Biophysical Chemistry, 35 (1990) 287–300
Elsevier

BIOCHE 01431

Ambazone as a membrane active antitumor drug

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Received 20 September 1989
Accepted 14 October 1989

Ambazone; DNA; Membrane; cyclic AMP; Antitumor effect

Ambazone (1,4-benzoquinone guanylhydrazone thiosemicarbazone) was found to be active against various transplantable tumors in mice as well as rats. When administered orally for 4–9 days, the effective therapeutic dose ranged between 60 and 125 mg/kg. The antineoplastic effect of ambazone appeared to be mediated, at least in part, by the immune system. In order to characterize the drug, biophysical and biophysicochemical studies were carried out using thin-layer chromatography, absorption spectroscopy and polarographic measurements. The distribution of ambazone in an *n*-octanol/water system indicated low hydrophobicity, thereby excluding the possibility of a preferential contribution from hydrophobic forces to the mode of action of ambazone. Ambazone undergoes three protonation reactions with pK values at 10.69 (equilibrium between the negatively charged and neutral forms), 7.39 (equilibrium between the neutral and singly positively charged form) and 6.22 (equilibrium between the singly and doubly positively charged form). Interaction of the drug with model membrane systems was monitored by spectrophotometric and fluorescence measurements. Using the fluorescence label 1-anilino-8-naphthalenesulfonic acid (ANS) as a probe pointed to the interaction of ambazone with the inner area of the phospholipid bilayer matrix of liposomes as being nonspecific. Ambazone induces an overall increase in the cellular cAMP content of leukemia cells and macrophages. So far, membrane interaction has provided a molecular basis for both immunological and antineoplastic activities of the drug. By performing DNA melting experiments, it was shown that neutral or singly positively charged ambazone species stabilize the secondary structure of DNA, while the doubly positively charged form binds more strongly and destabilizes the DNA. After oral administration to rats and mice, ambazone was found to be incompletely absorbed from the gastrointestinal tract, to an extent of about 35–50%. Absorbed ambazone binds only weakly to plasma proteins, whereas its binding to red blood cells is relatively strong. The mutagenic potential of ambazone shown in bacterial systems and human lymphocytes corresponds to its relatively weak interaction with DNA. The toxic action of ambazone on the intestine is believed to be due to inhibition by the drug of bacterial DNA, RNA and protein syntheses. It is assumed that the reported affinity of ambazone for different cellular targets, i.e., membranes, nucleic acids and proteins, contributes to the overall antibacterial effect. The weak antiviral activity of ambazone in the Sendai virus/chicken embryo fibroblast system is probably the result of the interaction with Sendai virus NH glycoprotein.

1. Introduction

In 1955, the antineoplastic activity of the antimicrobial agent 1,4-benzoquinone guanylhydrazone thiosemicarbazone (ambazone) was described for the first time [1]. Both the interesting activity against various transplantable tumors and the insignificant side effects demonstrated in recent animal studies prompted investigations on the molecular, cellular and organismic modes of action of this potentially cancerostatic compound.

In this review, we wish to outline data on physico-chemical studies of the affinity of ambazone for model membrane systems realized in phosphatidylcholine liposomes, deoxyribonucleic acid (DNA) and basic protein (bovine serum albumin). These were examined by using spectrophotometric, polarographic and chromatographic

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techniques. Interference by ambazone with the membrane-bound cyclic nucleotide system, and its antibacterial, antiviral and mutagenic activities are discussed in the light of interactions with different molecular targets. Furthermore, in vivo data are reported, taking into consideration the pharmacokinetic behavior of the compound, its pharmacodynamics and toxicity.

2. Chemical and physico-chemical characterization

2.1. Chemical properties

1,4-Benzoquinone guanylhydrazone thiosemicarbazone, ambazone [1], with the molecular formula $C_8H_{11}N_7S$ and a molecular mass of 255.32 g/mol, is a dark-brown, odorless and tasteless powder possessing the chemical structure given in fig. 1. Its melting point ranges between 192 and 194° C. The molecule contains one loosely bound molecule of water which was detected by Karl-Fischer titration (in methanol) or by means of reweighing after drying at 105° C for 2 h. The solubilities with respect to a volume of 100 ml are as follows: water, approx. 0.2 mg; chloroform, 0.3 mg; 1-butanol, 6.5 mg; ethyl acetate, 17 mg; acetone, 50 mg; ethanol (96%), 85 mg; methanol, 0.5 g; dimethyl formamide, 1.7 g; and dimethyl sulfoxide, 2.5 g [2].

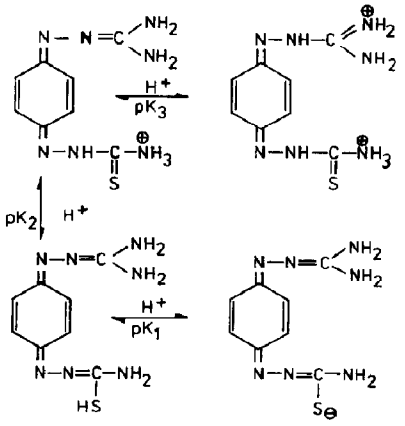

Fig. 1. Structure of the differently protonated forms of ambazone (taken from Gollmick and Stutter [4], with permission).

Table 1

Results obtained for ambazone by thin-layer chromatography [a]

Solvent system	R_f values		
	Silica gel C plates		Silufol plates (visual detection)
	Visual detection	Dragendorff's reagent	
1-Butanol/acetic acid/water (4:1:5, upper phase)	0.69 (±0.05)	0.69 (±0.05)	0.43 (±0.03)
2-Propanol/ethyl acetate/ammonia (59:25:16)	0.88 (±0.06)	0.88 (±0.06)	0.90 (±0.03)
1-Propanol/ethyl acetate/water/acetic acid (4:1:4:1)	0.85 (±0.05)	0.85 (±0.05)	0.80 (±0.05)

[a] Taken from Hesse et al. [2], with permission.

2.2. Thin-layer chromatography (TLC)

TLC was performed on silica gel and cellulose plates where distinct spots were obtained. Table 1 lists the results of TLC with three selected solvent systems on slurry-coated silica gel plates (10 × 20 cm; 250 μm) and commercially available silica gel sheets (Silufol, Kavalier Czechoslovakia) which contain starch as a binder. All spots could be detected by their yellow (as acetate) or light brown (as free base) color which darkened to brown overnight. On silica gel G (Merck, Darmstadt) the spots changed color to appear pink after spraying with Dragendorff's reagent [3]. No additional spot occurred after this detection step. In all runs, 5-μl samples containing 5 μg ambazone in methanol were applied to the plate 3 cm from the lower edge. The detection limits lie at 100 ng and lower to 50 ng per spot using visualization by irradiation with 350 nm light [2].

2.3. Spectrophotometric behavior

Ambazone strongly absorbs light with only a single maximum in the near-visible region. In water, a maximum appears at 403 nm which shifts to longer wavelengths in alcohols (methanol, 440 nm; 1-butanol, 445 nm), ethyl acetate (453 nm) and dimethyl sulfoxide (467 nm). In all solvents the values of the molar extinction coefficient ϵ are

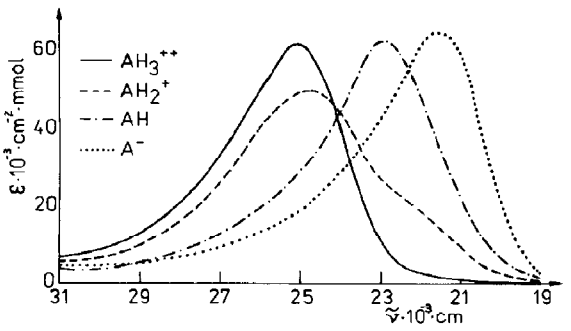

Fig. 2. Absorption bands in the visible region of the variously protonated forms of ambazone (taken from Gollmick and Stutter [4], with permission).

rather high (approx. 54000 M^{-1} cm^{-1} at pH 3 and approx. 48000 M^{-1} cm^{-1} at pH 5 in aqueous media, 46000–51000 M^{-1} cm^{-1} in various alcohols and 55200 M^{-1} cm^{-1} in dimethyl sulfoxide). Due to the high values of ϵ, absorption spectroscopy seems to be the most convenient method for the quantitative analysis of ambazone.

Solutions of ambazone in water, methanol, ethanol and dimethyl sulfoxide at a concentration of 1.5×10^{-5} M were measured spectrophotometrically as a function of the time. Within 24 h only slight decreases in absorbance could be observed which amount to 4% in H_2O, 2.4% in methanol and 0.9% in ethanol. A bathochromic shift of the absorption maximum of about 3 nm was observed on going from water to organic solvents.

The pH dependence of the absorption spectra is shown in fig. 2. The spectral changes clearly indicate that a minimum of three differently protonated forms must be responsible for the variation in the spectra. From fig. 2, one can expect that one protonation step occurs in the neutral region and another at about pH 11 in alkaline solutions.

As outlined previously [4], a reasonable fit of the experimental data is possible only with three protonation equilibria ($pK_I = 10.69 \pm 0.05$; $pK_{II} = 7.37 \pm 0.06$; $pK_{III} = 6.22 \pm 0.15$). Figs. 1 and 2 show the structures of the differently protonated forms and the corresponding absorption spectra of ambazone.

2.4. Polarographic half-wave potential

In electrochemical studies, ambazone shows both a cathodic and an anodic step, the latter not being separated from the former. The half-wave potential for the reduction lies within the range of -0.320 V (d.c. polarography (DCP) vs saturated calomel electrode, 0.04 M phosphate buffer, pH 7) and corresponds to the uptake of two electrons. The reduction is connected with the reversible electron- and proton-transfer processes taking place in the quinoid ring system. The oxidation process indicated by an anionic wave can be explained by a reaction of the $>C=S$ group with the mercury drop electrode.

During electrochemical reduction the yellow colored solution of ambazone becomes colorless. After this reduction, the resulting anodic current appears at the control half-wave potential and the same wave height. Following the end of reoxidation of ambazone with air, we obtain only a cathodic current: the cathodic wave shows that this part of the reaction is reversible. The loss of the anodic wave by reoxidation demonstrates the irreversibility of this part of the process. During this electrochemical reduction and oxidation process by air, hydrogen peroxide is generated. Probably the electrochemical reduction results in the formation of radicals similar to those described for the semiquinone part of the anthracycline antibiotics [5,6].

2.5. Distribution in n-octanol / water system

An important element for investigating the mode of action of a drug is its hydrophobic or lipophilic nature which can be determined by measuring its partition coefficient in a two-phase solvent system. The partitioning represents an equilibrium event like the protonation reaction but with a much longer time to achieve equilibration. The *n*-octanol/water system is frequently used and has been established as a standard tool for partitioning experiments [7,8]. The partition coefficient P represents the ratio c_o/c_w of the concentrations in the equilibrium state of a compound in the lipophilic *n*-octanol phase (c_o) and aqueous phase (c_w). The partition coefficients for

the pH values 6.72, 6.90, 7.11 and 7.33 are 0.144, 0.217, 0.265, 0.319 and 0.455, respectively [9]. This finding demonstrates a low degree of hydrophobicity for ambazone, so that the biological activity is not governed or preferentially directed by the hydrophobic nature of ambazone.

3. Interaction of ambazone with membrane systems

3.1. Spectrophotometric measurements

Using liposomes as membrane models, we attempted to determine the existence of possible interactions of ambazone with liposomes with the aid of absorption spectroscopy [10]. Based on the observation that weak monomer binding of a drug results in hypochromicity of the absorption spectrum and strong monomer binding in hypochromic and bathochromic effects, we were able to demonstrate a weak interaction of ambazone with liposomal phospholipid bilayers. Different liposomes were prepared, consisting of phosphatidylcholine (PC), phosphatidylcholine (PC)/cholesterol (Chol) (molar ratio 1 : 1), hydrogenated phosphatidylcholine (PC-hydrogenated) and phosphatidylcholine (PC)/cholesterol (Chol)/cardiolipin (molar ratio 10 : 7 : 1, negatively charged).

Depending on the pH of the solution, ambazone exists in various ionized forms AH_2^{2+}, AH^+, A and A^- with respective pK_a values of 6.22, 7.37 and 10.69. Within the range pH 3.8–8.5, mixtures of $AH^+ + AH_2^{2+}$ (low pH) and of $AH^+ + A$ (higher pH) with differing proportions of each component are present. The corresponding visible absorption spectra exhibit single peaks with maxima between 398 and 440 nm. Immediately after addition of PC liposomes to the ambazone solution, the absorbance decreases by 7.5, 6.5 and 6% at pH values of 3.8, 6.5 and 8.5, respectively (table 2). This hypochromic effect may be interpreted as being due to the interaction of the drug with the phospholipid matrix of the liposomes. The rather small differences in hypochromicity at various pH values indicate that, in agreement with the fluorescence measurements given below, the charge of the molecule does not play an important role in the interaction. Using liposomes prepared from catalytically hydrogenated lecithin without any double bonds of unsaturated fatty acids or cholesterol might have an effect on the binding of the drug. No influence on the magnitude of hypochromicity (7.5 %) could be observed.

Table 2

Liposome-induced hypochromic effect on the long-wavelength absorption spectrum of ambazone as a function of lipid composition and pH

Liposomes, neutral; salt concentration, 0.15 M [a].

Liposome composition	pH	Hypochromicity (%)
PC	3.8	7.5
PC	6.5	6.5
PC	8.5	6.0
PC (catalytically hydrogenated)	6.5	7.5
PC/Chol/cardiolipin [b]	6.5	5.0
PC/Chol	6.1	7.0
PC/Chol	6.5	6.6
PC/Chol	7.0	5.0
PC/Chol	8.0	6.5
PC/Chol [c]	6.5	9.5

[a] Taken from Hanschmann and Löber [10].
[b] Negatively charged.
[c] Salt concentration 0.0015 M.

Another set of experiments was performed with liposomes containing a negative charge due to cardiolipin. Since at pH 6.5, approx. 93% of the ambazone is present as mono- and dipositive species, a significant change in hypochromicity might be expected as a result of electrostatic effects involved in the interaction. Table 2, however, shows a decrease in absorbance of only 5%, demonstrating that the possibility of charge-induced interactions can be excluded as the dominant event. This result is supported by measurements at varying salt concentrations. Lowering the buffer/salt contents from 0.15 to 0.0015 M did not cause significant modification of the hypochromicity. The results presented here are consistent with a model where ambazone binds with different phospholipid model membrane systems. This finding offers the possibility of involving membrane-directed processes in the discussion of the cytostatic action of ambazone.

3.2. Fluorescence probe technique

Since ambazone itself displays no measurable fluorescence emission, fluorescence studies were performed with the membrane fluorescence label 1-anilino-8-naphthalenesulfonic acid (ANS), which is known to be localized within the polar head group region [12,13]. The labeled compound most likely occupies multi-member binding sites, thereby protruding partially into the hydrophobic fatty acyl domains [14]. In this environment, ANS is shielded from the surrounding water and exhibits a fairly high-yield blue-green fluorescence with a quantum efficiency of about 0.24 and a lifetime of 7–8 ns at room temperature [14–16]. The emission originates from an intramolecular charge-transfer type excited state [17,18]. In the presence of water, depopulation of the excited state rapidly decays along a unique, highly effective nonradiative pathway by photoionization. 'Pre-formed' three- or four-membered clusters of water molecules around the excited label act as electron acceptors [17,18]. In this case, a low-yield green emission with a fluorescence quantum yield of 0.003 and a fluorescence lifetime of 250 ps can be observed [19]. Thus, there is a possibility of unambiguously differentiating between bilayer-embedded and water-accessible ANS molecules by means of time-resolved fluorescence measurements. In the presence of any drug capable of interacting with membrane systems, one may detect changes in the fluorescence quantum yield of the shielded probe and/or variations in the ratio of both molecular populations.

The fluorescence decay kinetics of ANS bound to liposomes appear to be formally triply exponential. This is in accord with data for comparable systems [16,20]. The short-lived components of about 0.1 and 1.0 ns fluorescence lifetime originate from nonseparable increments of spectral relaxation effects and low-yield fluorescence emission of water-accessible probe molecules. The long-lived component of about 7 ns fluorescence lifetime corresponds to ANS molecules embedded in liposomal bilayers. In order to omit unwanted filter and spectral relaxation effects excitation was performed at wavelengths greater than 520 nm. As shown in table 3, the fluorescence decay parameters of samples derived from the compositionally modified reference system are characterized by the following properties [21].

Table 3

Modification of the long-wavelength edge fluorescence decay of ANS in PC liposomes by ambazone [a]

Reference		Ambazone	
τ_1 (ns) [b,c] τ_2 (ns)	γ_2/γ_1 [c]	τ_1 (ns) τ_2 (ns)	γ_2/γ_1
6.89 (±0.09) 0.30 (±0.02)	1.35 (±0.06)	6.36 (±0.07) 0.28 (±0.02)	2.00 (±0.09)
6.99 (±0.09) 0.26 (±0.01)	1.43 (±0.03)	6.28 (±0.10) 0.28 (±0.01)	2.15 (±0.09)
7.30 (±0.21) 0.29 (±0.01)	1.42 (±0.03)	6.48 (±0.19) 0.23 (±0.01)	2.26 (±0.08)

[a] Taken from Geller et al. [21].

[b] Measurement 20 h after sample preparation, storage at 4°C in the dark. Conditions: $\lambda_{em} > 520$ nm, $T = 20$°C; concentrations: PC, 2.54×10^{-4} M; Chol, 2.50×10^{-4} M; ANS, 3.66×10^{-5} M; ambazone, 5×10^{-5} M; molar ratios: PC/ANS = 7, PC/ambazone = 5; pH, 7.70.

[c] Best-fit parameter values for the equation:

$$I(t) = \sum_{i=1}^{3} \gamma_i \exp(-t/\tau_i).$$

The fit with three exponentials was preferred to that with two exponential terms, since a 4–5-fold decrease in the sum of deviation squares as well as a significant effect on the distribution of residuals was attained. $I(t)$, fluorescence intensity at time t; τ_i, fluorescence decay time of component i; γ_i, pre-exponential factor for component i which reflects the amount of the i-th component. $i = 3$ indicates unbound ANS with a decay time $\tau_3 \sim 0.1$ ns. $i = 1$ and $i = 2$ indicate bilayer-embedded and water-accessible bound ambazone, respectively.

(i) The main effect of the drug consists of changes in the ratio of the decay components γ_2/γ_1, in favour of solvent-accessible ANS species (γ_1, γ_2 denote pre-exponential factors in the fluorescence decay law, reflecting the amounts of bilayer-embedded and water-accessible ANS molecules, respectively). A small degree of quenching

of the embedded probe's high-yield fluorescence must be inferred from the drop in the τ_1 value.

(ii) There is no significant dependence of drug effects on pH, except for an occasional tendency to increase slightly on raising the pH value. The effects of ambazone become greater during a 20 h sample storage period with small successive changes thereafter (data not shown).

(iii) Under our experimental conditions, the quenching effect was independent of the ANS concentration.

On the basis of these findings, there is no indication for any kind of site binding, with definite localization of neutral or cationic ambazone species at or in the phospholipid bilayer matrix. A preferential surface association of, say, ambazone cations additionally to the ANS anions, thereby shielding the negative surface potential and favouring further probe incorporation, may be imagined. Such electrostatic interactions, however, would induce effects by ambazone on ANS fluorescence contrasting with the observed results.

Moreover, when considering the pK_a data of ambazone [4], any electrostatic effect should appear more pronounced at lower pH values. Site binding of neutral molecules would result in a concentration-dependent variation in fluorescence quenching of the embedded probe; such a dependence, however, was not found. Some conceivable external association of ambazone must be expressed as a modulation of the rapid decay process of ANS. Such ambazone association would be incompatible with the observed gradual changes of the very slow kinetics. Instead, our findings can be interpreted on the basis of the amphiphilic nature of ambazone. Its solubility in organic solvents has been found to be many times higher than in water [9]. The solute in the organic media is the neutral molecule HA [4]. Hence, the protonation equilibria existing in aqueous solution are shifted in favor of the neutral form. Supposing that ambazone preferentially penetrates into the fatty acyl core of the bilayer, we then obtain an explanation for the lack of significant pH effects as well as of the slow kinetics of the successive fluorescence changes during storage. It is assumed that a partitioning process implying all lamellar core domains needs some time to reach equilibrium (for compartment model calculations see ref. 22).

Whether or not ANS molecules belonging to the water-accessible fraction are still loosely associated with the bilayer or free in solution cannot be decided on the basis of fluorescence methods. On the other hand, the rise in the ratio of the decay components τ_2/τ_1 constitutes distinct evidence of drug-induced fractional release of the order of 30% of ANS from the liposome-embedded probe fraction. In conclusion, the results as a whole are indicative of a nonspecific interaction of the antineoplastically active drug ambazone with the phospholipid bilayer matrix of membrane systems.

3.3. Interaction of ambazone with the membrane-bound cyclic nucleotide system

The affinity of ambazone for binding with membrane systems prompted us to conduct investigations on membrane-bound enzymes in order to determine whether the functioning of such enzymes is modified by the drug. Enzymes belonging to this category include adenylate cyclase and cyclic AMP (cAMP) phosphodiesterase. Accordingly, steady-state levels of cAMP are balanced by the activity of both of these classes of enzymes [23]. Particular emphasis is currently being placed on the search for phosphodiesterase inhibitors as an approach to pharmaceutical agents which exert their action via the system of cyclic nucleotides [24,25].

Despite the central role of cAMP phosphodiesterase as well as adenylate cyclase in the maintenance and modulation of cAMP pools [26,27], so far no attention has been paid to investigations on the influence of 1,4-benzoquinone derivatives on the cyclic AMP system. Studies of the in vitro effect of ambazone on L1210 ascite tumor cells as well as on thioglycolate-elicited mouse peritoneal macrophages have been carried out [28–30]. The results demonstrate that the antileukemic agent ambazone is capable of increasing the cellular cAMP content triggered by the inhibition of the hydrolyzing enzyme phosphodiesterase as well as of stimulating the cAMP-producing enzyme cyclase (table 4).

Table 4

Alterations of cAMP phosphodiesterase activity, adenylate cyclase activity and cyclic AMP in L1210 supernatant and peritoneal macrophage homogenate as a function of ambazone concentration

Values represent the means of at least two experiments, each assayed in triplicate [a]. n.d., not determined.

	Concentration (μM)	cAMP phosphodiesterase activity (% decrease)	Adenylate cyclase activity (% increase)	Cyclic AMP (% increase)
Macrophage Homogenate	10	6.1	n.d.	n.d.
	50	29.7	11.2	0.8
	100	50.9	12.3	2.5
	150	53.2	24.0	15.7
	200	62.1	29.1	16.9
L1210 Supernatant	10	10.2	n.d.	n.d.
	50	33.4	10.2	6.4
	100	53.9	29.5	9.5
	150	65.7	31.7	21.1
	200	74.8	33.1	22.3

[a] Taken from Römer and Schulze [30], with permission.

The cAMP phosphodiesterase measured as high-affinity activity in leukemic and macrophage cells was inhibited by ambazone in a concentration-dependent manner. The IC_{50} value was 80–100 μM, i.e., the drug ambazone was about 10-times more potent than theophylline, a well-known phosphodiesterase inhibitor.

Taken together, the results suggest that in both leukemic cells and macrophages, at least part of the effect of ambazone might include an overall

Table 5

Changes in some in vivo parameters for $ABD2F_1$ mice following intraperitoneal injection of 5×10^6 L1210 lymphoma cells and treatment per os with two concentrations of ambazone [a]

Mode of treatment and time after intraperitoneal injection of 5×10^6 tumor cells	Spleen	Tumor cells		
	Weight (mg/mouse)	Number ($\times10^6$/ml)	cAMP (pmol/10^6 cells)	cAMP-PDE (pmol/10^6 cells)
Tumor control				
6 days post-injection	277	52.1 ± 3.8	6.1 ± 0.5	22.0 ± 4.0
8 days post-injection	314	47.7 ± 4.2	7.4 ± 1.8	27.3 ± 2.8
Tumor + drug [b]				
6 days post-injection	208	28.9 ± 3.3	9.4 ± 2.5	18.9 ± 0.8
8 days post-injection	264	28.6 ± 6.0	6.8 ± 0.8	18.3 ± 3.4
Tumor + drug [c]				
6 days post-injection	172	1.7 ± 0.3	18.4 ± 2.3	0
8 days post-injection	193	0.3 ± 0.2	19.7 ± 0.3	0
Control	63	–	–	–

[a] Taken from Mühlig et al. [31], with permission.
[b] Treatment with 62.5 mg/kg, 4 and 5 days after tumor injection.
[c] Treatment with 125 mg/kg, 4 and 5 days after tumor injection.

increase in the cellular cAMP content resulting from decreased cAMP phosphodiesterase and enhanced adenylate cyclase activities. The effect of ambazone on the cAMP system may be directed via the interaction of the drug with the enzyme-carrying membranes.

Moreover, this finding is consistent with the changes in some in vivo parameters for $ABD2F_1$ mice following intraperitoneal injection of L1210 lymphoma cells and oral administration of the drug to animals (table 5). In addition, scanning electron microscopic representation shows differences between ambazone-treated and untreated L1210 cells. The vasculation of the cell surface from damaged cells shows the mode of cell inactivation. This morphological effect of membrane disruption occurs in parallel with an increase in cAMP content and a decrease in cAMP phosphodiesterase activity of the cells [31].

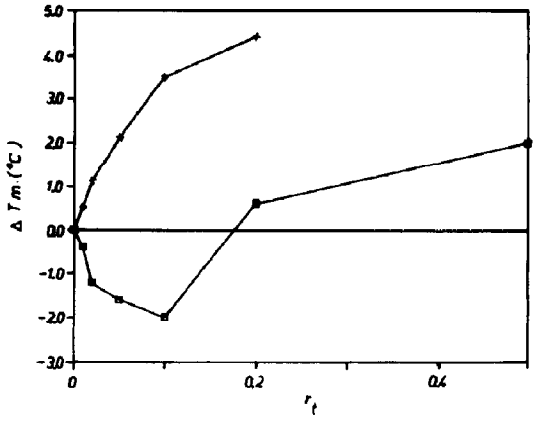

Fig. 3. Differences between melting temperatures of DNA-ambazone adducts and of pure DNA (ΔT_m) plotted as a function of the input ratio (r_t; molar ratio of drug to DNA phosphate). (+) pH 7.2; (□) pH 5.7. T_m for DNA in the absence of drug was 65.5° C. Denaturation experiments were performed on the day of mixing of adduct components (taken from Kleinwächter and Löber [32]).

4. Interaction of ambazone with deoxyribonucleic acid (DNA)

The effect of ambazone on chromosomes and on the synthesis of DNA and RNA in the bacterium *Escherichia coli* (section 6) poses the question of whether this drug interacts with nucleic acids.

After mixing solutions of DNA and ambazone, a small hypochromic shift was observed in the visible absorption band at 398 nm at both pH 7.2 and 5.7. The maximum decrease in absorbance was approx. 10%. Another piece of evidence in support of the formation of an ambazone-DNA adduct is yielded by thermal denaturation experiments.

We have been able to demonstrate in denaturation experiments that ambazone interacts with DNA and that the mode of interaction is dependent on the degree of ionization of ambazone [32]. The results obtained are summarized in fig. 3, which shows the dependence of the differences, ΔT_m, between the melting temperatures of the adduct and of pure DNA. At pH 7.2 the binding of ambazone moderately stabilizes DNA against thermal denaturation. On the other hand, in medium of pH 5.7, ambazone bound at $r_t = 0.1$ slightly destabilizes the DNA helix and only at higher concentrations of ambazone in the mixture does a small stabilization effect appear. In both cases, ambazone binding did not induce any cooperative renaturation.

The different effects of ambazone at pH 7.2 and 5.7 on the stability of the DNA double helix may be explained by assuming at least two distinct modes of binding in the DNA-ambazone interaction which depend on the charge. We suppose that the binding of neutral or singly positively charged ambazone does not disturb the secondary structure of DNA and that the moderate stabilization effect may be electrostatic in nature. The interaction of doubly positively charged ambazone is stronger and probably requires some distortion of DNA segments adjacent to the binding site, which is reflected in the observed destabilization of DNA. Only at a higher degree of saturation of the binding sites does the electrostatic stabilizing effect of doubly charged ambazone molecules become evident.

5. Interaction of ambazone with bovine serum albumin

Titration experiments were performed which started with pure solutions of ambazone at 1.2 and 2.3×10^{-5} M in phosphate buffer (pH 7.4) with 10% dimethyl sulfoxide (DMSO), to which a stock solution of bovine serum albumin (BSA) (1.2×10^{-3} M) was added [2]. The resulting parameters are $K = 1.1 \times 10^{3}$ M^{-1} with the absorption coefficient of free ambazone amounting to $\epsilon_f = 44\,440$ M^{-1} cm^{-1} and that of bound ambazone being $\epsilon_b = 38\,820$ M^{-1} cm^{-1}. This order of magnitude agrees well with data obtained in equilibrium dialysis studies. The low solubility and tendency toward decomposition for ambazone, the use of DMSO and the small difference between the ϵ_f and ϵ_b values limit the accuracy of the calculated binding constant. In any case, the binding of ambazone to BSA can be classified as being weak.

6. Antibacterial and antiviral activities of ambazone

The in vitro antibacterial activity of ambazone has previously been described by Petersen and Domagk [33]. Recently, the in vitro activity of the compound against bacterial reference strains without any primary resistance to antibacterial agents as well as against reference strains showing defined resistance mechanisms was reinvestigated [34]. The spectrum of antibacterial activity of ambazone was shown to be comparable to that of the sulfonamides. It is more active against Gram-positive cocci than against Gram-negative bacteria species. The activity of the agent is not affected by different defined mechanisms of resistance to various classes of antibacterial chemotherapeutics. Ambazone was shown to inhibit biomass production; it inhibits DNA, RNA and protein syntheses in both repair-proficient and -deficient strains of *Escherichia coli* [35]. While inhibition of protein synthesis occurred instantaneously, that of DNA and RNA syntheses was delayed. There are obviously multiple cellular targets for drug interactions with ambazone-directed, decreasing efficiency of cellular syntheses following the order protein > DNA > RNA.

Results obtained from an in vitro study with Sendai virus show that ambazone has a significantly cytotoxic effect on chicken embryo fibroblasts (CEF) and induces moderate alterations in the replication of the virus [36]. Moreover, the interaction of ambazone with membrane-directed processes of Sendai virus such as hemagglutination and neuraminidase activities was studied. Incubation of either Sendai virus or chicken erythrocytes with ambazone at concentrations ranging between 10^{-6} and 10^{-4} M resulted in a decrease in the hemagglutination titer. The inhibition of neuraminidase activity, however, amounted to only about 10% at an ambazone concentration of 5×10^{-5} M.

From these results, one may conclude that the interaction of the drug with Sendai virus NH glycoprotein responsible for neuraminidase and hemagglutination activities of the virus is relatively weak.

7. Pharmacokinetics of ambazone

Results of pharmacokinetic studies in small laboratory animals using the ^{14}C-labelled compound have been reported [37–40]. The total radioactivity (TRA) measured in serum, blood, tissues, urine, and feces was regarded as being representative for ambazone including its possible radioactive metabolites. In mice and rats, orally administered ambazone was found to be incompletely absorbed from the gastrointestinal tract, to an extent of about 35–50%. This may be explained by its physico-chemical properties: ambazone is freely soluble in water and its lipid solubility is only 4-times higher as indicated by the octanol/water partition coefficient of 4 at pH 7.4 [9]. Additionally, ambazone was shown to possess three pK values: 6.22, 7.37 and 10.67 [4]. Considering the neutral pH conditions in the small intestine, only a small fraction of the compound could be expected in the lipid-soluble unionized form.

After absorption or when administered intravenously, the major proportion of ambazone binds to blood cells as indicated by the red blood cell/plasma ratios of about 35.0 between 1 and 24

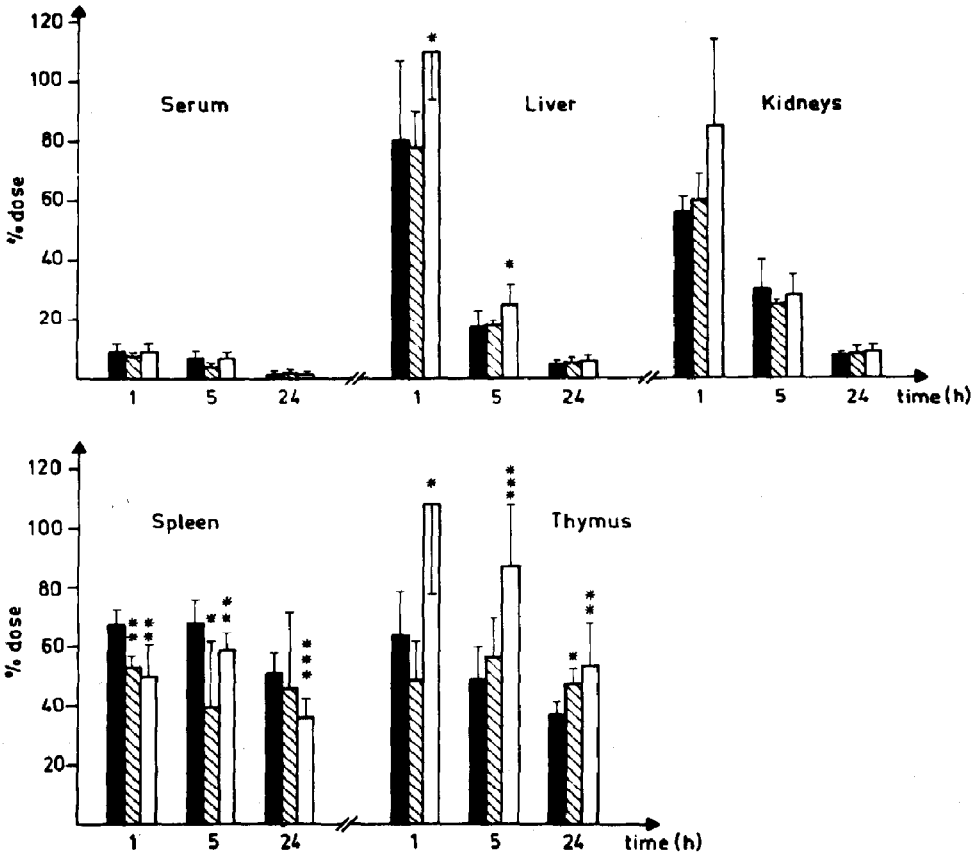

Fig. 4. Tissue distribution of total radioactivity following intravenous administration of 100 mg/kg [^{14}C]ambazone to mice. (Filled bars) Normal mice, leukemia P388-bearing mice at early (hatched bars) and advanced tumor (empty bars) stages. Means ± S.D., $n = 5–8$, significant differences vs normal mice: * $P < 0.05$; ** $= P < 0.01$; *** $P < 0.002$ (taken from Kühnel et al. [40], with permission).

h post-injection. On the basis of its physico-chemical properties, it seems likely that ambazone binds only to the membranes of erythrocytes and does not penetrate into cells. Plasma protein binding of the compound, on the other hand, is negligibly low, as indicated by the low binding affinity for BSA [2]. In contrast to its slow absorption, ambazone penetrates rapidly into tissues having a high blood supply, such as liver, kidney, and lung. Remarkably, in spleen and thymus, high and persistent TRA levels are measured (fig. 4). Higher TRA concentrations in liver, kidney, and thymus from P388 leukemia-bearing mice relative to controls could be demonstrated. The significantly lower TRA level in the spleen of tumor-bearing animals may be caused by progressive tumor cell infiltration of this organ [39,40].

After intravenous administration of ambazone, the process of decay of TRA is strongly biphasic in serum but not in blood (fig. 5). The half-life is calculated to be 6–7 h in rats and 11–15 h in mice. The greatest amount of TRA is excreted with urine, demonstrating that renal excretion is the preferential route for elimination of intravenously administered ambazone. Preliminary results in rats indicate that the compound seems to be metabolized to some extent. On the other hand, initial studies using ultraviolet-visible de-

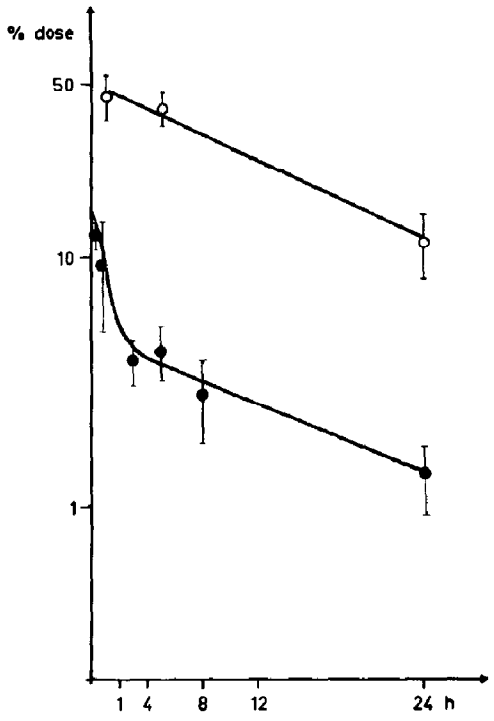

Fig. 5. Total radioactivity in blood (○) and serum (●) of mice after intravenous administration of 100 mg/kg [^{14}C]ambazone. Means ± S.D., $n = 3$ and 10, respectively (taken from Amlacher et al. [38], with permission).

termination of the parent agent demonstrate marked concentrations of unchanged ambazone in serum, tissues and urine after both intravenous and oral administration [41].

8. Pharmacodynamics of ambazone

8.1. Antineoplastic activity

The antineoplastic potential of ambazone was first reported by Petersen et al. [1] and was later characterized by others [42–46].

When administered orally, the compound was found to be active in 11 out of 14 tumor models (table 6), among them the murine leukemias L1210 and P388, the murine melanoma B16 and the Lewis lung carcinoma (LLC). Against B16 and LLC ambazone was as active as chlorambucil, and against P388 it was even more effective than the well-known drugs methyl-GAG and 5-thiouracil. The orally effective antitumor dose in mice ranged between 62.5 and 125 mg/kg given for 4–9 days. Effective doses of ambazone were capable of curing animals bearing transplantation leukemias. Interestingly, the antineoplastic effect of ambazone seems to be mediated, at least in part, by the immune system [47].

Treated animals recovering from leukemia were resistant to new leukemia challenge, and this resistance was transplantable via spleen cells to normal mice. Additionally, the antineoplastic effect of ambazone was found to be much smaller in congenitally athymic or neonatally thymectomized mice than in immunocompetent animals. These results suggest that predominantly T-lymphocytes and, possibly, immunocompetent cells of the macrophage lines are involved in the antitumor action of the compound. Comparison of young and adult mice showed that ambazone had reduced antitumor activity in 12-month-old mice [48] which are regarded as immunosenescent.

8.2. Influence on immunological functions

The effect of ambazone on various immunological parameters was studied in mice [49] in the dose range which was found to be effective in the case of murine leukemias and tumors [47]. The results are summarized in table 7. The complex tumoral and cellular responses were not inhibited or to only a slight extent by daily oral doses between 62.5 and 250 mg/kg of the compound. These findings correspond to in vitro studies in which ambazone was shown to decrease the nonspecific mitotic stimulation of cultured human lymphocytes by phytohemagglutinin [50]. It is tempting to conclude that the interaction of the drug with membrane systems, as demonstrated not only by absorption and fluorescence studies but also by investigation of the cAMP system (section 3), might be associated with its interference with immunological functions. So far, ambazone-induced physical alterations in membrane appear to be a common molecular basis for both the immunological and antineoplastic activities of

Table 6

Antineoplastic activity of ambazone in various experimental tumor models

Activity: +, strong; (+), moderate; −, none.

Tumor		Host	Drug administration route	Antineoplastic activity	References
Model	Inoculation route				
Leukemia P388	i.p.	$ABD2F_1$ mouse	i.p.	+	43, 55
	i.p.	$ABD2F_1$ mouse	p.o.	+	43, 55
	s.c.	$ABD2F_1$ mouse	p.o.	+	55
	i.v.	$ABD2F_1$ mouse	p.o.	−	55
	i.v.	$ABD2F_1$ mouse	s.c.	−	55
	i.p.	DBA/2 mouse	p.o.	+	55
	i.p.	C57Bl/6 nu/nu mouse	p.o.	(+)	47
Leukemia L1210	i.p.	$ABD2F_1$ mouse	i.p.	+	43, 55
	i.p.	$ABD2F_1$ mouse	p.o.	+	43, 55
	i.p.	$ABD2F_1$ mouse	s.c.	+	53
Lymphoma $ABDt_2$	i.p.	$ABD2F_1$ mouse	p.o.	+	46
Lymphoma $ABDt_5$	i.p.	$ABD2F_1$ mouse	p.o.	−	46
Lymphoma $ABDt_6$	i.p.	$ABD2F_1$ mouse	p.o.	−	46
Lewis-lung carcinoma	s.c.	C57Bl/6 mouse	i.p.	+	43, 55
	s.c.	C57Bl/6 mouse	p.o.	+	43, 55
Melanoma B16	i.p.	C57Bl/6 mouse	p.o.	−	45, 55
	i.p.	C57Bl/6 mouse	i.p.	+	45, 55
	i.m.	XVII Bln mouse	i.p.	+	44
Sarcoma 276 A	i.m.	XVII Bln mouse	i.p.	−	44
	i.v.	XVII Bln mouse	i.p.	(+)	44
Ehrlich-ascites carcinoma 'Bonn'	i.p.	$ABD2F_1$ mouse	p.o.	−	55
Murine mammary carcinoma Ca 755	s.c.	C57Bl/6 mouse	p.o.	+	55
Human mammary carcinoma 17035	s.c.	nu/nu mouse	i.p.	+	44
Benzpyrene-induced tumor	?	rat	?	+	55
Walker 256 carcinosarcoma	s.c.	Jelei-WIST rat	i.p.	(+)	43, 55
Melanoma A Mel 3	s.c.	hamster	p.o.	(+)	45

the cancerostatic. The poor immunodepressive side effects of ambazone may be regarded to be of particular significance, since cancer disease is associated with progressive immunodeficiency additionally aggravated by chemotherapy or radiation. The stimulation of certain specific or non-specific cellular responses such as ADCC, NK cell, cytotoxic macrophage or suppressor cell activities may be involved in the overall antineoplastic action of the compound.

8.3. Other pharmacological activities

The results of the pharmacological screening of ambazone have been reported [51]. Up to oral doses of 10^{-3} mol/kg (= 237 mg/kg) no central or autonomic actions were detected following a single-dose administration of the drug to mice and/or rats. After intravenous injection of 10^{-5} mol/kg into anesthetized rats the observed transitory alterations in blood pressure were regarded as

Table 7

Influence of ambazone at antineoplastically effective doses on several immunological functions of mice [a]

0, no reaction; ↓, inhibition; ↑, stimulation.

Complex humoral response	
Plaque-forming cell assay	0 or (↓)
Complex cellular response	
Graft-vs-host reaction	0
Allograft reaction	0 or (↓)
Specific/nonspecific cellular response	
Antibody-dependent cell-mediated cytotoxicity (ADCC)	↑
Suppressor cell activity	↑
Natural killer cell activity	↑
Cytotoxic macrophage activity	↑
Transfer of tumor immunity by spleen cells of long-time-survivors (cytotoxic T lymphocytes?)	
Human lymphocyte transformation	↓
Contact sensitivity (to picryl chloride)	↓

[a] Taken from Wisniewski et al. [49], Gutsche et al. [47] and Janke and Löber [50].

nonspecific. Using isolated guinea pig hearts, neither contraction nor coronary flow was affected by a single injection of 10^{-5}–10^{-4} M solutions. No effect on the intestinal transit time of mice was observed when the drug was given orally up to 10^{-3} mol/kg. In summary, neither the CNS functions nor cardiovascular, metabolic and gastrointestinal parameters were influenced by the compound at intravenous or intraperitoneal doses up to 10^{-5} mol/kg, and oral doses up to 10^{-3} mol/kg.

9. Toxicity of ambazone

Following oral administration, ambazone was relatively well tolerated by small laboratory rodents [52]. The single maximally tolerated dose (MTD) was shown to be about 1000 mg/kg body weight, in mice, and 750 mg/kg body weight in rats. On administration of ambazone once daily for 5 consecutive days, the MTD values fell within the ranges 125–250 mg/kg per day (mice) and 60–80 mg/kg per day (rats). In relation to the optimum antineoplastic dose in L1210- or P388-bearing mice (ED_{50} approx. 62.5–125 mg/kg body weight per os), the therapeutic index MTD/ED_{50} of ambazone is calculated to be 2–3. In two subchronic toxicity studies in rats (28 and 90 days oral treatment) 10–15 mg/kg per day was detected as the 'no-effect dose level' [53]. The gastrointestinal tract is the main target system for toxic side effects of the orally administered compound. The intestinal morphological findings such as catarrhal or hemorrhagical gastroenteritis, necrosis of enterocytes and inflammation were not identical with those found in the intestine after administration of commonly used cytostatics. Since ambazone is antimicrobially active, alterations of the intestinal bacterial flora of the animals including enterotoxin release may be involved in the toxic action of the agent on the intestine. This hypothesis is consistent with the toxic effect of ambazone on bacteria and the drug-mediated decrease in cellular macromolecule syntheses as described in section 5. As discussed above, it appears that multiple cellular targets, i.e., membranes, nucleic acids and proteins, contribute to the overall antibacterial effect. This interpretation is supported by the fact that the oral toxicity of ambazone is significantly reduced in germ-free rats [54].

The mutagenic potential of ambazone, if any, appears to be low. The results obtained in several bacterial test systems are inconsistent [56]. Using the AMES test without metabolic activation, ambazone was not mutagenic. A certain mutagenic potential was shown in DNA repair test in *Proteus mirabilis* including repair-defective mutants. However, the results cannot be regarded as representing real mutations because this test reflects single DNA strand breaks which are not heritable. Following metabolic activation, for example, via the host-mediated assay, the compound did not show any mutagenic activity. Only in the complete AMES test could a mutagenic effect be demonstrated. In summary, the obviously low mutagenic potential of ambazone is in accord with its poor affinity for binding with DNA [32]. Of course, studies in mammals are necessary to confirm the results obtained in bacterial test systems, especially since chromosome abnormalities have been reported in cultured human lymphocytes [50].

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