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# Identification of Ethylenediurea and Benzyl (Phenoxy) Acetic Acid Derivatives, a Potential Cocktail Therapy for Ischemia

*Alexandre Bridoux[1,3]*, Dhruba J Bharali[1], Murat Yalcin[1,2] and Shaker A Mousa[1,3]*

[1]The Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences, USA

[2]Department of Physiology, Uludag University, Turkey

[3]French Association of Pharmaceutical Chemistry, France

## Abstract

The cell-membrane integrin $\alpha_v\beta_3$ was shown to stimulate angiogenesis after binding the thyroid hormone and its analogue GC-1 (pM range). Ethylenediurea (EDU), a potent UV and ozone protector for plants, could show a potent antioxidant effect (low µM range) if modified as a catechol analogue. Probing the two potent low molecular weight drugs as a new combined treatment for limb ischemia has been addressed. As a first step toward this goal, the relative beneficial effect of each drug analog against models of angiogenesis and lipid peroxidation was assayed. The synthesized GC-1 derivatives QH-1- QH-8 (thyroid hormone-like compounds) and some catechol derivatives of EDU were tested in the Chick chorioallantoic membrane assay and the lipid peroxidation assay. The bioactivities of both classes of molecules are complimentary. Our results show that a GC-1 analogue and an EDU analogue could therefore be tested as candidates in preclinical studies as a pro-angiogenesis agent and its therapeutic adjuvant, respectively.

**Keywords: Ischemia; GC-1; Thyroid hormone analogs; Ethylenediurea; Antioxidant**

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***Correspondence:***
*Alexandre Bridoux, The Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences, 1 Discovery Drive, Rensselaer, NY, 12144 USA,*
*E-mail: abridoux@wanadoo.fr*

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## Introduction

Ischemia was reported as a deficient supply of blood to a body part (such as the heart or brain) [1], which is due to obstruction of the inflow of arterial blood. Results from studies on man and animals have linked poor coronary circulation [2], hypercholesterolemia [3], and hypothyroidism to a high risk of developing ischemia and related diseases defined hereafter [1,4]. The continuous ischemic state has also been found to lead to an accumulation of oxygen-free radicals, which have the potential for mediating cell injury and stimulating greater ischemic state in return [5-7]. Ways to remove the obstruction include chemotherapy using a catheter plus an auxiliary, ultrasound-enhanced thrombolysis device (the EkoSonic MACH 4e, EKOS, Bothel, WA) [1]. Traditional surgery has not shown great success at restoring the blood flow in a timely manner and with respect to quality of life. Research has identified five parameters that could restore blood flow: thrombolysis, restoring normal thyroid-stimulated activity and cholesterol concentration in blood, stimulating the lines of defense against oxygen-free radicals, and stimulating new blood capillaries.

During the human body's detoxification process, the first line of defense against reactive oxygen radicals is the enzyme Superoxide Dismutase (SOD), which catalyzes the dismutation of $O_2$- to $H_2O_2$ [8]. Research has already found a small-molecular weight molecule, the heterocyclic compound ethylenediurea, EDU, (N-[2-(2-oxo-1-imidazolindinyl) ethyl]-N'-phenylurea) (4 in Figure 1), which was found to induce total activities of SOD both *in vitro* and *in vivo* [9]. It has also been tested successfully to protect a wide variety of plants from ozone injury and has recently been used to monitor air quality and to assess the effect of ambient ozone on plants in India [10,11].

The primary focus of this reported work is the topical stimulation of blood capillaries using derivatives of the most common synthetic molecule given for the treatment of hypothyroidism: Synthroid (Abbott Laboratories), i.e., synthetic L-thyroxine sodium (L-$T_4$ sodium) and of GC-1 (3 in Figure 1) [2,12], a synthetic selective TRβ agonist [13]. GC-1 emerged from research on biomimetism and has been proposed to efficiently treat hypercholesterolemia [14]. This molecule has also proven to have beneficial effects on hypothyroid-induced illness and to induce angiogenesis at low concentration in the same way as L-$T_4$ did. Thus both abnormal thyroid activity and

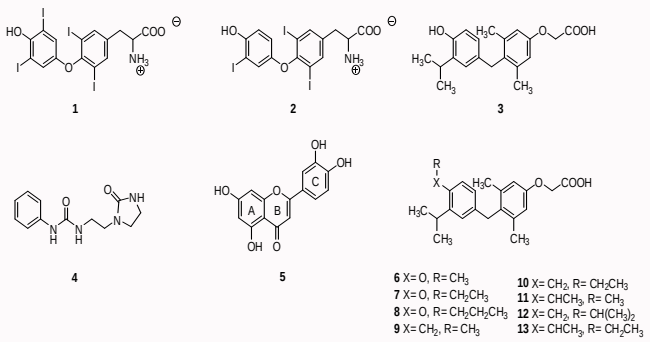



**Figure 1:** Structures of L-T4 (1), L-T3 (2), GC-1 (3), EDU (4), a flavone: structure of apigonin (5), GC-1 analogues (6-13).

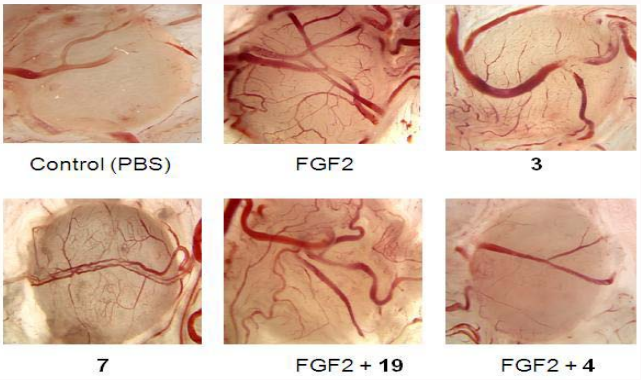



**Figure 2:** Representative chick chorioallantoic membrane (CAM) images showing the effect on angiogenesis of FGF2, GC-1 (3), and 7 (pro-angiogenic); FGF2+19 (no disturbance of the pro-angiogenic state); FGF2+4 (anti-angiogenic).

angiogenesis issues could be addressed at the same time [15].

In parallel, derivatives of the antioxidant EDU molecule were probed for a dual bioactivity; i.e., antioxidant effect and angiogenesis activity. Because EDU was seriously considered for potential use as an adjuvant therapy to the GC-1 analogs' chemotherapy, this preliminary study allowed us to determine which GC-1 and EDU analogs could be used in the interference and complimentary trials that represent fundamental issues in combination therapy. Thus, these trials were not the focus of this work. The final goal is to study the importance of the amino substituent of the L-thyroxine molecule for its angiogenesis activity by testing the activities of amino-deprived GC-1 analogs in comparison to previously reported work.

## Medicinal Chemistry

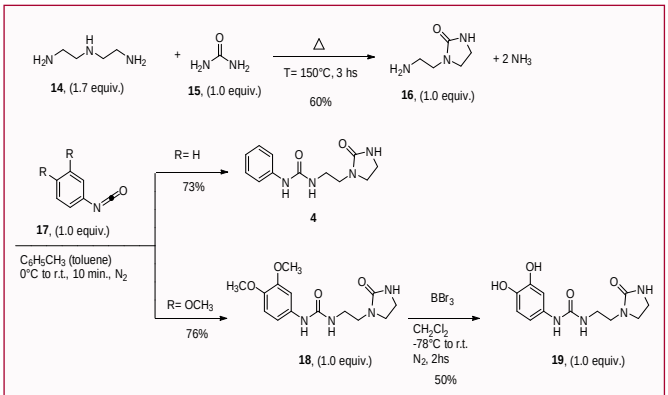
The concept of using a mix of analogs of these drugs to target ischemia was considered. Indeed, this work was started with the aim of using GC-1 derivatives with EDU analogs. The rationale was that a strong therapy to treat ischemia, consisting of a strong angiogenesis agonist, could benefit from the effects induced by a strong antioxidant adjuvant. This adjuvant would therefore not interfere with angiogenesis. Thus the combination of both therapy agents should be more effective as a treatment. As a first step toward this aim, amino group-free GC-1 analogs 6-13 (Figure 1) were synthesized in the laboratory of our research partner, Professor Thomas S Scanlan, Ohio State University, and tested for their topical pro-angiogenesis activity [1,16].

Antioxidant effects of EDU have been reported many times [17]. The rationale behind the choice of the catechol-like EDU analogs used here is the long-time reported beneficial antioxidant effect of vicinal OH groups on aromatic rings [18]. These protective effects have been attributed to the abundance of antioxidants, including flavonoids available in green tea, for example. The flavonoid compounds (5 in Figure 1 as a general example) have been generally sub-classified according to the level of oxidation of the C-ring, while individual family members exhibit variations in the degree of hydroxylation, substitution, and conjugation of the B and C rings.

EDU (4) and catechol-like analogs (18 and 19, Scheme 1) were made using isocyanate chemistry on 2-(2-oxo-l-imidazolidinyl) ethylamine (16) in good yield [19]. Compound 16 was the result of the condensation of liquid diethylenetriamine 14 (1.7 equiv.) with solid urea 15 (1.0 equiv.) at reflux (T=150°C) over three hours. The reaction proceeded with the release of 2 ammonia molecules. For the next step, toluene ($C_6H_5CH_3$) was used instead of $CH_2Cl_2$ because it was thought to better solvate the phenyl isocyanate derivatives 17 (R=H or OCH3) due to possible stacking interactions. EDU (4) was synthesized with 73% yield, and analog 18 was obtained within the same yield range. Deprotection of the two methoxy groups of 18 afforded 19 with 50% yield using $CH_2Cl_2$ miscible $BBr_3$ from -78°C to r.t. over two hours [20].

## Biology

Compounds were tested against two biological models: the chick Chorioallantoic Membrane (CAM) assay for angiogenesis, and the lipid peroxidation assay for antioxidant activity. The CAM assay was used previously by us to validate the angiogenic activity of GC-1, L-$T_4$, and analogs [21]. Here, some GC-1 analogs were synthesized and then first-run tested on angiogenesis. These analogs were previously tested on TRβ receptors and TRβ mutant receptors, which are usually expressed in the genetic disease Resistance to Thyroid Hormone (RTH); compound 7 was 61-fold more potent than $T_3$ (2) and 778-fold more potent than the parent analog GC-1 (3) with the mutant TRβ in these tests [16]. Here, the effect of each compound was compared to Fibroblast-Growth-Factor (FGF2, 1 µg/mL) and GC-1 (10 ng/CAM) using the CAM model (Table 1, Figure 2). Compounds were considered strong agonists if they induced at least 50% stimulation in comparison to the level of stimulation by the reference molecules. FGF2 stimulated angiogenesis (up to 186-188 new branch points), and GC-1, used as a reference, also stimulated angiogenesis (147-160 branch points). In the first batch, compounds 6-10 were tested, and GC-1 stimulated an average of 147.1 branch points (77% stimulation in comparison to FGF2 activity (100%)). In the second batch, compounds 11-13 were tested; GC-1 stimulated an average of 160.0 branch points (86% stimulation in comparison to FGF2 (100%)). Activities of compounds 6-13 were rationalized by comparing their activities to that of GC-1, not FGF2. The different activities observed came from the difference in the functional groups ortho to the isopropyl of GC-1, e.g., the modulation of the phenol's OH. These results allowed us to separate molecules into two groups. One group was considered as strong agonists (compounds 6, 7, 11, 13) and a second group of molecules that were not efficient in this assay (compounds 8, 9, 10, 12), with compound 8 having the lowest stimulation activity and compound 6 the highest. Looking at the activities of 6 and 9 allowed us to conclude that an oxygen atom is better than a methylene at the X position when X is substituted by small R groups (Figure 1). The use of longer n-alkyl chains at the R position was not tolerated by the receptor, which could be the integrin




**Scheme 1:** Synthesis of EDU (4) and EDU analogs 18 and 19.

**Table 1:** Pro-angiogenesis efficacy of GC-1 analogs in the CAM model.

| Treatment[a] | Branch pts ± SEM[b] | Mean % of stimulation[c] |
|---|---|---|
| PBS | 83.0 ± 6.9 | - |
| FGF2 (1 µg/mL) | 188.4 ± 11.4[d] | 100 ± 6[d] |
| GC-1 (**3**) | 147.1 ± 11.3[d] | 77 ± 6[d] |
| **6** | 154.4 ± 11.8[d] | 86 ± 6[d] |
| **7** | 136.7 ± 9.7[d] | 65 ± 5[d] |
| **8** | 103.6 ± 13.1[d] | 25 ± 3[e] |
| **9** | 121.4 ± 16.5[d] | 46 ± 6[e] |
| **10** | 127.5 ± 13.4[d] | 54 ± 6[d] |
| PBS | 85.8 ± 6.7 | - |
| FGF2 (1 µg/mL) | 186.0 ± 17.8[d] | 100 ± 9[d] |
| GC-1 (3) | 160.0 ± 10.2[d] | 86 ± 5[d] |
| 11 | 146.8 ± 8.7[d] | 71 ± 4[d] |
| 12 | 137.8 ± 7.8[d] | 61 ± 3[d] |
| 13 | 148.3 ± 12.8[d] | 73 ± 6[d] |

[a] All GC-1 analogs were tested at a concentration of 10 ng/CAM. [b] Data represent the means ± SEM, n=8. [c] Compared to PBS. [d] P<0.01 as compared to PBS (control). [e] P<0.05

$\alpha_V\beta_3$ [21], most probably due to the total length of the analogs 7, 8, 10 and the length and bulk of analog 12.

Interestingly, 11 and 13, which are also long and bulky, showed an unexpected agonist activity. The presence of the isopropyl and isobutyl substituent's may prevent the second isopropyl, the one meta relative to the benzylic carbon atom, from rotating freely and thereby lock the conformation in that region of the compound. This peculiar setting could be enough to induce a special conformational change or be more suitable to the receptor active site for a better ligand-receptor interaction.

Thus the active site of the receptor that is linked to angiogenesis (which could be integrin $\alpha_V\beta_3$) could accommodate substitutions up to three chained carbon atoms on the aromatic hydrocarbon ring. This research completed the first goal, which was the investigation of the power of compounds structurally related to the thyroid hormone to induce angiogenesis.

A summary of the antioxidant effects of EDU analogs 18 and 19 is listed in (Table 2). In the lipid peroxidation assay [22], compounds 18 and 19 were tested at two concentrations (1 µM and 10 µM), and their activities were compared to two reference antioxidant molecules, ascorbic acid and Pyrroloquinoline Quinine (PQQ). At 100 µM, the

**Table 2:** Measurements of the formation of Malondialdehyde (MDA) as the end product of lipid peroxidation in tissue homogenate (1 mg/mL after centrifugation at 2500 rpm). Quantification of the oxidation damage is with and without Ethylenediurea (EDU) analogs 18 and 19.

| Treatment[a,d] | MDA [pmoles/mg tissue/min] | % Inhibition |
|---|---|---|
| (+) control PQQ[b] (100 µM) | 0.4 ± 0.1 | 97.1 ± 0.4 |
| (+) control ascorbic acid (100 µM) | 0.7 ± 0.1 | 94.8 ± 0.4 |
| PBS | 13.1 ± 0.2 | 0.0 ± 0.8 |
| 18 (1 µM) | 7.1 ± 0.1 | 45.8 ± 0.4 |
| 18 (10 µM) | 2.9 ± 0.1 | 77.9 ± 0.4 |
| 19 (1 µM) | 6.1 ± 0.2 | 53.1 ± 0.8 |
| 19 (10 µM) | 1.2 ± 0.1 | 90.9 ± 0.4 |

[a] 60 min incubation time. [b] pyrroloquinoline quinine. [c] Data represent the means ± SEM, n=2. [d] Compared to PBS (control)

**Table 3:** Anti-angiogenesis efficacy of ethylenediurea (EDU, 4) analogs in the CAM model.

| Treatment | Branch pts ± SEM[a] | % Inhibition ± SEM |
|---|---|---|
| PBS | 76.0 ± 8.5 | - |
| FGF2 (1.25 µg/mL) | 137.9 ± 7.5 | - |
| L-$T_4$ (1) (100 ng) | 137.0 ± 8.0 | - |
| FGF2 + 4 (10 µg) | 77.0 ± 5.0 | 98 ± 8[b] |
| FGF2 + 18 (10 µg) | 120.6 ± 6.1 | 28 ± 10[c] |
| FGF2 + 19 (10 µg) | 149.7 ± 9.2 | - |

[a] Data represent the means ± SEM, n=8. [b] P<0.05. [c] P<0.001

references completely inhibited Malondialdehyde (MDA) expressed by tissue homogenate following incubation (60 min). Compound 18 proved to be a potent inhibitor at 10 µM, whereas it showed average inhibition at 1 µM. Compound 19 (at 10 µM) clearly showed almost complete inhibition at a concentration 10 times lower than that used for the references and at 1 µM reduced the generation of peroxides by half at a concentration 100 times lower than that used for the references. The tests were repeated twice. Undoubtedly, the catechol function induced a large beneficial effect to the antioxidant of the EDU compound. These results were suggestive enough to consider subsequent assays in the angiogenesis model to detect whether they could induce any disturbance of regular blood capillary growth promoted by FGF2.

In another set of experiments (Table 3), EDU (4) and EDU analogs (18 and 19) were applied to FGF2-stimulated CAM to evaluate whether they interfered with simulated physiological pro-angiogenesis effect. L-$T_4$ was used as a second reference and showed a more potent pro-angiogenic effect in comparison to that of FGF2. In these tests, while 4 and 18 counteracted FGF2-stimulated angiogenesis (98% and 28% inhibition, respectively), 19 showed no effect on angiogenesis (Table 3). This proved that modifying the structure of EDU into a catechol-like molecule should be a transformation of choice for the future phase of our project, that is, the consideration of interference between the two drugs.

From previous work, it was found that the carboxylate substituent in thyroid hormone derivatives is the most important feature for any pro-angiogenesis activity [21,23-25]. The thyroid hormone shows an amino substituent that reinforces the pro-angiogenesis effect. Nevertheless, this function was hypothesized to be non-mandatory for pro-angiogenesis activity [21]. Here the results from the CAM assays revealed that most of the GC-1 analogs, which are deaminated analogs, stimulated angiogenesis at least by 50% in comparison to

the reference macromolecule FGF2. These results taken together with those of previous work allow us to conclude that the presence of the amino functional group in such molecules to obtain strong angiogenesis activity is relatively non-important [21,23-25].

## Conclusion

Results from the CAM assays on GC-1 analogs clearly showed that a α-amino group such as that of L-$T_4$ is unnecessary to stimulate angiogenesis and that in addition to being a TRβ mutant receptor agonist [22], compound 6 is a potent angiogenesis stimulating agent. It also bears a $CH_3$ protecting group on the phenol's OH, which may slow down the process of detoxification, e.g., the metabolism of GC-1 by the body, thereby increasing the half-life of the compound. This molecule could therefore be used in combination with an ultrasound-enhanced thrombolysis device to develop new capillaries where a thrombus is occluding a coronary artery and to revest the inflow of blood to a body part. GC-1 analog 6 and EDU analog 19 could therefore be assayed as candidates in studies on mutual interferences as a pro-angiogenesis agent and its therapeutic adjuvant, respectively. Further development could yield to a "cocktail" adjuvant therapy to thrombolysis for hypoxic ischemic encephalopathy and ischemic-induced renal tissue hypoxia.

Experimental details are provided in the Appendix.

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