TYROSINE KINASE INHIBITORS AND ANGIOGENESIS: MOLECULAR MECHANISM OF ACTION AND *IN VIVO* EFFECT IN THE RABBIT CORNEAL NEOVASCULARISATION ASSAY

DOCTORATE THESIS

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Neovascularisation in the eye is associated with various disorders, and is the leading cause of blindness in the world. Thus, the identification of the molecular mechanisms of angiogenesis and the development of sufficient anti-angiogenic treatment is very critical. VEGF (Vascular Endothelial Growth Factor) is considered the main angiogenic factor in the ocular angiogenesis.

In an attempt to identify phytochemicals contributing to the well-documented preventive effect of plant-based diets on cancer incidence and mortality, we have previously shown that certain flavonoids inhibit *in vitro* angiogenesis. Here, we show that the flavonoid, luteolin, inhibited VEGF-induced in vivo angiogenesis in the rabbit cornea assay. In agreement, luteolin inhibited both VEGF-induced survival and proliferation of human umbilical vein endothelial cells (HUVECs) with a half maximum concentration of 5 μM. The effect of luteolin on survival and proliferation correlated with inhibition of VEGF-induced phosphatidylinositol 3-kinase (PI3K) activity, whereas inhibition of VEGFR-2 phosphorylation was observed at higher concentrations. Luteolin inhibited VEGF-induced phosphorylation of Akt, a downstream target of PI3K that mediates survival and proliferative signals, and enhanced VEGF-induced phosphorylation of p38, a pathway that is suppressed by Akt and is known to cause apoptosis in endothelial cells (ECs). Overexpression of a constitutively active form of Akt rescued HUVECs from luteolin-induced apoptosis, but had a minimal effect in reversing the inhibition of VEGF-induced proliferation. Regarding the anti-mitotic activity, luteolin did not inhibit VEGF-induced ERK1/2 phosphorylation, but inhibited VEGF-induced phosphorylation of p70 S6K, a downstream effector of PI3K responsible for G1 progression Indeed, VEGF-induced proliferation of HUVECs was sensitive to rapamycin, an inhibitor of p70 S6K activation. Thus, inhibition of PI3K by luteolin mediates the inhibitory effects of luteolin on VEGF-induced survival and proliferation of HUVECs. The anti-survival

effects are mediated via PI3K/Akt-dependent pathways, whereas the anti-mitotic effects via PI3K/p70 S6K-dependent pathways.

Finally in order to determine the molecular mechanism underlying the anti angiogenic effect of luteolin, we tested the effect of luteolin on VEGF-induced transcription genes in endothelial cells using cDNA microarrays technology. From the 17000 genes tested, VEGF regulated the expression of 123 genes and luteolin reversed VEGF effect in 16 genes. A2-macroglobulin and ORP150 (Oxygen Regulated Protein) are both induced by VEGF and downregulated by luteolin. The role of the differently regulated genes in angiogenesis and the molecular mechanism underlying the effect of luteolin on their regulation is yet to be defined.

1: Bagli E, Stefaniotou M, Morbidelli L, Ziche M, Psillas K, Murphy C, Fotsis T. Related Articles, Links

Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3'-kinase activity.
Cancer Res. 2004 Nov 1;64(21):7936-46.
PMID: 15520200 [PubMed - indexed for MEDLINE]

2: Kotanidou A, Xagorari A, Bagli E, Kitsanta P, Fotsis T, Papapetropoulos A, Roussos C. Related Articles, Links

Luteolin reduces lipopolysaccharide-induced lethal toxicity and expression of proinflammatory molecules in mice.
Am J Respir Crit Care Med. 2002 Mar 15;165(6):818-23.
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Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3'-kinase activity.

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In an attempt to identify phytochemicals contributing to the well-documented preventive effect of plant-based diets on cancer incidence and mortality, we have previously shown that certain flavonoids inhibit in vitro angiogenesis. Here, we show that the flavonoid luteolin inhibited tumor growth and angiogenesis in a murine xenograft model. Furthermore, luteolin inhibited vascular endothelial growth factor (VEGF)-induced in vivo angiogenesis in the rabbit corneal assay. In agreement, luteolin inhibited both VEGF-induced survival and proliferation of human umbilical vein endothelial cells (HUVECs) with an IC(50) of about 5 mumol/L. Luteolin inhibited VEGF-induced phosphatidylinositol 3'-kinase (PI3K) activity in HUVECs, and this inhibition was critical for both the antisurvival and antimitotic affects of the compound. Indeed, luteolin abolished VEGF-induced activation of Akt, a downstream target of PI3K conveying both survival and mitotic downstream signals. Because overexpression of a constitutively active form of Akt rescued HUVECs only from the antisurvival effects of luteolin, the result indicated that luteolin targeted mainly the survival signals of the PI3K/Akt pathway. With regard to its antimitotic activity, luteolin inhibited VEGF-induced phosphorylation of p70 S6 kinase (S6K), a downstream effector of PI3K responsible for G(1) progression. Indeed, VEGF-induced proliferation of HUVECs was sensitive to rapamycin, an inhibitor of p70 S6K activation. Surprisingly, luteolin did not affect VEGF-induced phosphorylation of extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases, a pathway that is considered important for the mitotic effects of VEGF. Thus, blockade of PI3K by luteolin was responsible for the inhibitory effects of the compound on VEGF-induced survival and proliferation of HUVECs. The antisurvival effects of luteolin were mediated via blockage of PI3K/Akt-dependent pathways, whereas inhibition of the PI3K/p70 S6K pathway mediated the antimitotic effects of the compound.

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Luteolin reduces lipopolysaccharide-induced lethal toxicity and expression of proinflammatory molecules in mice.

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Luteolin is a flavonoid that has been shown to reduce proinflammatory molecule expression in vitro. In the present study, we have tested the ability of luteolin to inhibit lipopolysaccharide (LPS)- induced lethal toxicity and proinflammatory molecule expression in vivo. Mice receiving LPS (Salmonella enteriditis LPS, 32 mg/kg, intraperitoneally) exhibited high mortality with only 4.1% of the animals surviving seven days after the LPS challenge. On the contrary, mice that had received luteolin (0.2 mg/kg, intraperitoneally) before LPS showed an increased survival rate with 48% remaining alive on Day 7. To investigate the mechanism by which luteolin affords protection against LPS toxicity we measured intercellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor-alpha (TNF-alpha) production in response to LPS in the presence or absence of luteolin pretreatment. Treatment of animals with LPS increased serum TNF-alpha levels in a time-dependent manner. The increase in peak serum TNF-alpha levels was sensitive to luteolin pretreatment. Luteolin pretreatment also reduced LPS-stimulated ICAM-1 expression in the liver and abolished leukocyte infiltration in the liver and lung. We conclude that luteolin protects against LPS-induced lethal toxicity, possibly by inhibiting proinflammatory molecule (TNF-alpha, ICAM-1) expression in vivo and reducing leukocyte infiltration in tissues.

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