An indazole derivative, YC-1, was identified in this study to be capable of reversibly and effectively inhibiting proliferation of rat A10 vascular smooth-muscle cells (VSMCs) in vitro. YC-1 (1–100 μM) dose-dependently inhibited [3H]thymidine incorporation into DNA in rat A10 VSMCs that were synchronized by serum depletion and then restimulated by addition of 10% foetal calf serum (FCS), whereas FCS-induced [3H]thymidine incorporation into rat synchronized endothelial cells was unaffected by this agent. The dose of YC-1 required to cause inhibition of FCS-induced proliferation was similar to that necessary for the formation of cellular cyclic GMP (cGMP). Guanylate cyclase activity in soluble fractions of VSMCs was activated by YC-1 (1–100 μM), whereas cGMP-specific phosphodiesterase activity was unaffected by this compound. The anti-proliferative effect of YC-1 was mimicked by 8-bromo-cGMP, a membrane-permeable cGMP analogue, and was antagonized by KT 5823 (0.2 μM), a selective inhibitor of protein kinase G. The anti-proliferative effect of YC-1 was also antagonized by Methylene Blue (50 μM), a guanylate cyclase inhibitor, and was potentiated by 3-isobutyl-1-methylxanthine (500 μM), a phosphodiesterase inhibitor. These results verified that YC-1 is a direct soluble guanylate cyclase activator in A10 VSMCs, and the anti-proliferative effect of YC-1 is mediated by cGMP. YC-1 still inhibited FCS-induced DNA synthesis even when added 10–18 h after restimulation of the serum-deprived A10 VSMCs with 10% FCS. Flow cytometry in synchronized populations revealed an acute blockage of FCS-inducible cell-cycle progression at a point in the G1/S-phase in YC-1 (100 μM)-treated cells. The inhibition of proliferation by YC-1 was demonstrated to be independent of cell damage, as documented by several criteria of cell viability. In conclusion, YC-1 reversibly and effectively inhibited the proliferation of VSMCs, suggesting that it has potential as a therapeutic agent in the prevention of vascular diseases.

INTRODUCTION

Proliferation of vascular smooth-muscle cells (VSMCs) is a prominent feature of vascular response to mechanical injury [1,2]. Abnormal proliferation of VSMCs is a major component of vascular disease, including atherosclerosis, vascular rejection and restenosis after angioplasty [3,4]. Therefore modulation of proliferation has critical therapeutic implications for vascular disease. An important therapeutic aim is inhibition of VSMC proliferation without interfering with endothelial or other cell proliferation. Theoretically, a selective inhibitor of VSMC proliferation is therefore required [5]. Several agents that suppress VSMC proliferation have already been identified: prostaglandins E1, E2 [6,7], heparin [8], interferon α [9], β-transforming growth factor [10] and protein kinase C activator [11]. However, no significant inhibition of atherosclerosis or restenosis has been achieved with the above agents [5].

Much of the work involving inhibitors of VSMC proliferation has been carried out on VSMCs in culture. This technique is ideally suited to the study of action mechanisms of inhibitors and the cell cycle. A10 cells, isolated and characterized by Kimes and Brandt [12] from rat aorta, have many of the properties of smooth-muscles cells in culture. These cells have some advantages over primary cultured cells, i.e. flexibility in handling and morphological and biochemical stability after multiple passages [13]. In our studies, the characteristics of stimulation of DNA synthesis by mitogens and the proliferative properties of serum remained stable from passage 16 through to passage 32.

It has recently been discovered that NO and nitrovasodilators have an inhibitory effect on the proliferation of VSMCs [14,15] by a cyclic GMP (cGMP)-mediated mechanism. In a large-scale screening test, we found that 1-benzyl-3-(5'-hydroxyethyl-2'- furyl)indazole (YC-1), an indazole derivative (Figure 1), exerted vasorelaxing effects in rat aorta as well as anti-aggregatory effects in platelets, both of which protect the vascular wall against arteriosclerotic changes. The vasorelaxation and anti-aggregatory effects of YC-1 were mediated by a cGMP-controlled pathway [16]. In this study, YC-1 was found to inhibit proliferation of rat A10 VSMCs; an attempt was therefore made to characterize its modes of action. This is the first report to demonstrate that YC-1 potentially inhibits proliferation of VSMCs by activating soluble guanylate cyclase.

![Figure 1 Chemical structure of YC-1](image-url)
MATERIALS AND METHODS

Materials

YC-1 (Figure 1) was chemically synthesized as described previously [17]. Dulbecco’s modified Eagle’s medium (DMEM), foetal calf serum (FCS) and all other tissue culture reagents were obtained from Grand Island Biological Co. (Gibco-BRL). [3H]Thymidine, [3H]-cGMP, [α-32P]GTP and cGMP e.l.i.s.a. kit were from Amersham (Bucks., U.K.). KT 5823 was obtained from Biomol. Protein dye reagents were purchased from Bio-Rad. Sodium mitoprazide (SNP), 3-isobutyl-1-methylxanthine (IBMX), 8-bromo-cGMP (8-Br-cGMP), Trypan Blue, Methylene Blue and all other chemical reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell cultures

Rat A10 VSMCs were obtained from the American Type Culture Collection (ATCC). A10 cells were grown in DMEM containing 10% FCS, 100 units/ml penicillin G and 100 μg/ml streptomycin sulphate at 37°C in a humidified 5% CO₂ in air atmosphere. Rat aortic endothelial cells were isolated from the thoracic portion by digestion in collagenase in serum-free M199 medium for 7 min at 37°C [18]. The cells were collected by centrifugation and the pellet was resuspended in M199 containing 10% FCS.

3HThymidine incorporation

Cells were finally grown in 24-well plates (2.5 × 10⁴ cells/well), washed with Krebs–Henseleit solution (KHS) (117.5 mM NaCl, 5.6 mM KCl, 1.18 mM MgSO₄, 1.2 mM NaH₂PO₄, 25.0 mM NaHCO₃, 5.5 mM glucose, 25.0 mM Heps and 2.5 mM CaCl₂), then incubated in KHS containing 2% FCS for 48 h to induce quiescence at 37°C. To investigate the effect of YC-1 on proliferation and cell cycle, quiescent cells were cultured for 20 h in medium supplemented with or without 10% FCS, in the presence or absence of experimental agents. Finally, cells were incubated for 2 h in freshly prepared media that were additionally supplemented with [3H]thymidine (1 μCi/ml) to measure DNA synthesis by thymidine incorporation [19,20]. The experiments were terminated by washing cells with KHS, precipitation of acid-insoluble material with 10% trichloroacetic acid and extraction of DNA with 0.1 M NaOH. The precipitates were collected on Whatman GF/B filters, which were cut up and counted in a scintillation counter.

Measurement of cGMP

cGMP concentrations in A10 VSMCs were assayed as previously described by Kariya et al. [21]. At confluence, monolayer cells were incubated with YC-1 in the presence of 0.1 mM IBMX for 10 min. Incubation was terminated by the addition of 0.1 M HCl. After four extractions with ether, the supernatant was assayed by using a cGMP e.l.i.s.a. kit (Amersham).

Determination of soluble guanylate cyclase activity

A10 cells were resuspended in ice-cold 50 mM Tris/HCl buffer (pH 7.4) and sonicated at 4°C for 10 s bursts (Sonicator W-220F). Homogenates were centrifuged at 40000 g for 10 min and the resulting supernatant fraction was used for guanylate cyclase determination [22,23]. The incubation mixture (100 μl total volume) contained 0.2 mM GTP, 1 × 10⁶ c.p.m. of [α-32P]GTP, 5 mM MgCl₂, 2.5 mM cGMP, 10 mM dithiothreitol, 15 mM creatine phosphate, 0.2 mg/ml creatine kinase, with or without YC-1 in a 50 mM Tris/HCl buffer, pH 7.4. Incubations were carried out at 37°C for 10 min with 50 μl (1 mg/ml) of enzyme preparation, and terminated by adding 0.5 M HCl and 1 mM imidazole. The tubes were then heated to 100°C for 3 min in an electric heating block and cooled in an ice-bath. The supernatant was applied to a neutral alumina column to isolate cGMP for radioassay of 32P and 3H. Protein was determined by using Bradford reagent (Bio-Rad) [24].

Determination of cGMP-specific phosphodiesterase activity

The supernatant fraction of A10 VSMC lysate was used for cGMP-specific phosphodiesterase determination [25,26]. The crude cytosolic enzyme (8.4 mg/ml) was incubated with cGMP (10 μM, containing 0.1 μCi of [3H]-cGMP) and compound in a final volume of 0.4 ml (buffer was 50 mM Tris/HCl, 5 mM MgCl₂, pH 7.4). After 30 min at 25°C, the samples were heated to 100°C for 1 min before cooling. Snake (Ophiophagus hannah) venom (0.1 ml; 1 mg/ml) was then added for 30 min to convert the 5’-GMP into the uncharged nucleoside, guanosine. Anion-exchange resin (Dowex 1; 1 ml) was added to bind all of the unconverted cGMP. After centrifugation, an aliquot (0.5 ml) of the supernatant was removed for quantification in a liquid-scintillation counter.

Cell-cycle analysis

To estimate the proportions of cells in different phases of the cell cycle, cellular DNA contents were measured by flow cytometry as described by March et al. [27]. Briefly, cells (2 × 10⁶ cells/ml) were fixed in 70% ethanol (in PBS) in ice for 30 min and then resuspended in PBS containing 40 μg/ml propidium iodide and 0.1 mg/ml RNAase (Boehringer, Mannheim, Germany). After 30 min at 37°C, 2 × 10⁶ cells were analysed on a FACstar cytometer (Becton-Dickinson, San Jose, CA, U.S.A.) exciting at 488 nm and sensing at 585 nm.

Cell growth

To determine the effect of YC-1 on cell growth, cells (2.5 × 10⁶/well) were cultured for 48 h in FCS-free medium to induce quiescence. They were then cultured for 6 days in medium supplemented with 10% FCS, in the presence or absence of YC-1. Culture medium and YC-1 were changed daily and cell numbers were determined by dissociation of adherent cells with trypsin and counting in a haemocytometer.

Data analysis

Results are expressed as means±S.E.M. A one-way analysis of variance was used for multiple comparison, and, if there was significant variation between treatment groups, the mean values for inhibitors were compared with those for controls by using Student’s t test; P values of less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Effects of YC-1 on mitogen-induced [3H]thymidine incorporation

Initial studies were designed to characterize the ability of mitogens to stimulate incorporation of [3H]thymidine into DNA as an indicator of proliferation [28,29]. YC-1 (1–100 μM) dose-dependently inhibited [3H]thymidine incorporation into DNA in rat A10 VSMCs that were synchronized by 48 h serum depletion and then stimulated by addition of 10% FCS, with IC₅₀ values of 10.5 ± 2.9 μM (Figure 2). The inhibitory effect of YC-1 was
Table 2 Effects of IBMX and YC-1 on VSMC cGMP-specific phosphodiesterase activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conc. (µM)</th>
<th>Phosphodiesterase activity (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (basal)</td>
<td>10655 ± 599</td>
<td></td>
</tr>
<tr>
<td>IBMX</td>
<td>100</td>
<td>8933 ± 413*</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>7888 ± 299**</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>5000 ± 261**</td>
</tr>
<tr>
<td>YC-1</td>
<td>30</td>
<td>10196 ± 188</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10385 ± 117</td>
</tr>
</tbody>
</table>

Fully reversible, and [H]thymidine incorporation recovered within 24 h of the removal of 100 µM YC-1 (results not shown). YC-1 also caused inhibition of platelet-derived growth factor/BB (10 ng/ml)-, 5-hydroxytryptamine (10 µM)- and ADP (10 µM)-stimulated DNA synthesis in A10 VSMCs with similar IC₅₀ values: 14.6 ± 4.7, 16.4 ± 3.7 and 18.1 ± 4.0 µM respectively. These results demonstrate that YC-1, an indazole derivative, reversibly and effectively inhibits proliferation of rat A10 VSMCs, which is a prominent feature of vascular response to mechanical injury.

YC-1 (1–100 µM) did not inhibit [H]thymidine incorporation in FCS-stimulated rat aortic endothelial cells (Figure 2). However, high concentrations of YC-1 (300 µM) did inhibit [H]thymidine incorporation in FCS-stimulated endothelial cells by 23 ± 4%. SNP (1 mM) did inhibit [H]thymidine incorporation in FCS-stimulated endothelial cells by 26 ± 5%. It has been postulated that one of the functions of endothelial cells is to maintain the mitogenic quiescence of the underlying medial VSMCs [15,32]. A major therapeutic aim is to inhibit VSMC proliferation without interfering with endothelial-cell proliferation [30,31]. YC-1 (1–100 µM), which selectively inhibits A10 VSMC proliferation without affecting endothelial-cell proliferation, might therefore be expected to prevent or inhibit the progress of vascular diseases. Yang et al. [33] have reported that donors of NO inhibit the proliferation of cultures of bovine foetal aortic, human umbilical-vein and mouse lymph-node- vessel endothelial cells, but Ziche et al. [34] reported that exogenous NO enhances the proliferation of this cell type cultured from bovine coronary post-capillary venules. The exact reason for the discrepancy between these results is unclear, but it may be due to differences in the cell lines and experimental conditions used.

Table 1 Stimulation of guanylate cyclase activity in the soluble fraction of VSMCs by YC-1

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conc. (µM)</th>
<th>Guanylate cyclase activity (pmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (basal)</td>
<td></td>
<td>26.5 ± 7.3</td>
</tr>
<tr>
<td>SNP</td>
<td>10</td>
<td>50.5 ± 6.9*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>70.8 ± 8.6**</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>94.0 ± 7.6***</td>
</tr>
<tr>
<td>YC-1</td>
<td>1</td>
<td>33.0 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>47.6 ± 5.1*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>61.4 ± 7.6**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>77.3 ± 6.9**</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>92.9 ± 8.8***</td>
</tr>
</tbody>
</table>

The dose of YC-1 required to inhibit FCS-induced proliferation was similar to that necessary for cGMP formation (Figure 3). The site of action of YC-1-induced cGMP formation was clarified by determining the activities of guanylate cyclase and cGMP-specific phosphodiesterase. As shown in Table 1, dose-dependent increases in soluble guanylate cyclase activity were observed in YC-1 and SNP-treated VSMCs. cGMP-specific phosphodiesterase activity was inhibited by IBMX, whereas YC-1 (100 µM) had no effect on this enzyme activity in A10 VSMCs (Table 2). These results demonstrate that YC-1 is an activator of soluble guanylate cyclase in VSMCs.
Effects of 8-Br-cGMP, KT 5823, Methylene Blue and IBMX on 
YC-1-induced anti-proliferation

The anti-proliferative effect of YC-1 was mimicked by 8-Br-cGMP (10–1000 μM), a membrane-permeable cGMP analogue, as shown in Figure 4(a) and antagonized by KT 5823 (0.2 μM), a selective inhibitor of protein kinase G [35] (Figure 4b), which by itself did not affect cell proliferation. Methylene Blue (50 μM), a guanylate cyclase inhibitor [36], by itself had only a slight effect on FCS-induced proliferation. However, as indicated in Figure 4(b), the dose–response curve for YC-1-induced inhibition of proliferation was shifted to the right, indicating antagonism of the anti-proliferative activity of YC-1 by Methylene Blue. IBMX (500 μM), a non-selective cyclic nucleotide phosphodiesterase inhibitor, alone caused a 5% decrease in FCS-stimulated thymidine incorporation; however, as shown in Figure 4(b) it caused significant potentiation of the anti-proliferative activity of YC-1, particularly at low concentrations of the latter. These results verify that guanylate cyclase was the effector of YC-1, and the anti-proliferative effect of YC-1 was mediated by cGMP. cGMP has been reported to inhibit the proliferation of VSMCs [32,37], but the molecular mechanisms of this anti-proliferative action remain to be clarified. Inhibition of proliferation of cGMP-elevating agents (e.g. SNP and 3-morpholinosydnonimine) at concentrations higher than those required for vasorelaxation [15,38,39] was also observed in this study. Reasons for this discrepancy may include instability of these agents [40] and binding to serum [41]. However, inhibition of proliferation by YC-1 at concentrations of 1–100 μM was similar to that causing vasorelaxation in the rat aorta. This phenomenon demonstrates that YC-1 is fairly stable over a 24 h culture period and has very weak plasma-protein-binding properties.

Effects of YC-1 on the cell cycle in synchronized populations

To ensure that the cells were capable of synchronously re-entering the cell cycle after 48 h of serum starvation, cellular stimulation of [3H]thymidine incorporation was determined (Figure 5). DNA synthesis increased slowly, reached its maximum within 16 h, and declined soon after. The G1-phase can be confined to the first 8 h, the S-phase between 10 and 18 h, and the G2/M-phase between 20 and 26 h. To clarify the mode of anti-proliferative action of YC-1, the relationship between the time of addition of YC-1 and its inhibitory action on FCS-induced DNA synthesis was examined. As shown in Figure 5, YC-1 still inhibited FCS-induced DNA synthesis even when added 10–18 h after the addition of FCS. This result implies that YC-1 inhibits FCS-induced proliferation by inhibiting progression from the G1- to the S-phase of the cell cycle. Furthermore, the present flow-cytometric results demonstrate that YC-1 inhibits progression from the G1/S-phase of the cell cycle. After a 48 h exposure to serum-free medium, most VSMCs remained in the G1-phase of the cell cycle (Figure 6a). After replacement of serum-free medium with DMEM containing 10% FCS, the emergence of cells into and through the S-phase was observed by flow cytometry using quantitative DNA staining with propidium iodide (Figure 6b). The majority of cycling cells were found to progress through the S-phase approximately 18 h after serum repletion (Figure 6b). YC-1-treated cells showed an acute blockage of cell cycle progression occurring at the G1/S-phase; no increase in DNA content was observed in cells stimulated by serum in the presence of YC-1 (100 μM) (Figure 6c). This result is consistent with cGMP-elevating vasodilators exerting their anti-proliferative action by inhibiting progression from the G1- to the S-phase of the cell cycle [15,21,32]. Our results are also consistent with previous work [7,11,32] showing that elevated cGMP concentrations at a latter stage of the G1-phase of the cell cycle would be necessary to inhibit proliferation.
Effects of YC-1 on cell growth

Cell counting was used to evaluate the effects of YC-1 on cell growth. This revealed prolonged stasis of growth of subconfluent cells caused by YC-1 (100 μM) (Figure 7); a lethal effect on the cell population was discounted as ascertained by Trypan Blue exclusion.

To examine the possibility that YC-1 might be toxic to VSMCs, the following series of experiments was performed. (a) The number of the cells present in the supernatant was determined daily for 4 days, in both the presence and absence of 100 μM YC-1. Although this concentration of YC-1 caused significant inhibition of cell growth (more than 90%), less than 5% of the cells were found to be present in the supernatant during any portion of the culture period. Thus detachment and loss of cells did not account for the inhibition of cell proliferation. (b) Incubation of A10 VSMCs for 24 h with YC-1 (100 μM) also caused no significant cell loss: the number of cells was 2.8(±0.2)×10^4 in control wells and 3.0(±0.3)×10^4 in YC-1 (100 μM)-treated wells. Furthermore, no significant release of lactate dehydrogenase was observed in this experiment, again indicating that, in short-term incubations, even higher concentrations of YC-1 did not induce cell damage. (c) Less than 1% of the cells treated with YC-1 (100 μM) for 24 h stained with Trypan Blue.

In conclusion, this study demonstrates that YC-1 inhibits VSMC proliferation via the guanylate cyclase/cGMP pathway, and by inhibiting progression from the G1- to the S-phase of the cell cycle. The anti-proliferative effect of YC-1 is unlikely to cause cell toxicity, therefore it may have potential as a therapeutic agent for the prevention of vascular diseases.

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