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Structure–Activity Relationships Studies of the Anti-Angiogenic Activities of Linomide

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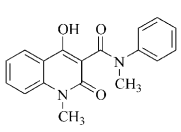
Abstract—The synthesis and anti-angiogenic activities of linomide and its analogues are reported. Three of the analogues are 3.3–69 times more potent than linomide at inhibiting blood vessel formation in the CAM angiogenesis assay. These compounds possessed considerable anti-proliferative activity against isolated HUVEC cells with no activity against epithelial-derived prostate tumor cells.

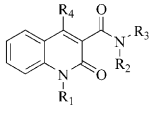
Linomide (*N*-phenylmethyl-1,2-dihydro-4-hydroxyl-1-methyl-2-oxo-quinoline-3-carboxamide) has been shown to be an immunomodulator. In clinical trials, it has been shown to have potential in the treatment of autoimmune disease such as rheumatoid arthritis, systemic lupus erythematosis and multiple.[1–3] Recently, linomide has also been reported to have antiangiogenic activity.[4,5] Angiogenesis is the formation of new blood vessels from pre-existing ones. It has been shown that without angiogenesis a tumor can only reach the size of 1–2 mm^3, small enough to be easily resected or treated with conventional cytotoxic chemotherapeutic agents. In vitro, linomide inhibited endothelial cells proliferation and migration[4,6] as well as invasion through basement membrane at high concentration (>100 μg/mL).[4] In vivo, linomide has been demonstrated to cause a dose dependent, antiangiogenic activity using a Matrigel-based quantitative in angiogenic assay in rats.[4,7] In addition, linomide has been reported to have in vivo antitumor effect against rat and human prostatic cancers.[8–10]

The antiangiogenic activity of linomide has been known for more than 10 years. Surprisingly, no structure–activity relationship studies of the antiangiogenic activity of linomide have been reported. In our continued effort to search for potent antiangiogenic agents, we synthesized several analogues (**2**–**7**) of linomide to investigate its structure–activity relationships (Fig. 1), particularly on the importance of 1-methyl (R_1), 4-hydroxy (R_4), *N*-methyl (R_2) and *N*-phenyl (R_3) group in regard to their antiangiogenic activity.

Linomide **1**, compounds **2** and **3** were prepared according to the procedure described by Edgar et al., as outlined in Figure 2.[11] Briefly, stirring *N*-methylisatoic anhydride **9** with dimethylmalonate **11** in the presence of sodium hydride and DMF at 120 °C yielded the hydroxyquinoline carboxylic ester **12**. Linomide **1**, compounds **2** and **3** were obtained by coupling the ester **12** with *N*-methylaniline, dimethylamine and aniline, respectively. The syntheses of compounds **4** and **5** were similar to the syntheses of linomide and compound **3** except isatoic anhydride **10** was used as the starting material.



Linomide, **1**



2. $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = CH_3$,, $R_4 = OH$
3. $R_1 = CH_3$, $R_2 = H$, $R_3 = Ph$, $R_4 = OH$
4. $R_1 = H$, $R_2 = CH_3$, $R_3 = Ph$, $R_4 = OH$
5. $R_1 = H$, $R_2 = H$, $R_3 = Ph$, $R_4 = OH$
6. $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = Ph$, $R_4 = H$
7. $R_1 = H$, $R_2 = H$, $R_3 = Ph$, $R_4 = H$

Figure 1. Structures of linomide and its analogues.

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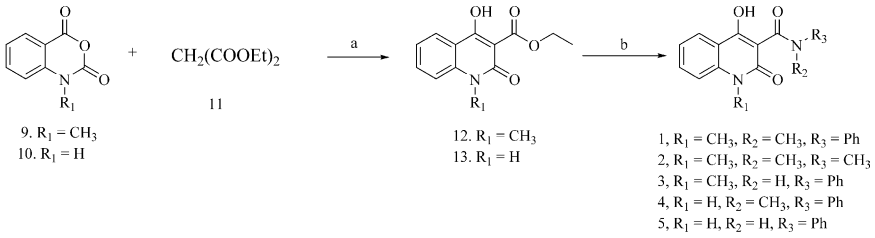


Figure 2. Syntheses of linomide **1** and compounds **2–5**. Reagents and conditions: (a) NaH, DMF, 120 °C, 2.5 h, 44.2–72.7%; (b) R_2R_3NH, toluene, reflux 4 h, 55.4–80.5%.

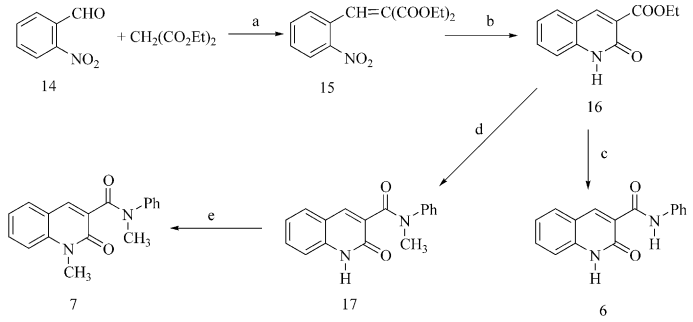


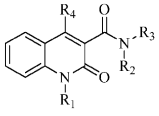
Figure 3. Syntheses of compounds **6** and **7**. Reagents and conditions: (a) TsOH, toluene, 36 h, 70.5%; (b) Fe/AcOH, 100 °C, 6 h, 66.6%; (c) (i) NaOH/MeOH/H_2O, 60 °C, 12 h, 93%; (ii) (1) $SOCl_2$; (2) aniline/DMF, rt, overnight, 56.2%; (d) (i) NaOH/MeOH/H_2O, 60 °C, 12 h, 93%; (ii) (1) $SOCl_2$; (2) *N*-methylaniline/DMF, rt, overnight, 53.1%; (e) CH_3I/NaH/DMF, rt, overnight, 85.6%.

The syntheses of compounds **6** and **7** (Fig. 3) have never been reported. Quinolinecarboxylate **16** is the common intermediate which was prepared from compound **14** according to the reported procedure.[12] Compound **6** was obtained by reacting **16** with thionyl chloride followed by condensation with aniline. Substituting aniline with *N*-methylaniline yielded **17** which was methylated at the N-1 position to yield compound **7**. The compounds were purified by column chromatography and recrystallization and their purities were confirmed by elemental analyses.

The antiangiogenic activities of linomide and its analogues were evaluated using two established angiogenic assays. The first assay we used was an in vivo chicken chorioallantoic membrane (CAM) assay.[13,14] In the CAM assay, human VEGF-165 and bFGF (200 ng each) were added to saturation to a microbial testing disk and placed onto the CAM of 10-day-old chicken embryos.[15] Eight h after the growth factor treatment, antiangiogenic compounds were added to the same microbial testing disk and the growth factors and embryos allowed to incubate an additional 40 h. CAMs were removed, paraformaldehyde fixed, placed onto Petri dishes, and digital images taken at 7.5× using a dissecting microscope. A 1 × 1-cm grid was then added to the digital CAM images and the number of vessels within each grid counted as a measure of vascularity. Table 1 represents the CAM assay data of linomide and its analogues and dose in μg/embryo of compound necessary to reduce the blood vessel number to 50% that of the VEGF/bFGF treated group. Linomide is weakly active with ED_{50} of 60.1 μg/embryo. It appears that the 4-OH group (R_4) is critical for anti-angiogenic activity since both compounds **6** and **7**, with 4-OH replaced by H, are devoid of anti-angiogenic activity (> 100 μg/embryo). Interestingly, replacing the 1-methyl (R_1) or *N*-methyl (R_2) with H resulted in significantly increased anti-angiogenic activity. The ED_{50} of compounds **3** and **4** are 18 and 7.5 μg/embryo, respectively 3.3 and 8 times more potent than linomide. Eliminating both of the methyl groups in linomide yielded compound **5** which is 69 times more potent than linomide with an ED_{50} of 0.87 μg/embryo. In addition, the *N*-phenyl group is not optimal for anti-angiogenic activity. Replacing the phenyl group in linomide with a methyl moiety to form compound **2** increases the anti-angiogenic activity (ED_{50} = 8.5 μg/embryo).

Because we found that linomide and its analogues were active in a functional angiogenesis assay, we next sought to define the cellular target for the compounds. First, we

Table 1. Effect of linomide and its analogues on CAM angiogenesis and human umbilical vein endothelial cell (HUVEC) proliferation



Compd	R_1	R_2	R_3	R_4	ED_{50} (μg/embryo)	IC_{50} HUVEC proliferation (μM)
Linomide **1**	CH_3	CH_3	Ph	OH	60.1	13.95
2	CH_3	CH_3	CH_3	OH	8.5	16.47
3	CH_3	H	Ph	OH	18.0	7.20
4	H	CH_3	Ph	OH	7.5	6.55
5	H	H	Ph	OH	0.87	4.0
6	CH_3	CH_3	Ph	H	> 100	20.58
7	H	H	Ph	H	> 100	22.26

examine the anti-proliferative activity of the compounds on HUVEC cells as previously described.[15] We chose HUVEC cells because they are a standard line for anti-angiogenic screening, as set by the National Cancer Institute in the Angiogenesis Resource Center (http://www.dtp.nci.nih.gov/). Linomide and its analogues exhibited substabtial anti-proliferative activities with IC_{50} values range from 4.0 to 22.26 μM (Table 1). Except compound **2**, there seems to be a direct correlation between the inhibition of CAM blood vessel formation and inhibition of HUVEC cells proliferation (Table 1).

In order to ascertain whether linomide and its metabolites are selective for endothelial cells, the effects of the compared on prostate cancer cells (PC-3) were also examined. The PC-3 cell line was chosen because linomide has been reported to have in vivo antitumor effect against human prostatic cancers.[8–10] Linomide and its analogues had no antiproliferative activities against PC-3 prostate cancer cells with IC_{50} of all the compounds >100 μM (data not shown).

In conclusion, this is the first report on the structure–activity relationship studies of the antiangiogenic activity of linomide. Several linomide analogues exhibited higher antiangiogenic activity than linomide. This study also points to the fact that linomide may not be the true antiangiogenic moiety in vivo. Compound **4**, a known metabolite of linomide in vivo,[16] is 8 times more potent than linomide at inhibiting blood vessel formation in the CAM angiogenesis assay. Furthermore, the cellular target for the linomide analogues appears to be the endothelial cells lining the blood vessel since these compounds possessed considerable anti-proliferative activity against isolated HUVEC cells with no activity against epithelial-derived prostate tumor cells. Detailed structure–activity relationships studies of linomide are currently in progress.

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