STRUCTURAL FEATURES OF CYTOCHALASINS RESPONSIBLE FOR GRAM-POSITIVE BACTERIAL INHIBITIONS

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A study of the relative effectiveness of some eighteen natural and synthetically modified cytochalasins on the uptake of glucose by the Gram-positive bacterium *Arthrobacter sialophilus* showed that cytochalasins B, C or D and aspochalasins A, C or D were inactive natural congeners. The presence of an α,β-unsaturated carbonyl group in the macrolide moiety of these compounds with appropriate bioisosteric placement, as exemplified by cytochalasin A and aspochalasin B, are requisite molecular features. The transmembrane inhibitory index of active compounds was enhanced by increasing their lipophilicity. Thiol adducts of CA were around 20% as active in solute uptake inhibition as was the free drug. Radioactive 7-*O*-acetyl CA and its thiol adduct were each rapidly taken up by *A. sialophilus* and remained firmly bound to cellular components even after denaturant manipulations. These findings provide strong evidence for a stable association between CA and presumptive macromolecular receptors in transport and related processes.

The antagonistic effects of cytochalasins on bacteria was initially indicated by the work of BETINÁ *et al.*[1] We further demonstrated that CA* (as opposed to CB) inhibits growth, metabolism, transport, and enzyme inductions in several Gram-positive species[2]; a finding which was subsequently confirmed[3]. Whereas CB was noted to inhibit motility of the myxobacterium *Cystobacter fuscus* it did not, at 10^{-5} M, inhibit its growth[4]. Furthermore, CE at relatively high concentrations appeared to exhibit no antagonism to the growth of a large variety of representative microorganisms[5]. It has also been reported[6] that "asposterol", in fact a mixture of aspochalasins[7], shows antibiotic effects on a variety of microorganisms including *Bacillus subtilis* and the yeast *Saccharomyces pombe*.

Our initial survey[8] of the mechanism of these phenomena implicated, in terms of molecular structure, the essentiality of the conjugated α,β-keto unsaturated group in the macrolide moiety of CA. The present communication extends this observation by way of a structure-function analysis of a variety of natural and semi-synthetic cytochalasins in relation to their inhibition of glucose uptake in *Arthrobacter sialophilus*. We conclude, for activity, that effective cytochalasins must not only contain this indispensable function, but that it must be present in an appropriate regiospecific apposition to presumed macromolecular ligates. Along these lines, we also present evidence in support of the formation of a highly stable association between radioactive CA and its derivatives and several protein components of this microorganism. These findings are compared to models for cytochalasin inhibition of glucose transport in the erythrocyte[9], and to emerging structure-activity patterns for drug anti-contractility manifestations[10].

* Abbreviations used: CA, cytochalasin A; CB, cytochalasin B; CD, cytochalasin D; CE, cytochalasin E; rbc, red blood cell.

Materials and Methods

Chemicals

CB was isolated from chloroform extracts of *Helminthosporium dematoideum* and was oxidized to CA using MnO_2 as described by ZABEL *et al.*[11] Compounds **6**~**12** (see Table 1) were synthesized in this laboratory. They were purified by HPLC and met appropriate microanalytical, mass and NMR spectral criteria. Compounds **14**~**18** were kindly provided by Dr. KELLER-SCHIERLEIN[7]. Reaction of CB with phenyltrimethylammonium perbromide in tetrahydrofuran followed by MnO_2 oxidation and resolution of the reaction mixture by HPLC provided homogeneous dibromocytochalasin A (compound **5**). Its elemental analysis was consistent with the presence of two atoms of bromine; by analogy to parallel work in this laboratory on CD derivatives[12] the placement of these bromines is tentatively assigned to the 6, 12 positions. Stock solutions of cytochalasins were prepared in reagent grade dimethylsulfoxide (DMSO). A final DMSO concentration of 0.25% was adjusted in all experiments; in controls, this concentration did not affect glucose uptake. D-[^{14}C]glucose (213 mCi/mmole) and [^{3}H]acetic anhydride (104 mCi/mmole) were commercial preparations.

[^{3}H]-7-*O*-Acetylcytochalasin A

Reaction of CA with [^{3}H]acetic anhydride in pyridine, followed by repeated preparative TLC (benzene - acetone, 3: 2) on silica gel provided the homogeneous radiolabeled derivative with constant specific activity (48 mCi/mmole). Quantitative dose-response experiments showed that it had the same biological activity in inhibiting glucose uptake in *A. sialophilus* as did its non-radioactive analog. 7-*O*-Acetyl-CA was converted by reaction with 2-mercaptoethanol to its MICHAEL adduct, by an analogous procedure to that reported for CA[10].

Glucose Uptake

Growth of *A. sialophilus* (ATCC 31253) and protocols for D-[^{14}C]glucose uptake by this organism were carried out with modifications of earlier protocols[2]. In essence, the organism was inoculated into 500 ml Erlenmeyer flasks containing 50 ml of 1% (w/v) Tryptone (Bacto) and 0.5% (w/v) yeast extract (TYE). After 18 hours growth at 30°C with rotary shaking, the cells (5 ml) were diluted into 50 ml of TYE medium and were grown for an additional 3 hours at 30°C. Two ml cell aliquots (*ca.* 10^8 cells/ml) were then transferred to 50 ml Erlenmeyer flasks and 25 μg/ml CA (or its derivatives) were added. After 10 minutes, D-[^{14}C]glucose (0.20 μCi/ml) together with 2.0 mM unlabeled D-glucose was added. Culture samples were removed at 3 minutes intervals (see Fig. 2), filtered through membrane filters[13] (pore size, 0.45 μm; MF-Millipore, Millipore Corp.), washed with 10 ml of 0.15 M LiCl, dried 1 hour at 60°C, and were placed in vials containing 10 ml of scintillation fluid. For each probe, initial velocities were determined by linear regression. Percent inhibitions are reported as related to 0.25% DMSO controls.

[^{3}H]-7-*O*-Acetyl-CA Binding

Bacterial cultures were prepared as above with the alternate addition of either [^{3}H]-7-*O*-acetyl-CA or its mercaptoethanol adduct. After 16 minutes incubation, the cells were centrifuged, washed eight times with 2 ml of 0.9% NaCl containing 1% DMSO, and once again with water. The harvested cells were added to 20 ml of 4% boiling sodium dodecylsulfate, were refluxed for 25 minutes and then were kept overnight. After extraction (three times) with chloroform, the organic solvent was concentrated by reduced pressure, dried with $MgSO_4$, and its radioactivity was assayed. Residual activity in aqueous samples (0.2 ml) was determined after appropriate dilution with scintillation fluid.

Results

The relative effectiveness of some twenty cytochalasins, their synthetic modifications and related compounds, is shown in Table 1 (see Fig. 1, for representative structures). These data were extrapolated from initial velocities of glucose uptake, taken over timed intervals, for up to 15 minutes (Fig. 2). Of the natural congeneric drugs tested, only CA and aspochalasin B were active. Thus, the nature of the

Fig. 1. Structures of cytochalasins and aspochalasins used in this study.
The numbers refer to those assigned to the compounds in Table 1.

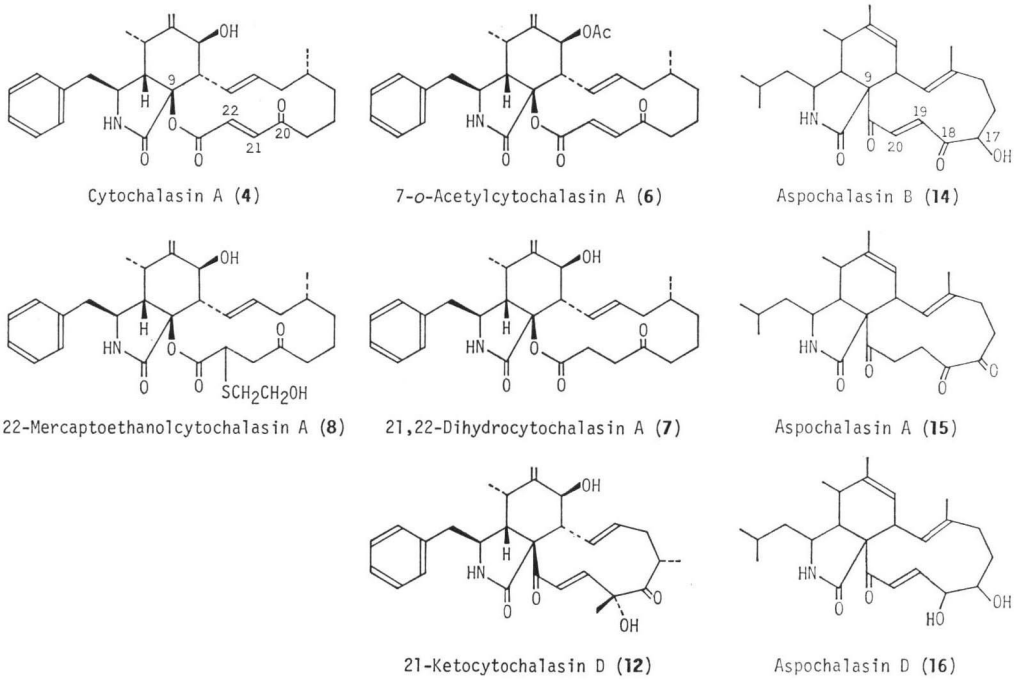


Fig. 2. Effect of CA and 7-*O*-acetyl-CA on the uptake of D-glucose in *A. sialophilus*.

Cultures were prepared as described in Materials and Methods. The cells were pre-incubated with the drug for 10 minutes before the addition of D-glucose (0.2 μCi of [^{14}C]glucose plus 2 mM unlabeled D-glucose). Samples were removed at the indicated times and radioactivity was determined as described in the text.

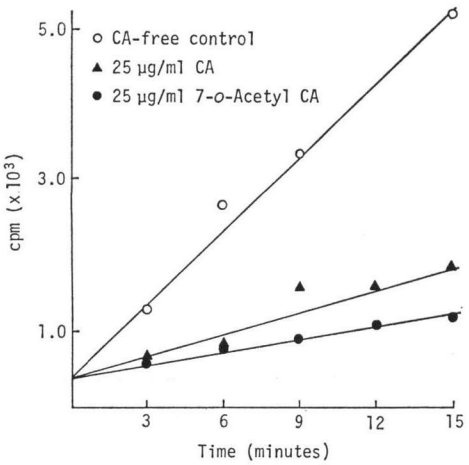


Table 1. Relative effectiveness of cytochalasins for inhibition glucose transport in *A. sialophilus*.

No.	Compound	Percent inhibition
1~3	Cytochalasins B, C, or D	0
4	Cytochalasin A	75
5	6,12-Dibromocytochalasin A	88
6	7-*O*-Acetylcytochalasin A	85
7	21,22-Dihydrocytochalasin A	0
8	22-β-Mercaptoethanolcytochalasin A adduct	21
9	22-β-Dithiothreitolcytochalasin A adduct	7
10	7-*O*-Acetyl-22-β-mercaptoethanol CA adduct	31
11	1-Methoxy-1,2-iminocytochalasin A	0
12	21-Ketocytochalasin D	0
13	7-Ketocytochalasin D	0
14	Aspochalasin B	40
15	Aspochalasin A	0
16	Aspochalasins C or D	0
17	17-*O*-Acetylaspochalasin B	70
18	19,20-Dihydro-18-hydroxyaspochalasin B	0
19	Ethacrynic acid	8

substituent at C_{10} of the cytochalasin core structure, and hence its participation in receptor binding, appears to be irrelevant. Increasing the lipophilicity of these two compounds, *i.e.*, by acetylation (compounds **6** and **17**) or by bromination (compound **5**), appeared to increase their potency. Since aspochalasin B lacks a C-7 hydroxyl group (see Fig. 1), this finding further indicates the dispensability of this particular function for inhibition of bacterial transmembrane movement. Blockage of the lactam function in CA by conversion to the 1-methoxy-1,2-imino derivative (compound **11**) completely abolished its effectiveness. Furthermore, modifications to the macrolide α,β-unsaturated carbonyl moieties of CA or of aspochalasin B by reduction (to compounds **7**, **15** and **18**, respectively) completely negated their antagonistic properties. Derivatization of CA to its 22-β-mercaptoethanol or dithiothreitol adducts (compounds **8** and **9**), while considerably lowering inhibitory potency, still allowed for 21 and 7%, respectively, of parental compound activity. The latter value corresponds, in this system, to that found for the non-specific sulfhydryl reagent, ethacrynic acid (compound **19**). Attempts to transform the inactive congener CD into a potential bacterial transport inhibitor were carried out by regiospecific modifications to provide two α,β-unsaturated carbonyl analogs. While the first of these derivatives, 7-ketocytochalasin D (compound **13**) was, as anticipated, still inactive; the second, 21-ketocytochalasin D (compound **12**), was also surprisingly devoid of activity. Aspochalasins C and D, stereoisomers, each of which contain regiospecifically displaced α,β-unsaturated carbonyl functions in their macrolide portions, were also ineffective inhibitors.

The enhancement of CA activity by acetylation provided an entree into the preparation of a bioactive, radiolabeled drug. Exposure of *A. sialophilus* to 25 μg/ml of [^{3}H]-7-*O*-acetyl-CA led to its instantaneous partitioning into the cell. A similarly rapid uptake of CB into eukaryotic cells has also been earlier reported[14]. Since the expression of drug action, as measured by inhibition of glucose uptake (Table 1) was found to be time-dependent (data not shown), an arbitrary time point of 16 minutes exposure was chosen as the baseline for the operations given in Table 2. Under these conditions, about 40% of the total administered radioactivity was sequestered by *A. sialophilus*. Even after exhaustive washing of the bacteria with medium containing 1% DMSO, a constant amount of radioactive 7-*O*-acetyl-CA or of its thiol adduct, remained affiliated with the cells (Table 2). This observation suggests a rather stable association of the drug with cellular components; a surmise further strengthened by dissolving the cells in 4% SDS followed by subsequent extraction with chloroform. Since all of the radioactive material remained in the aqueous phase (Table 2; stage 3B), it is highly likely that a covalent linkage between CA and protein receptors took place.

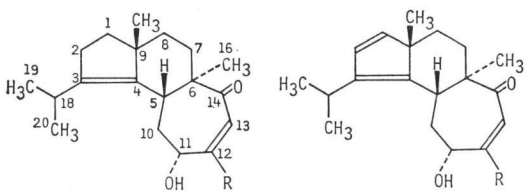
Table 2. Radioactivity distribution of [^{3}H]-7-*O*-acetyl-CA and its 22-mercaptoethanol adduct with *A. sialophilus*.

Stage and protocol	Residual total radioactivities (cpm/operational stage)	
	7-*O*-Acetyl-CA	7-*O*-Acetyl-CA-22-β-ME-adduct
1. Cells incubated with 25 μg/ml of drug (4.13×10^6 cpm, total) for 16 minutes	1.55×10^6	1.80×10^6
2. Cell pellet washed 8× with 2 ml salts medium plus 1% DMSO	1.05×10^4	0.38×10^4
3. Cells disrupted with 4% SDS: extracted 3× with 20 ml $CHCl_3$;		
(A) Aqueous layer	1.50×10^4	0.40×10^4
(B) Organic extract	0	0

Discussion

The molecular parameters for cytochalasin (or aspochalasin) inhibition of Gram-positive bacterial solute uptake would appear to include an unhindered macrolide α,β-unsaturated carbonyl function at an appropriate bioisosteric distance from the points of juncture with the 9-bridgehead carbon, and the unsubstituted lactam (C_1-N) function. This can best be envisioned by the "right-hand", as opposed to the "left-hand" carbonyl adjacent to the olefinic carbons groups at the lower faces of the molecular representations of CA, 21-keto-CD and aspochalasins B and D (Fig. 1). The ineffectiveness of the α,β-unsaturated analogs 7-ketocytochalasin D (compound **13**) and aspochalasin D (compound **16**) is consistent with the apparent requisite regiospecific placement of the enone function, as similarly reported for the congeneric, fungal diterpenoid cyathins. Here again, only one isomeric α,β-enone within each given structural pair (compounds **2** and **4**, Fig. 3) is highly active in inhibiting the growth of Gram-positive bacteria[15]. The fact that 21-keto-CD is an inactive analog is in keeping with this pattern; although it is also conceivable in this instance that the presence of the 18-methyl substituent interferes with tight liganding at complementary receptor regions. The retention of radioactivity within the water-soluble cell fraction after administration of labeled 7-*O*-acetyl-CA and denaturant solvent extraction (Table 2), supports not only the foregoing suppositions, but also provides evidence for drug function[8] as a natural affinity-type reagent. The inhibitory activity of the 7-*O*-acetyl-22-β-mercaptoethanol-CA adduct (compound **10**, Table 1) and the distribution pattern of its radioactivity into fractionated cell extracts (Table 2) are compatible with its likely participation in *retro*-MICHAEL reactions. A precedent for such a process comes from whole animal experiments[16] which established ethacrynic acid liberation from its thiol adducts. In the *A. sialophilus* hexose uptake system, it is noteworthy that this non-selective thiol reagent (compound **19**, Table 1) is of marginal inhibitory activity.

Fig. 3. Structures of active and inactive cyathins[15].



1 R=$^{15}CH_2OH$ **3** R=$^{15}CH_2OH$
2 R=CHO **4** R=CHO

In terms of cytochalasin structure-activity correlations with eukaryotes, we have already established for a model contractility system from Ehrlich ascites tumor cells that protein interactions with the α,β-unsaturated moiety of CA is not an obligatory step[10]. Further in contrast to the emerging structure-activity drug pattern for inhibitions of eukaryotic contractile processes[12], neither the presence of the C-7 hydroxyl group nor the necessity for a 10-benzyl side chain appears necessary for bacterial transport antagonism. From a structure-activity correlation for several cytochalasins as inhibitors of glucose transport in the erythrocyte based upon X-ray diffraction analyses, it has also been inferred that hydrogen bonding at the C_1-N_2 and C_{23}-O domains may be important[9]. In contrast to the findings reported here (Table 1), a C_7-O atom appears to be essential for interaction with the rbc glucose carrier. This factor, along with the transport inhibitory potencies of CB and CD[9], would preclude the drawing of any parallel between molecular susceptibility drug parameters of eukaryotic and prokaryotic transport systems. This point is even more strongly borne out by the recent demonstration by CZECH and coworkers[20] of the equivalence of the rbc membrane transporter with the CB-binding protein. That membrane components are presumed primary targets for cytochalasin action can be inferred from the increased inhibitory activity as related to increased lipophilicity of active cytochalasin congeners (compounds **6** and **17**, Table 1). This observation is in keeping with patterns earlier established for acyl derivatives of 16-membered macrolides[17], and is also convergent with the known selective permeability and enzyme derepression changes which are produced in bacteria by local anesthetics[18,19].

We have previously demonstrated that cytochalasins can simultaneously inhibit the uptake of simple hexoses, amino acids and nucleosides in Gram-positive bacteria[2]. One key question that can be addressed is how a single congener can cause such manifestations, since it is known that each process involves an array of proteins. It can be speculated that the focal cytochalasin receptors would be a class of membrane-bound proteins with which each of these primary transport proteins could, in turn, react. In mechanistic terms, evidence from many investigations[13,21] has implicated active site thiols as essential

compounds in this process. The functioning of such thiol groups on critical bacterial transmembrane movement proteins may thus be neutralized by selective electrophilic attack of reactive cytochalasins, such as CA. Attempts to identify such juncture receptors in *A. sialophilus* are currently under exploration.

Acknowledgments

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