin and fatty acid free) from Sigma. Plasma and protein-depleted plasma were obtained as described previously.[15] Albumin concentrations were measured immunoturbinometrically on a Cobas Integra 700 instrument.

HPLC analyses

These were performed on a Waters 2000 instrument consisting of a UV-detector ($\lambda = 267$ nm) and a reversed phase column (Hypersil C18, 4.6 × 240 mm, particle size 6 μm). Mobile phase: Buffer A = 0.1 M ammonium acetate, Buffer B = 0.1 M ammonium acetate in 50% (v/v) aq. acetonitrile. The following solvent systems were used as the eluents. *System 1*: isocratic elution of 50% A (pH rate profile, the effect of Cl^-, SCN^- and GSH on the rate of PAM decomposition). *System 2*: Gradient: from 0 to 10 min, 100% A; from 10 to 40 min, a linear gradient from 100% A to 100% B (product analyses, reactions of PAM in human fluid matrixes and in the presence of purified HSA). In all the cases, flow rate was 1.0 ml min^{-1}.

Kinetic measurements

These were performed as described previously,[13] except in the case of reactions in the presence of proteins, which were precipitated with a known amount of ethanol prior to HPLC analyses.

Qualitative product analyses

These were performed on a Perkin-Elmer Sciex API 365LC/MS/MS triple quadrupole mass spectrometer in negative detection mode (HPLC *System 2*; 0.01 M buffer concentration). Reaction mixtures containing 0.005 M GSH or SCN^- and 0.1 mM PAM were analyzed at $t = 2t_{1/2}$ (pH 6.8; $T = 37$ °C).

Quantitative product analyses

These were performed *via* HPLC at $\lambda = 267$ nm. This wavelength was chosen since it is the isosbestic point of the reactions of PAM with non-chromophoric nucleophiles, as judged on UV spectrophotometry. At this wavelength all reaction intermediates and end products have the same ε, and the mole fractions of each reaction component are proportional to the integrals of their HPLC signals. For details, see ref. 11.

Effect of pH and nucleophiles on the rate of PAM hydrolysis

If the intermediates I and IH^+ are assumed to be very unstable and their concentrations reach steady state, then at steady state eqn. (1) applies,

$$\frac{d[I_{tot}]}{dt} = 0 \quad (1A)$$

$$\Rightarrow k_{diss1}[SH] + k_{diss2}[S^-] - k_{ass1}[IH^+][Cl^-] - k_{ass2}[I][Cl^-] - k_1[IH^+][Nu] - k_2[I][Nu] = 0 \quad (1B)$$

where

$$[I_{tot}] = [IH^+] + [I]$$

$$[IH^+] = \frac{[I_{tot}]}{1 + \frac{K_{a3}}{H^+}}; [I] = \frac{[I_{tot}]}{1 + \frac{[H^+]}{[K_{a3}]}}$$

$$[S_{tot}] = [SH_2^+] + [SH] + [S^-]$$

$$[S^-] = \frac{[S_{tot}]}{1 + \frac{[H^+]}{K_{a2}} + \frac{[H^+]^2}{K_{a1}K_{a2}}}; [SH] = \frac{[S_{tot}]}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}}$$

and K_{a1}, K_{a2} and K_{a3} are the equilibrium constants.

$$K_{a1} = \frac{[SH^+][H^+]}{[SH_2^+]}; K_{a2} = \frac{[S^-][H^+]}{[SH^+]}; K_{a3} = \frac{[I][H^+]}{[IH^+]}$$

Substituting $[S^-]$, [SH], $[IH^+]$ and [I] into eqn. (1B), the total concentration of the reaction intermediates is given by eqn. (2).

$$[I_{tot}] = \frac{\frac{k_{diss1}}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}} + \frac{k_{diss2}}{1 + \frac{[H^+]}{K_{a2}} + \frac{[H^+]^2}{K_{a1}K_{a2}}}}{\frac{k_1[Nu] + k_{ass1}[Cl^-]}{1 + \frac{K_{a3}}{[H^+]}} + \frac{k_2[Nu] + k_{ass2}[Cl^-]}{1 + \frac{[H^+]}{K_{a3}}}} [S_{tot}] \quad (2)$$

The observed rate constant is then given by eqn. (3), where $[I_{tot}]$

$$\left[\frac{dP}{dt}\right] = (k_1[IH^+] + k_2[I])[Nu] = \left(\frac{k_1[Nu]}{1 + \frac{K_{a_3}}{[H^+]}} + \frac{k_2[Nu]}{1 + \frac{[H^+]}{K_{a_3}}}\right)[I_{tot}] \quad (3)$$

is given in eqn. (2). If $[Cl^-] = 0$ (or in general $k_{1,2}[Nu] \gg k_{ass1,2}[Cl^-]$), then eqn. (3) is simplified to eqn. (4) at constant pH and

$$\frac{d[P]}{dt} = \frac{k_{diss1}}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}} + \frac{k_{diss2}}{1 + \frac{[H^+]}{K_{a2}} + \frac{[H^+]^2}{K_{a1}K_{a2}}} \quad (4)$$

[Nu] eqn. (3) is simplified to eqn. (5), *i.e.* the rate constant is linearly dependent on $\frac{1}{[Cl^-]}$.

$$\frac{dP}{dt} = \frac{\text{constant}_1}{\text{constant}_2 + \text{constant}_3[Cl^-]} \quad (5)$$

$$\Rightarrow \frac{1}{k_{diss}} \propto [Cl^-]$$

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References

1 C. B. Faguet, *J. Clin. Oncol.*, 1994, **12**, 974.
2 W. B. Pratt, R. W. Ruddon, W. D. Ensminger and J. Maybaum, *The Anticancer Drugs*, 2nd edn., Oxford University Press, New York, 1994, ch. 6.
3 A. McLean, A. D. Newell, G. Baker and T. Connors, *Biochem. Pharmacol.*, 1980, **29**, 2039.
4 N. H. Greig, E. M. Daly, D. J. Sweeney and S. I. Rapaport, *Cancer Chemother. Pharmacol.*, 1990, **25**, 320.
5 M. M. Oppitz, E. Musch, H. P Malek, H. Rüb, G. E. von Unruh, U. Loos and B. Mühlenbruch, *Cancer Chemother. Pharmacol.*, 1989, **23**, 208.
6 L. Bastholt, C.-J. Johansson and P. A. Pfeiffer, *Cancer Chemother. Pharmacol.*, 1991, **28**, 205.
7 W. R. Owen and P. J. Stewart, *J. Pharm. Sci.*, 1979, **68**, 992.
8 D. C. Chatterji, R. L. Yager and J. F. Gallelli, *J. Pharm. Sci.*, 1982, **71**, 50.
9 G. C. Kundu, J. R. Schullek and I. R. Wilson, *Pharmacol. Biochem. Behav.*, 1994, **49**, 621.
10 P. M. Cullis, R. E. Green and M. E. Malone, *J. Chem. Soc.*, *Perkin Trans. 2*, 1995, 1503.
11 J. Hovinen, T. Pettersson-Fernholm, M. Lahti and J. Vilpo, *Chem. Res. Toxicol.*, 1998, **11**, 1377.
12 D. M. Dulik, O. M. Colvin and C. Fenselau, *Biomed. Environ. Mass Spectrom.*, 1990, **19**, 248.
13 J. Hovinen, R. Silvennoinen and J. Vilpo, *Chem. Res. Toxicol.*, 1998, **11**, 91.
14 *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th edn., ed. J. G. Hardman and L. E. Limbird, McGraw-Hill, New York, 1995.
15 T. Peters, Jr., *All About Albumin*, Academic Press, San Diego, 1996.
16 K. Löf, J. Hovinen, P. Reinikainen, L. M. Vilpo, E. Seppälä and J. A. Vilpo, *Chem.-Biol. Interact.*, 1997, **103**, 187.
17 A. R. Green and J. K. Guillory, *J. Pharm. Sci.*, 1989, **78**, 427.
18 M. J. Lind and C. Ardiet, *Cancer Surv.*, 1993, **17**, 157.
19 J. Hovinen, *Acta Chem. Scand.*, 1996, **50**, 1174.

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