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MECHANISTIC ASPECTS OF THE SYNERGISTIC ANTIVIRAL EFFECT OF XANTHATES AND MONOCARBONIC ACIDS

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Abstract—The xanthate tricyclodecan-9-yl-xanthogenate (D609) displays antiviral and antitumoral properties that are inversely proportional *in vitro* to the serum concentration. Accordingly, it has been found that D609 binds to serum albumin. Recently, we have reported that D609, in combination with undecanoic acid, has a synergistic antiviral effect, which appears, as shown here, to be due to competition for the same binding domain on serum albumin. Furthermore, undecanoic acid fosters the binding of D609 to the cell. Both the competition of D609 with monocarbonic acid for binding on serum albumin and the enhanced binding of xanthate to the cell are dependent, in accordance with previously reported results, on the chain length of the fatty acids. Eleven to 14 C-atoms (undecanoic, lauric and myristic acid) were found to be appropriate while shorter (C6) and larger (C18) monocarbonic acids were shown to lack synergistic properties.

In a number of reports we have dealt with the pharmacologically relevant activities of D609. The replication of particular viruses from various genera, including herpes, papova, picorna, rhabdo and human retrolentiviruses, can be inhibited *in vitro* and under conditions that leave uninfected control cultures mitotically active [1]. Furthermore, antitumoral effects have been noticed both *in vitro* and *in vivo* [2]. An important prerequisite to render the compound D609 antivirally active *in vitro* is an acidic pH (between pH 6.5 and 7.0); an alkaline milieu was found to reduce the activity of the compound increasingly (between pH 7.2 and 7.4) [3].

In a recent publication we have shown that the addition of ionic detergents (sodium deoxycholate, sodium dodecylsulfate and fatty acids of chain lengths between 10 and 14 C-atoms), which are *per se* antivirally inactive, leads to a synergistic effect [4]. Moreover, the joint application of both components *in vivo* and *in vitro* has an overriding effect on the pH restriction. It was now possible to apply the xanthate together with the detergent, also under physiological pH conditions, without loss of activity [4]. This discovery enabled us also to utilize the antitumorally active xanthate under systemic conditions in experimental tumor-bearing animals [2]. The mechanism through which certain monocarbonic acids, e.g., undecanoic and lauric acid, interacted synergistically with D609 remained obscure. In view of the implications on potentially novel systemic antiviral (e.g., HIV; cf. Ref. 5) and antitumoral chemotherapeutic approaches, we attempted to provide experimental data that might help elucidate the mechanism that would explain the synergism.

MATERIALS AND METHODS

Cells. Rita cells (Italdiagnostics, Rome) were grown in a 5% CO_2/95% air atmosphere in Eagle's basal medium with Earle's salts (BME) and 2 × standard concentration of amino acids and vitamins, 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin.

Virus. Vesicular stomatitis virus (VSV, Indiana strain, obtained from the American Type Culture Collection).

Medium. BME 2.2 g/l $NaHCO_3$, 5% FBS, 1% penicillin and 1% streptomycin, pH 7.4, was used for the experiments. Prior to use the medium was stabilized at the desired pH value by incubation for 24 hr at 37° in a 5% CO_2/95% air atmosphere.

Compounds. The xanthate D609 was supplied by Merz, Frankfurt/Main. [^{3}H]-D609 was custom-labeled by Amersham & Buchler (sp. act.: 11.4 Ci/mmol). Hexanoic acid, undecanoic acid and lauric acid were purchased from Merck (Darmstadt), myristic acid from Serva (Heidelberg); [^{14}C]-lauric acid (sp. act.: 58 mCi/mmol) was purchased from Amersham & Buchler. The compounds were freshly solubilized in 20 mM Tris–HCl, pH 7.4. For the binding studies of xanthate on cells, the compounds were prepared as 1% stock solutions (w/v) in 80% acetone/20% H_2O.

Infection of cells. Rita cells in 6 cm petri dishes (1.3×10^6 cells/dish) were infected at a multiplicity of infection of 0.01 plaque-forming units (PFU) per cell. After 1 hr adsorption at 37° in a 5% CO_2/95% air atmosphere, cells were incubated in medium, with or without D609, containing 0%, 5% or 10% FBS. The viral progeny was harvested 24 hr after infection. The virus yield was determined by plaque assay on Rita cells in Linbro plates.

Gel filtration. We tested whether the binding of D609 on blood compounds is dependent on indole, aspirin, bilirubin or lauric acid. Bovine serum albumin (BSA) and γ-globulin (Sigma) were solubilized in 20 mM Tris–HCl, pH 7.4, as 1% solutions (w/v).

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Table 1. Effect of serum and D609 on replication of vesicular stomatitis virus

FBS (%)	Virus yield (PFU/ml) Control (untreated)	Treated* (15 μg/ml D609)	Inhibition factor
0	1.2×10^8	4.1×10^3	2.93×10^5
5	7.65×10^7	1.5×10^6	5.1×10^1
10	8×10^7	3.6×10^7	2.2

* Infected Rita cells in duplicate were treated with 15 μg/ml D609 1 hr after adsorption for 24 hr. Virus was harvested and titrated in duplicate plaque assays.

The protein solution was incubated with D609 and with appropriate compound concentrations, as indicated in the figure legends, for 1 hr at 37°. The probes were passed by Sephadex G25 columns (1 ml volume). The eluated probes were accumulated in 12 fractions, with 0.1 ml volume.

Dialysis. To study the effect of monocarbonic acids on the binding of D609 to BSA, 0.1 mg/ml (375 μM) D609, 10 mg/ml BSA and increasing concentrations of fatty acids were incubated in 20 mM Tris–HCl, pH 7.4, for 1 hr at 37°. The probes (5 ml) were dialyzed against 5 l of 20 mM Tris–HCl, pH 7.4, at 4° for 24 hr. The buffer was changed three times after 20 min and once after 4 hr. The dialysates were measured by UV adsorption.

Binding of [^{3}H]-D609 on cells. Rita cells (1.4×10^6 cells/petri dish) were incubated with medium containing 0.625 μCi/ml [^{3}H]-D609 (13.8 ng/ml), 10 μg/ml D609 and 40 μg/ml monocarbonic acid. The concentrations were chosen in accordance with those that had been used routinely in antitumoral experiments *in vitro* [6]. After various time periods, the medium was removed and cells were washed with ice-cold PBS (2×3 ml). Subsequently, the cell cultures were treated with 2×1 ml (3 min) ethanol in order to remove, as previously shown [1, 4], the xanthate from the cell. The radioactivity was measured by using a liquid scintillation counter.

RESULTS

We have noticed empirically that the antiviral activity of D609 (in the absence of ionic detergents) is inversely related to the concentration of fetal bovine serum in tissue culture medium. When studying the antiviral activity of D609 on the replication of vesicular stomatitis virus (VSV) at pH 7.4, it was found that the inhibitory effect of D609 was almost entirely lost upon elevation of the serum concentration from 5% to 10%, without altering the pH (Table 1). Still higher serum concentrations led to abolishment of the D609 antiviral activity. The same was observed when other virus systems were included in this study (herpes, simian virus 40; unpublished data). As albumin is the most frequent type of protein in serum, it was suspected that the binding of xanthate might account for loss of its antiviral activity. In fact, in addition to maintenance of the colloidal osmotic pressure, albumin serves as a transport vehicle for several compounds [7]. Substances like bilirubin, indole and certain pharmaceutical compounds, as well as fatty acids, are known to bind specifically to albumin [7], which possesses for each one of these compounds certain binding domains (Fig. 1). Some compounds such as bilirubin and lauric acid may use one and the same binding domain not only of bovine serum albumin (BSA) [7, 8] but also of human serum albumin [9].

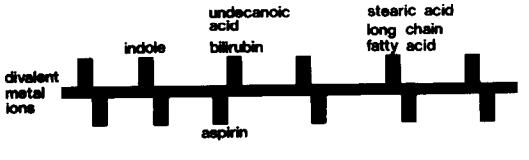

Fig. 1. Binding domains on bovine serum albumin.

In view of the data in Table 1 we wondered whether D609 might also belong to the BSA binding compounds. Therefore, BSA was incubated with D609 in buffer for 1 hr at 37°, and the reaction mixture was separated subsequently by gelfiltration chromatography. The distribution of D609 and BSA was then monitored spectrometrically at 300 nm (the maximum of absorption of D609). While free D609 appeared only at fractions 11 and 12 (Fig. 2c), the D609 that was bound to BSA was contained in fractions 3 to 6 (Fig. 2a). For comparison the elution pattern of BSA, determined at 280 nm is displayed in Fig. 2d. In order to assess the specificity of the D609 binding, BSA was replaced by the serum component γ-globulin.

The data are shown in Fig. 2b, where it can be seen that there is no association of the xanthate compound with γ-globulin. Hence, the binding appears to be specific to serum albumin.

Furthermore, the binding is reversible since acetone is capable of releasing D609 from the complex. Both D609 (Fig. 3a) and BSA (Fig. 3b) display different maxima of UV adsorption (300 nm and 280 nm, respectively) which are being maintained also after formation of a complex (Fig. 3c). Upon precipitation with acetone, D609 was liberated out of the complex (Fig. 3c).

As already mentioned, certain compounds may compete for the same binding domain on serum albumin. Therefore, increasing concentrations of D609 were reacted with indole, aspirin or bilirubin, together with constant amounts of BSA, for 1 hr at 37° in buffer. Subsequently, column chromatography was carried out and the fractions were measured spectrometrically. As Fig. 4 shows, D609 had no competitive effect on the binding of indole (data not shown) or of aspirin on BSA. In contrast, however, bilirubin and D609 competed for the binding on BSA. D609 was capable of replacing bilirubin on BSA at a concentration of 0.75 mM up to 18% (Fig. 4). Elevation of the D609 concentration to 3 mM led to replacement of bilirubin of up to 63%.

As it is known that bilirubin and lauric acid (12 C-atoms) compete for the same binding site on BSA [7, 8], the appropriate competition experiment was performed, the result of which is shown in Fig. 5. Lauric acid that was bound to BSA appeared in

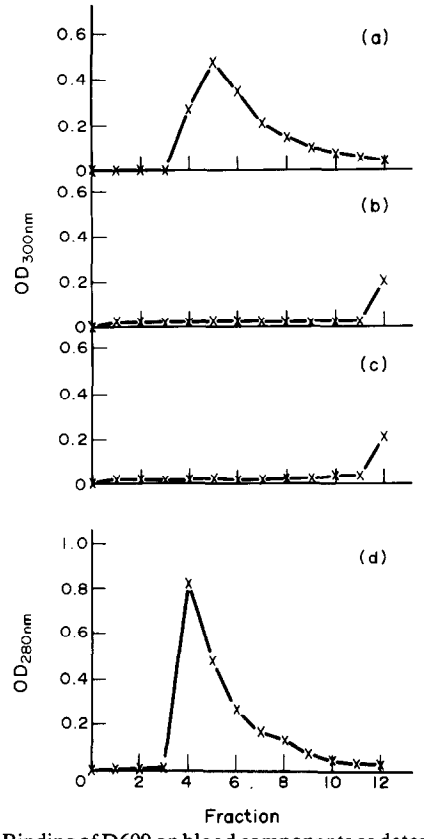

Fig. 2. Binding of D609 on blood components as determined by chromatographic analysis. D609 (0.1 mg/ml) (c) was incubated with 10 mg/ml BSA (a) or 10 mg/ml γ-globulin (b). The elution profile of BSA was determined at 280 nm (d). The probes were chromatographed on Sephadex G25 columns.

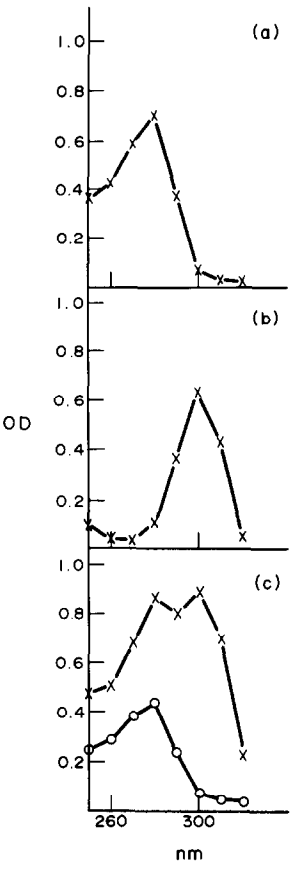

Fig. 3. The reversible binding of D609 to BSA. BSA (10 mg/ml) (a) and D609 (0.1 mg/ml) (b) were incubated, dialyzed and measured by UV-adsorption. The BSA-D609-complex, as shown in Fig. 3c (×), was treated with 3 vol acetone for 30 min at −20°. The protein pellet (O) was dissolved in buffer and monitored spectrometrically (c).

fractions 4 to 7 while free lauric acid which had been replaced by D609 was contained in fractions 10 to 12.

We have recently shown [4] that undecanoic acid (11 C-atoms) is, among various other monocarbonic acids, the most efficient adjuvant to D609 with regard to anti-herpes activity. Therefore, undecanoic acid was also employed for competition with D609 on the BSA binding domain. In addition, hexanoic acid (6 C-atoms) and stearic acid (18 C-atoms) (data not shown) were included in this set of competition experiments (Fig. 6). Neither hexanoic nor stearic acid was capable of competing as efficiently as undecanoic acid for the same binding domain with D609. Both compounds replaced D609 only to approximately 20%, while undecanoic acid competed for up to 70% with D609. These results show that bilirubin, undecanoic acid, lauric acid and D609 occupy the same binding domain on BSA.

We also studied the effect of undecanoic acid on the binding of D609 to the cell. These studies were conducted with custom labeled [^{3}H]-D609. Rita cell cultures were incubated with D609 and various monocarbonic acids in order to examine the effect of the co-incubation on the binding of radiolabeled xanthate. After incubation for increasing periods of

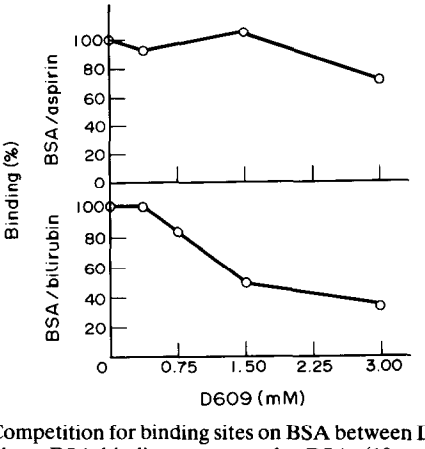

Fig. 4. Competition for binding sites on BSA between D609 and various BSA-binding compounds. BSA (10 mg/ml) was incubated either with aspirin (0.1 mg/ml) or bilirubin (0.1 mg/ml) and increasing concentrations of D609. The probes were eluted from Sephadex G25 columns and the optical density was determined (D609, O.D.$_{300}$; bilirubin, O.D.$_{420}$).

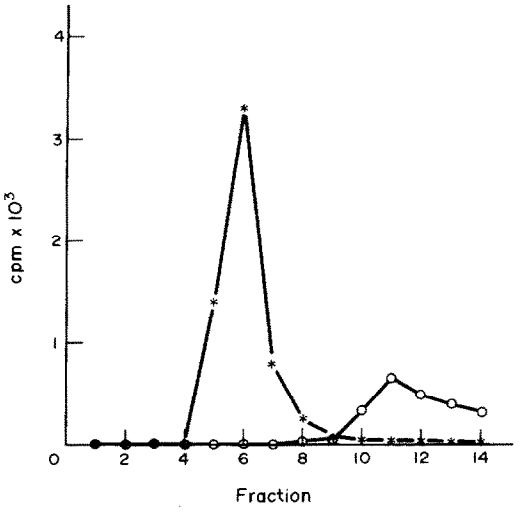

Fig. 5. Competition for binding sites on BSA between D609 and lauric acid. BSA (1 mg/ml) was incubated with 0.1 μCi (345 ng) [^{14}C]-lauric acid (*) and, in addition, with 50 mg/ml D609 (○) (187.5 mM).

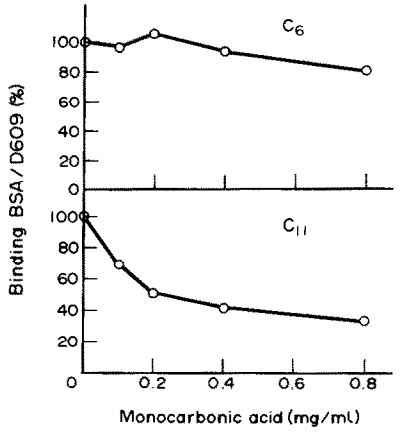

Fig. 6. Competition for binding sites on BSA between D609, hexanoic and undecanoic acids. D609 (0.1 mg/ml) and BSA (10 mg/ml) were incubated with increasing concentrations of hexanoic acid (C6) or undecanoic acid (C11). The mixtures were dialyzed and measured by UV adsorption.

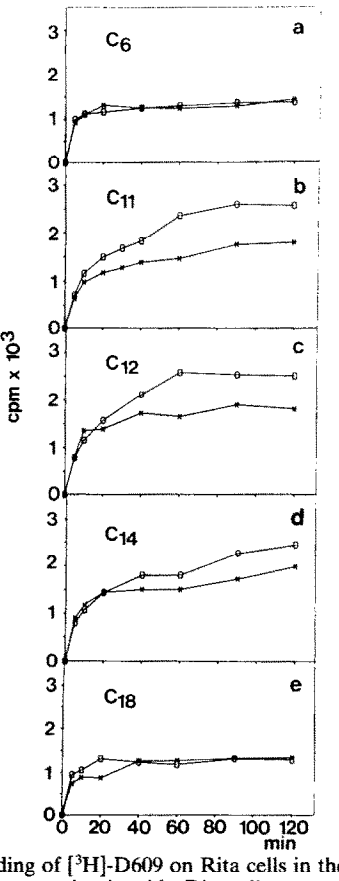

Fig. 7. Binding of [^{3}H]-D609 on Rita cells in the presence of various monocarbonic acids. Rita cells were treated with [^{3}H]-D609 (0.625 μCi/ml) and various monocarbonic acids (40 μg/ml of each): (a) hexanoic acid; (b) undecanoic acid; (c) lauric acid; (d) myristic acid; (e) stearic acid. After washing with PBS the cells were treated with ethanol and the radioactivity was determined in a liquid scintillation counter. (*): D609; (○): D609/monocarbonic acid.

time the tissue culture medium was replaced by phosphate-buffered saline to remove unbound labeled D609. The cells were treated subsequently in accordance with previous experiments [1, 4] with ethanol to remove D609 from the cell, and the radioactivity was determined in a liquid scintillation counter. Both hexanoic and stearic acid failed to display an effect on the binding of D609 (Fig. 7a, e). In contrast, undecanoic acid was capable of increasing the stable binding of D609 on the cell by up to 50% (Fig. 7b). The same effect was exerted by lauric acid (Fig. 7c). With an increasing number of C-atoms, a reduction in the binding of D609 on Rita cells was noted (Fig. 7d, e). Thus, in agreement with previously published data [4], the chain length of the monocarbonic acid turned out to be of paramount importance for the synergistic effect on D609: the longer the monocarbonic acid chain, the less the effect on D609.

DISCUSSION

In this work we attempted to provide an explanation for the synergism between the xanthate compound D609 and monocarbonic acids of chain lengths between 10 and 14 C-atoms [4]. It was found that the antiviral effect of the D609 decreased with increasing serum concentration of the tissue culture medium, owing to binding of D609 to BSA. The latter serves as a vehicle for the transport of various substances such as bilirubin to the respective excreting organs [7]. The unavailability of pharmacological compounds owing to their serum binding properties is a common problem [10]. Some of these BSA-binding compounds occupy one and the same binding domain on BSA. We were able to show that D609 and bilirubin prefer the same site, and the same effect was also found with various monocarbonic acids

between 10 and 14 C-atoms. In particular, undecanoic, lauric and myristic acid compete with D609 for binding on BSA. As a corollary thereof D609 is being displaced from the BSA complex. Additionally, the presence of these particular monocarbonic acids led to an increased binding of D609 to the cell such that up to 50% more xanthate became cell-associated (Fig. 7).

Currently the phenomenon of the enhanced binding of D609 in the presence of the appropriate monocarbonic acid is not clearly understood. Conceivably the entire BSA-D609 complex might become integrated into the cellular membrane with the aid of the monocarbonic acid. It has been established, that various proteins for example, the sarcoma virus encoded transforming protein $pp60^{src}$, can be associated with myristic acid which is responsible for the anchorage in the membrane [11, 12].

We think it more likely, however, that D609 was liberated from the transporting BSA by the competition with the appropriate monocarbonic acid. Upon its release D609 becomes available for the incorporation into the cell. As previously shown [1, 4] D609 may be integrated in the cell membrane where it exerts its antiviral and antitransforming effect [13].

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