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Reversal of Doxorubicin Resistance by Hydrophobic, But Not Hydrophilic, Forskolins

SCOTT WADLER and CHIA-PING HUANG YANG

Department of Oncology, Montefiore Medical Center, Bronx, New York 10467 (S.W.), the Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10467 (C.P.H.Y.), and the Albert Einstein Cancer Center, Bronx, New York 10461

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SUMMARY

The plant diterpene forskolin reverses acquired resistance to doxorubicin in variants of the murine sarcoma S180 cell line. Because forskolin is known to elevate intracellular cAMP levels, investigations were performed to determine whether this reversal of resistance resulted from effects on signal transduction. Two analogues of forskolin, dideoxyforskolin, which does not elevate cAMP, and a water-soluble analogue, were also investigated. Although all three diterpenes elevated levels of either cAMP or protein kinase C, these effects were not consistently associated with reversal of doxorubicin resistance. Likewise, all three diterpenes were capable of displacing [^{3}H]azidopine from P-glycoprotein, but reversal of doxorubicin resistance was observed only with forskolin and dideoxyforskolin, suggesting that binding to P-glycoprotein may be a necessary, but not sufficient, condition for reversing doxorubicin resistance. The hydrophobicity of the compounds appeared to be the single factor most consistently related to reversal of doxorubicin resistance in this cell system, with the hydrophilic compound water-soluble forskolin failing to produce this result, even at concentrations 10-fold higher than effective concentrations of the hydrophobic diterpenes.

Acquired resistance to the anthracycline antibiotic doxorubicin represents an important clinical problem. In patients with malignancies that are initially responsive to doxorubicin, tumors almost invariably recur, having become refractory not only to doxorubicin but also to multiple other unrelated compounds.

One preclinical model sharing features of acquired doxorubicin resistance has been termed MDR. This phenomenon is characterized by resistance to multiple natural product cytotoxic drugs (1–3), by impaired intracellular retention of drug (4, 5), by the presence of a 140–180-kDa plasma membrane-associated glycoprotein (P-glycoprotein) (6, 7), and by defined genetic abnormalities (8, 9). The detection of P-glycoprotein and *mdr1* mRNA in human tumor samples suggests that this model may have relevance to the clinical situation (10).

MDR is partially reversed by a variety of agents, including calcium channel blockers and calmodulin inhibitors, although the mechanism of action of these agents has not been fully established (11–13). To determine whether MDR could be reversed by agents that affect transmembrane signal transduction, the adenylate cyclase agonist forskolin (Fig. 1) and an inactive analogue, DF, were investigated in two MDR variants of the murine sarcoma S180 cell line. These plant diterpenes were capable of partially reversing MDR in these cell lines; however, the mechanism of action was obscure and did not appear to be related to their ability to affect transmembrane signal transduction (14). Because recent studies have suggested that hydrophobicity may be an important determinant of reversal of MDR (15, 16), WSF, which is also an adenylate cyclase agonist, was investigated (Fig. 1).

Materials and Methods

Cells. The S180 murine sarcoma cell line and two doxorubicin-resistant variants, A5 and A5-2.5 (17), were the generous gift of Dr. Thomas Tritton, University of Vermont School of Medicine (Burlington, VT). These cell lines were modified slightly by continuous exposure to doxorubicin (either 0.8 or 2.5 μg/ml), in RPMI 1640 with 10% horse serum (GIBCO, Grand Island, NY) in 5% CO_2.

Drugs. Forskolin, DF, and WSF [7-β-deacetyl-7-β-(γ-*N*-methylpiperazino)butyryl forskolin] were purchased from Calbiochem (La Jolla, CA). Because ethanol can reverse the adenylate cyclase agonist properties of forskolin (18), solutions of forskolin and DF were prepared in dimethyl sulfoxide and stored at −80°. WSF was dissolved in water, and stock solutions were kept at −80%. Doxorubicin was obtained from Adria Laboratories (Columbus, OH).

mRNA analysis. S180, A5, or A5-2.5 cells, in midexponential growth, were incubated with effectors for 1 hr. Total mRNA was isolated by the guanidium/cesium chloride method (19). Samples were electrophoresed on a 1.5% formaldehyde gel, transferred to nitrocellu-

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ABBREVIATIONS: MDR, multidrug resistance; WSF, Water-soluble forskolin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DF, 1,9-dideoxyforskolin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C.

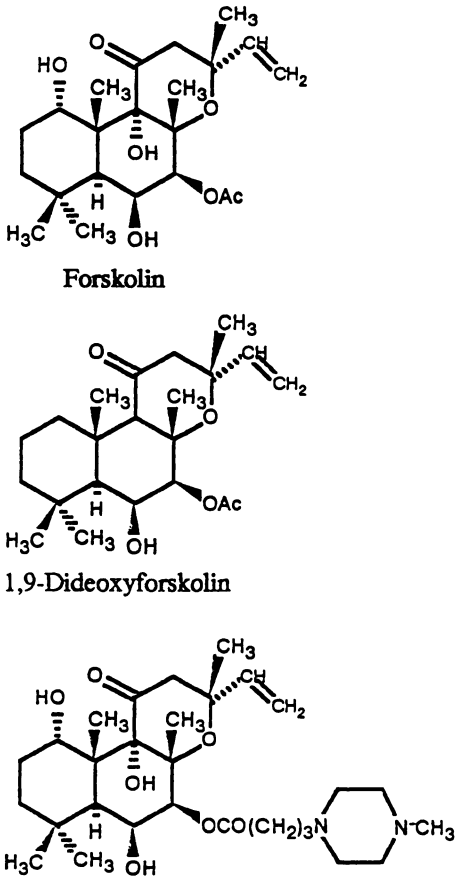

Fig. 1. Molecular structure of the plant diterpenes forskolin, DF, and WSF.

lose (Schleicher and Schuell), and hybridized with the random-primed ^{32}P-labeled pCHP1 probe (8). Blots were washed in 20% standard saline citrate at 55° and exposed to Kodak X-Omat film at −85°.

Immunoblot analysis of P-glycoprotein. Membranes were prepared from S180, A5, or A5-2.5 cells. Fifty micrograms of protein were analyzed by SDS-PAGE on 7% gels, electroblotted onto nitrocellulose filters, and probed with the R3 antibody, a specific polyclonal antibody prepared against the P-glycoprotein isolated from J7.V1-1 cells (20).

Preparation and photoaffinity labeling of microsomal membranes. Confluent cells were homogenized and microsomal membranes were prepared exactly as described (21). The pellet was resuspended (1 μg/μl) and photolabeled in 50 mM Tris·HCl, pH 7.4, containing protease inhibitors and 50 nM [^{3}H]azidopine. The mixture was preincubated for 1 hr at 25° and then irradiated for 10 min at 4° with a UV lamp (254 nm), at a distance of 8 cm (20). Photolabeled membranes were analyzed by SDS-PAGE, on a 7% gel, and detected by fluorography.

TABLE 2

Effects of plant diterpenes on PKC activity

PKC activity was measured as calcium/phosphatidylserine-dependent incorporation of [^{32}P]ATP into lysine-rich histones, by the method of Kreutter *et al.* (22). Cells in midexponential growth were incubated with effectors at 20 μM for 1 hr and then rapidly washed twice with ice-cold Dulbecco's PBS. Cells were disrupted in lysis buffer (see Materials and Methods), treated with Triton X-100, and then purified by DEAE-cellulose chromatography. The assay was performed as described in Materials and Methods. Values are the means of at least three experiments.

	cAMP level			
	Control	Forskolin	DF	WSF
	pmol/mg of protein			
S180	58.5 ± 3.9[a]	59.8 ± 3.2	82.6 ± 8.2[a]	112.7 ± 6.7[a]
A5	41.7 ± 3.0[b]	69.0 ± 13.2	99.8 ± 13.3[b]	140.6 ± 6.2[b]
A5-2.5	79.3 ± 9.7[c]	202.0 ± 8.4[c]	169.9 ± 17.8[c]	227.5 ± 11.6[c]

[a] $p < 0.0009$; [b] $p < 0.015$; [c] $p < 0.0009$; S180 vs A5 vs A5-2.5.

PKC assay. PKC activity was assayed as previously described (22). S180, A5, or A5-2.5 cells, in midexponential growth, were incubated with effectors for 1 hr, washed twice with ice-cold Dulbecco's PBS, and then sonicated for 15 × 3 sec in lysis buffer (20 mM Tris·HCl, 0.1 mM EGTA, 10% sucrose, 50 mM 2-mercaptoethanol, pH 7.5), using a Fisher Sonic Dismembrator, model 300 (Fisher Scientific Products, Pittsburgh, PA). Triton X-100 (0.3% final concentration) was added for 15 min before purification of extracts by DEAE-cellulose chromatography. Enzyme activity was measured by incorporation of [^{32}P]ATP into lysine-rich histones, in the presence of phosphatidylserine and diolein, in a standard reaction mixture (22). The final free calcium was set at 0.5 mM at pH 7.5, using a calcium-EGTA buffer system, exactly as described (22). Calcium- and phospholipid-independent activity was measured in the absence of calcium, phosphatidylserine, and diolein and in the presence of EGTA and usually represented <5% of total activity. Incubations were for 8 min at 30° and were terminated by the addition of 50 μg of bovine serum albumin and 1 ml of 20% trichloroacetic acid at 4°. Precipitated protein was collected on Millipore 0.45-μm type HA filters, washed thoroughly with 5% trichloroacetic acid, dried, and quantitated by liquid scintillation counting. All incubations were performed under conditions determined as linear for time and enzyme concentrations.

cAMP assay. S180, A5, or A5-2.5 cells, in midexponential growth, were exposed to effectors for 1 hr and extracted with acidified ethanol. cAMP levels were assayed by radioimmunoassay, by the method of Gilman (23). Protein levels were determined by the method of Lowry *et al.* (24).

Clonogenic assay. S180, A5, or A5-2.5 cells, in midexponential growth were exposed to effectors for 1 hr, washed three times with ice-cold PBS, and then cloned in soft agar as previously described (25).

Results and Discussion

As shown in Table 1, basal levels of cAMP did not differ between wild-type and resistant cells. Incubation of cells with forskolin (20 μM) for 1 hr resulted in a 5–20-fold increase in

TABLE 1

Effects of plant diterpenes on cAMP levels in S180 cells and resistant variants

Cells in exponential growth were adjusted to 2 × 10^6/ml in RPMI 1640 and then incubated, with or without effectors at the indicated concentrations, for 1 hr at 37°. Cells were washed with ice-cold PBS and then extracted with acidified ethanol. Levels of cAMP were determined by radioimmunoassay (23). Protein content was determined by the method of Lowry *et al.* (24). Values are the mean of three experiments.

	Control	Forskolin (20 μM)	DF (20 μM)	WSF		
				20 μM	200 μM	1 mM
	pmol/mg of protein					
S180	2.89 ± 0.56	15.83 ± 1.13	2.89 ± 0.34	4.43 ± 1.29	22.41 ± 0.0	47.05 ± 1.68
A5	3.30 ± 1.58	61.28 ± 12.72	6.00 ± 0.48	7.33 ± 1.35	67.34 ± 2.35	111.34 ± 3.30
A5-2.5	1.73 ± 0.29	35.19 ± 12.13	<1.00	3.87 ± 0.80	9.15 ± 1.58	23.95 ± 8.73

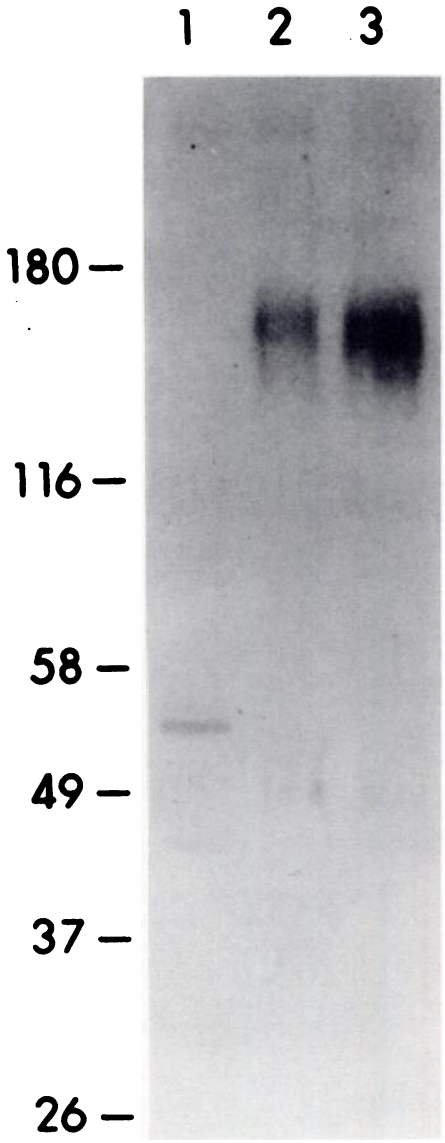

Fig. 2. Doxorubicin-resistant cell lines express proportionally higher levels of P-glycoprotein. A Western blot of membranes from S180 (*lane 1*), A5 (*lane 2*), or A5-2.5 (*lane 3*) cells demonstrates the presence of a 170-kDa membrane protein that binds an antibody specific for P-glycoprotein. Levels of resistance in A5 and A5-2.5 cells are 100-fold and >500-fold, respectively, compared with the wild-type (S180) cell line.

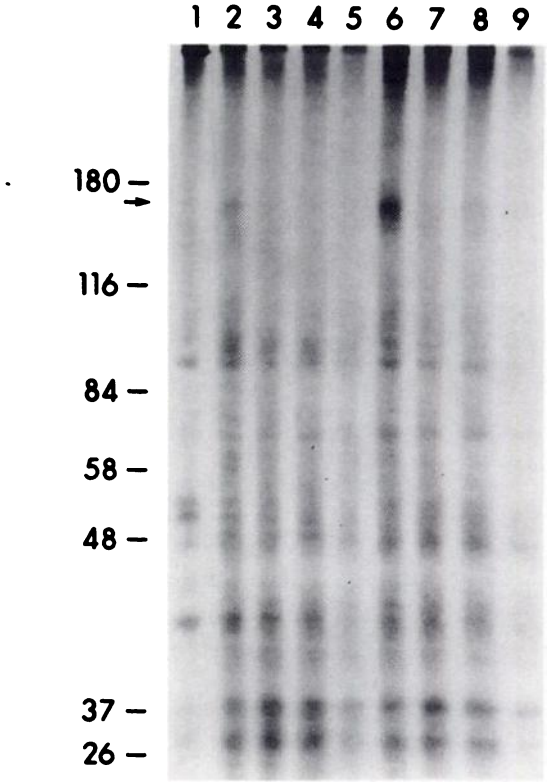

Fig. 3. Effect of diterpenes on [^{3}H]azidopine photoaffinity labeling of membrane proteins from wild-type and resistant cell lines. Cells were incubated in the absence (*lanes 1*, *2*, and *6*) or presence of forskolin (*lanes 3* and *7*), DF (*lanes 4* and *8*), or WSF (*lanes 5* and *9*) (all 50 μM). [^{3}H]Azidopine photoaffinity-labeled membrane proteins (25 μg) from S180 (*lane 1*), A5 (*lanes 2–5*), or A5-2.5 cells (*lanes 6–9*) were prepared as described in Materials and Methods. Photolabeled membranes were analyzed by SDS-PAGE on a 7% gel. Equal amounts of protein were added to each lane; equal loading was confirmed by staining blots with Coommassie Blue and comparing the intensity of the bands. Resistant variants expressed a 170-kDa protein that binds the radiolabeled compound (*arrow*).

intracellular levels of cAMP. Comparable effects were observed with WSF, but only at 10–50-fold higher concentrations of drug. In contrast, incubation of cells with DF (20 μM) resulted in no increase in intracellular cAMP levels. As shown in Table 2, basal levels of PKC activity did not differ significantly between the wild-type and resistant cell lines. Incubation of cells with either DF or WSF (20 μM) for 1 hr resulted in augmentation of PKC activity by 1.4–3.4-fold in all three cell lines. In contrast, incubation with forskolin (20 μM) resulted in increased PKC activity only in the A5-2.5 cells.

Cells made progressively more resistant to doxorubicin expressed proportionally higher levels of a high molecular weight protein consistent with P-glycoprotein, as detected by a polyclonal antibody, R-3, made against P-glycoprotein isolated from J7.V1-1 cells (20) (Fig. 2). This membrane protein could be photoaffinity labeled with [^{3}H]azidopine under the conditions described (Fig. 3). Treatment of A5 or A5-2.5 cells with either of the plant diterpenes (20 μM) inhibited binding of [^{3}H]azidopine to P-glycoprotein. Although these effects were specific to P-glycoprotein from cells treated with forskolin or DF, for cells treated with WSF there was a general inhibition of azidopine binding to many membrane proteins. As shown in Fig. 4, the resistant cell lines, but not the wild-type cells, expressed the *mdr* gene product. Levels of *mdr*-specific mRNA were not affected by treatment of cells with forskolin, DF, or WSF (20 μM) for 1 hr.

Doxorubicin resistance in A5 and A5-2.5 cells was partially reversed in the presence of forskolin or DF (20 μM) (14). To determine whether the hydrophilic analogue WSF was capable of reversing resistance, a clonogenic assay was performed using two concentrations of WSF. No effects of WSF on cell survival were observed (Fig. 5).

Forskolin is a derivative of the labdane family of plant diterpenes, closely related to the phorbol esters (26). In addition to its function as a generalized activator of adenylate cyclase (27), forskolin also has direct anesthetic properties on cell membranes (28), inhibits erythrocyte hexose transport (29), and alters voltage-dependent potassium channels in a clonal pheochromocytoma cell line (30). To distinguish effects of forskolin related to stimulation of adenylate cyclase, we used an inactive analogue of forskolin, DF, which was also capable of reversing doxorubicin resistance in the S180 variants at concentrations identical to those used with forskolin. With the

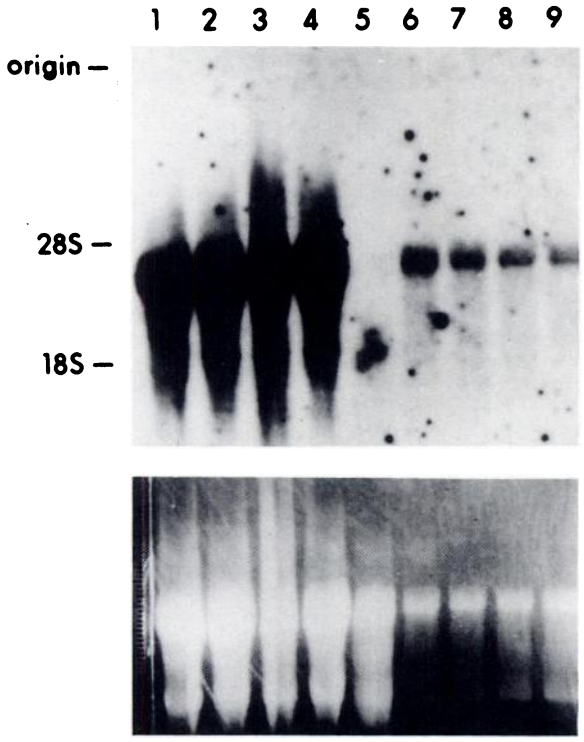

Fig. 4. Absence of effect of plant diterpenes on expression of *mdr*. S180 (*lane 5*), A5 (*lanes 1–4*), or A5-2.5 (*lanes 6–9*) cells were incubated with forskolin (*lanes 2* and *7*), DF (*lanes 3* and *8*), or WSF (*lanes 4* and *9*) (20 μM) for 1 hr. *Lanes 1, 5,* and *6*, untreated controls. Total mRNA was extracted from cells in the presence of guanidium isothiocyanate. Twenty micrograms (*lanes 1–5*) or 7 μg (*lanes 6–9*) were loaded and fractionated on formaldehyde/agarose gels. The mRNA was transferred to nitrocellulose and hybridized with the random-primed, ^{32}P-labeled, pCHP1 probe. The *mdr1* gene product is not detected in wild-type cells. *Bottom*, ethidium bromide-stained gel under UV light.

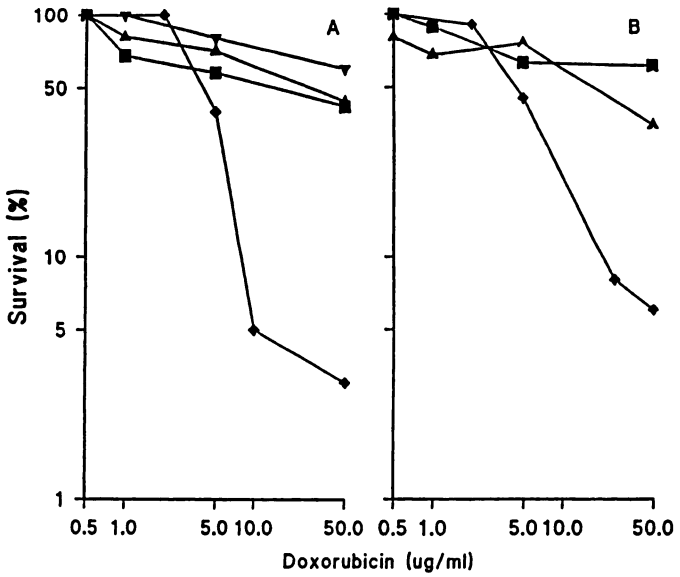

Fig. 5. Cell survival of A5 (A) or A5-2.5 (B) cells incubated with doxorubicin in the absence (■) or presence of WSF (20 μM) (▼) or WSF (200 μM) (▲). Incubation with WSF resulted in no reversal of doxorubicin resistance. In contrast, treatment with forskolin (20 μM) (♦) partially reversed resistance in both A5 and A5-2.5 cells. The effect of DF on reversal of doxorubicin resistance has been described (14).

exception of the absence of stimulation of adenylate cyclase, the effects of DF were nearly indistinguishable from those of forskolin, including displacement of [^{3}H]azidopine from P-glycoprotein, stimulation of PKC activity, and failure to inhibit *mdr* expression. This strongly suggests that stimulation of adenylate cyclase is neither necessary nor sufficient for reversal of doxorubicin resistance.

MDR is reversible by a variety of agents; one of the more important families of compounds is the indole alkaloids, lipid-soluble organic bases with planar aromatic rings and a basic nitrogen atom (15). Because the hydrophobicity of these compounds was postulated to be central to their action as ligands for P-glycoprotein, a hydrophilic analogue of forskolin, WSF, was investigated for its ability to reverse doxorubicin resistance. WSF, a partial agonist of adenylate cyclase, shared other properties with forskolin, including stimulation of PKC activity, failure to inhibit levels of *mdr*-specific mRNA, and displacement of [^{3}H]azidopine from P-glycoprotein, although, unlike forskolin, the displacement was not specific to P-glycoprotein, as observed in Fig. 3. Furthermore, unlike forskolin, WSF failed to reverse doxorubicin resistance in A5 or A5-2.5 cells, even at 10-fold higher concentrations than used with forskolin. Thus, although stimulation of PKC activity and binding to P-glycoprotein may be important factors in reversal of doxorubicin resistance, neither is sufficient for this phenomenon. In contrast, the failure of the hydrophilic analogue WSF to reverse doxorubicin resistance supports the view that hydrophobicity is critical.

Although P-glycoprotein has been postulated to act as an energy-dependent membrane efflux pump, many questions remain about the role of this molecule, including the mechanism by which reversal of its action occurs. MDR cells have been noted to have altered membrane transport systems, including transport of both ions (31) and sugars (32), either of which may be affected by forskolin and its analogues. Our investigations suggest that reversal of doxorubicin resistance by plant diterpenes is critically associated with the hydrophobicity of the compounds, but not their ability to alter signal transduction. Other actions of these compounds should be investigated as a mechanism for reversal of doxorubicin resistance.

Note Added in Proof

A recent paper (Morris *et al. Biochemistry* **30**:8371–8379 (1991)) reported on the interaction of forskolin with P-glycoprotein.

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Send reprint requests to: Scott Wadler, Department of Oncology, Montefiore Medical Center, 111 E. 210th Street, Bronx, NY 10467-2490.