Raxofelast, (±)5-(Acetyloxy)-2,3-dihydro-4,6,7-trimethyl-2-benzofuranacetic Acid: A New Antioxidant to Modulate the Inflammatory Response During Ischemia-Reperfusion Injury and Impaired Wound Healing

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Abstract: Raxofelast, also named IRFI 016 or (±)5-(Acetyloxy)-2,3-dihydro-4,6,7-trimethyl-2-benzofuranacetic acid, belongs to a family of novel molecules designed with the aim to maximize antioxidant potency of phenols related to Vitamin E (α-tocopherol). This review will focus on the antioxidant and radical scavenging activity of this new promising compound.

Key Words: Raxofelast, inflammation, ischemia/reperfusion injury, wound healing, diabetes, safety.

INTRODUCTION

Over the last decades free radicals have attracted a great deal of attention for their potentially harmful role. They are mainly derived from oxygen (reactive oxygen species/ROS) and are generated by various endogenous systems or during pathophysiological state. Free radicals can adversely alter lipids, proteins and DNA and have been implicated in a number of human diseases. Lipids are highly prone to free radical damage resulting in lipid peroxidation that can lead to adverse alterations. Cellular antioxidant mechanisms are represented by glutathione, superoxide dismutase (SOD), catalase, vitamin E (tocopherols and tocotrienols), glutathione peroxidases and reductase, vitamin C and others [1].

Several evidences support the idea that an excessive formation of toxic oxygen metabolites in ischemic reperfused tissue is present [2]. Tissues are equipped with both an intracellular and extracellular antioxidant defense system. The defense system can also be divided into enzymatic and nonenzymatic defenses. Important components of a nonenzymatic antioxidant include alpha-tocopherol, ascorbic acid, and beta-carotene as well as other compounds that can react with radicals to form less reactive products such as sulfur-containing amino acids.

Extracellular fluid comprises a second line of defense against oxidant injury. These extracellular antioxidants include ceruloplasmin, albumin, transferrin, haptoglobin, and uric acid. The oxidant injury can potentially occur during ischemia and reperfusion due to an excess production of oxygen free radicals, or a decrease in antioxidant defenses, or both.

Involvement of oxygen free radicals in ischemia-/reperfusion injury is confirmed by the increased lipid peroxidation after organ ischemia and restoration of blood flow during surgical operations and reperfusion of organ transplants. Because antioxidants act removing the toxic oxygen metabolites, prevention of lipid peroxidation and the subsequent improvement in organ function could be highly effective in reducing ischemia-reperfusion injury [3].

A preventive activity against oxidative stress generation can justify a large utilization and association of antioxidant molecules for preventing complications in diabetic patients, where antioxidant defenses have been shown to be defective [4, 5]. In fact in diabetes hyperglycemia directly promotes an endothelial dysfunction inducing process of overproduction of superoxide and consequently peroxynitrite that damages cellular membranes. Classic antioxidants, like vitamin E, failed to show beneficial effects on diabetic complications probably due to their only "symptomatic" action. In this review we will focus our attention on new synthetic derivatives of vitamin E, namely Raxofelast.

CHEMISTRY

Raxofelast ($C_{15}H_{18}O_5$), also named IRFI 016 or (±)5-(Acetyloxy)-2,3-dihydro-4,6,7-trimethyl-2-benzofuranacetic acid, belongs to a family of novel compounds designed with the aim to maximize antioxidant potency of phenols related to α-tocopherol (Fig. **1**). This novel 2-substituted 2,3-dihydro-5-benzofuranols optionally protected on 5-OH group have been prepared *via* a simple two step route to racemic 2,3-dihydro-5-hydroxy-2-benzofuranacetic acids from hydroquinones and 4-bromocrotonates [6] (Fig. **2**). After oral administration to animals and humans, raxofelast is rapidly absorbed and quantitatively converted to the deacetylated active metabolite IRFI 005 (Fig. **3**). The antioxidant and radical scavenging activity of IRFI 005 has been demonstrated in

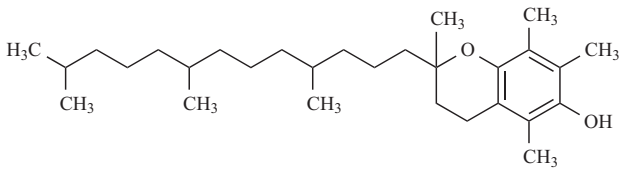

Fig. (1). Chemical structure of α-tocopherol.

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1389-5575/07 $50.00+.00

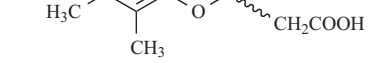
several *in vitro* systems [7], and its inhibition rate constant for reaction with peroxyl radicals was shown to be greater than that of vitamin E or ascorbic acid [8].

The powerful antioxidant activity of IRFI005 is at least in part mediated by a chain breaking mechanism as it is an efficient peroxyl radical scavenger. During the oxidation process IRF1005 is consumed with the formation of the benzoquinone oxidation product [9].

Raxofelast displays a good oral bioavailability: the active metabolite distributed to most tissues with the exception of the brain and is cleared mainly by renal route. Because of a carboxyl group on the side chain in position 2, these compounds are soluble in an aqueous environment at physiological pH, thus potentially enabling them to exert additional scavenging activity against reactive oxygen species and other water-soluble radical chain initiators in interstitial fluids [10].

RAXOFELAST PROTECTS AGAINST ISCHEMIA/ REPERFUSION INJURY

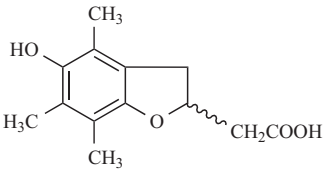
Ischemia/reperfusion damage is strongly sustained by free radicals especially the oxygen-derived ones derived from the reoxygenation of tissues. Although reperfusion is essential for the survival of ischemic tissue, it can also generate additional cell injury, which has been attributed to neutrophil infiltration and generation of reactive oxygen species. When ROS generation exceeds the defence mechanisms, an oxidative stress is generated and contributes to a reversible or irreversible cell injury. Reactive oxygen species are also involved in the pathophysiology of myocardial injury after reperfusion [11]. In rats subjected to a 6-hour ischemic injury raxofelast treatment prevented the loss of creatine-phospho-kinase (CPK), which decrease has been related to the extent of tissue necrosis, when compared to ischemic tissue from untreated animals [12]. Furthermore free radicals stimulate leukocyte chemotaxis and in turn neutrophil accumulation in the ischemic myocardium releasing cytotoxic oxygen metabolites, enzymes and cytokines responsible for the worsening of this pathological condition. IRFI 016 administration reduced cardiac infarct size, limited the amount of neutrophil infiltration into the necrotic zone, blunted CPK depletion, reduced the cytokine content in the ischemic tissue and increased the resistance of rats to ischemia/reperfusion injury [13, 14].



2,3-dihydro-5-acetyloxyl-4,6,7-trimethyl-2-benzofuranacetic acid (IRFI-016)

Fig. (2). Chemical structure of Raxofelast or IRFI-016 (2,3-dihydro-5-acetyloxyl-4,6,7-trimethyl-2-benzofuranacetic acid).

Raxofelast was also tested in a model of transient cerebral ischemia induced by bilateral carotid occlusion in Mongolian gerbils. The compound was able to reduce in a dose-dependent manner (up to 200 mg/kg), oxidative stress in the brain delaying neuronal death of CA1 cells and preventing the onset of the typical behavioural changes observed in this situation such as hyperactivity [15].



2,3-dihydro-5-hydroxy-4,6,7-trimethyl-2-benzofuranacetic acid (IRFI-005)

Fig. (3). Chemical structure of IRFI-005 (2,3-dihydro-5-hydroxy-4,6,7-trimethyl-2-benzofuranacetic acid).

Our group also tested Raxofelast in an experimental model of testicular ischemia/reperfusion injury, where oxidative stress and the consequent irreversible damage are present in either the ipsilateral testis and in the contralateral one. Antioxidants would theoretically have a dual effect in test ischemia-reperfusion injury: limiting damage evolution decreasing free radical generated by lipid peroxidation and counteracting ROS mediated activation of inflammatory reaction. In fact, our study has demonstrated that antioxidant raxofelast reduces oxidative stress and the morphological changes that follows ischemia-reperfusion of the testis [16]. Moreover is known that reperfusion plays a key role in the activation of two proteins of the family of Mitogen Activated Protein Kinases (MAPKs), namely ERK and JNK, also in the testis [17]. Both kinases are also triggered by the oxidative stress-lipid peroxidation phenomenon, as previously shown in another experimental model [18]. Our results demonstrated that Raxofelast was able to lower conjugated dienes levels (2.8+/-0.2 ΔABS/g protein on the ischemic testis and 1.9+/-0.1 ΔABS/g protein in the contralateral one), significantly reduced histological damage in both testes [16], and decreased the activation of both ERK and JNK (unpublished data). These data suggest that the hydrophilic vitamin E-like antioxidants are good candidates for designing a novel therapeutic strategy to halt the oxidative stress that follows acute testis torsion.

Furthermore in a model of carrageenan-induced pleurisy raxofelast reduced morphological injury and neutrophil infiltration in carrageenan-induced inflammation and reduced nitrotyrosine immunostaining, an indicator of peroxynitrite formation in inflammation. The authors hypothesized that the reduced neutrophil infiltration into the inflamed tissue may be related to a prevention of endothelial oxidant injury by raxofelast and to a preservation of endothelial barrier function [19].

RAXOFELAST AMELIORATES IMPAIRED WOUND HEALING

Wound healing is a natural restorative response to tissue injury, a complex process that involves at least 3 phases: an inflammation phase, a cellular proliferation phase, and a remodeling phase.

The healing process is the interaction of a complex cascade of cellular events that generate resurfacing, reconstitution and restoration of injured skin [20]. Injury damages blood vessels causing platelet activation and initiation of the

coagulation cascade. Granulocytes, infiltrate the wound within the first 24 hours, destroy invading microorganisms and remove cellular and matrix debris [21]. Neutrophil infiltration slows down as monocyte infiltration increases. On migration into the wound site, monocytes undergo phenotypic changes to become macrophages. These macrophages continue to destroy bacteria as well as debride the wound; moreover, they release growth factors such as vascular endothelial growth factor (VEGF), which promotes angiogenesis. Therefore, the normal wound healing process implies the occurrence of a robust inflammatory reaction. However, the persistence of the inflammatory phase has detrimental consequences for the process of wound repair. The healing during thermal injury is stalled in the inflammatory phase. It has been shown that the pathophysiology of burns is characterized by exaggerated inflammatory reactions leading to rapid edema formation, additional tissue necrosis, and delayed wound healing [21].

Burn wounds derived from a thermal injury show a marked lipid peroxidation accompanied by a dramatic depletion of reduced glutathione, with a local reduction in the endogenous scavenging capacity. The inhibition of lipid peroxidation was a successfully strategy to improve wound healing in an experimental model of thermal injury [22]. The administration of Raxofelast, in fact, blunted the increased lipid peroxidation and prevented the consumption of the endogenous tissue glutathione. Furthermore Raxofelast markedly increased VEGF in skin wounds. VEGF governs the controlled and regulated phenomenon of angiogenesis. The marked effect of raxofelast on angiogenesis was also indicated by the robust increase in CD31 expression observed in the skin samples obtained by the mice administered with the lipid peroxidation inhibitor. Therefore it might be speculated that the "key" consequence of quenching oxidative stress-lipid peroxidation is the acceleration of neo-angiogenesis. In agreement with hypothesis, the increased healing of burn wounds following raxofelast administration was accompanied by a marked production of nitrite in the wounds and by an increases expression of both eNOS and iNOS which have been related to VEGF production [22].

The molecular effects of raxofelast also correlated well with all histological parameters which were considered. As a matter of fact the lipid peroxidation inhibitor enhanced burn wound repair by reducing inflammatory infiltration and burn edema, and by stimulating dermal and epidermal regeneration, proliferation of fibroblasts, and formation of new well-structured capillary vessels [22].

Another condition of impaired wound healing is represented by diabetes [23], in fact it has been demonstrated that in diabetes there is a defective production of VEGF [24]. In diabetes mellitus the main feature is hyperglycemia which engenders free radicals and also impairs the endogenous antioxidant defense system in many ways. Antioxidant defense mechanisms involve both enzymatic and non-enzymatic strategies. Hydroperoxides have toxic effects on cells both directly and through degradation to highly toxic hydroxyl radicals. Peroxyl radicals can remove hydrogen from lipids, producing hydroperoxides that further propagate the free-radical pathway. Generally, nitric oxide at physiological levels produces many benefits to the vascular system. However, nitric oxide may react with superoxide anion radical to form reactive peroxyl nitrite radicals. The increased oxidative stress and the subsequent activation of the transcription factor NF-κB have been linked to the development of late diabetic complications [25, 26]. NF-κB enhances nitric oxide production, which is believed to be a mediator of islet beta-cell damage.

In our experimental model, raxofelast was able to reverse the effects of diabetes on wound healing by reducing lipid peroxidation and edema and by stimulating re-epithelization, neovascularization, proliferation of fibroblasts, and synthesis and maturation of extracellular matrix [27]. The degree of wound healing in diabetic mice treated with raxofelast was approximately the same as that in control mice. The beneficial effects of raxofelast on wound healing were also stressed by the increase in breaking-strength measurements. Furthermore, the inhibition of lipid peroxidation normalized the pattern of VEGF mRNA expression and secretion in diabetic mice, thus strongly supporting the idea that there might exist a close link between the deleterious phenomenon of lipid peroxidation and a defect in VEGF production. In fact in a previous study our group demonstrated a deficient production of VEGF by diabetic animals compared to normoglycemic [28]. Indeed, the improvement in VEGF expression after raxofelast administration does not seem to be a consequence of a direct effect of the drug on the angiogenic factor. In fact, the vitamin E analog did not enhance VEGF expression in nondiabetic mice; furthermore, *in vitro* raxofelast (50 μmol/l) did not change the ability of murine macrophages to secrete VEGF in response to lipopolysaccharide (unpublished observations). The mechanism by which increased lipid peroxidation impairs VEGF expression in diabetic mice remains, at the moment, a matter of speculation. One may speculate that the large production of unstable reactive intermediates and hydroxyperoxides that occurs during lipid peroxidation could cause structural DNA changes that lead to an impairment in the transduction mechanism.

These results suggests that lipid peroxidation inhibition have beneficial effects in those conditions of impaired wound healing characterized by an altered pattern of VEGF expression.

HUMAN STUDIES ON RAXOFELAST

Diabetes is one of the most serious health problems facing the healthcare systems of developed countries and its prevalence is expected to dramatically grow in the next future, with estimates of about 300 million people that will be affected in the next ten years. These patients are at risk for developing severe chronic complications including retinopathy, neuropathy, nephropathy, cardiovascular disease and foot ulcers.

Diabetic foot ulceration is a problem that impairs the quality of life of the patient, leading to prolonged hospitalization, and resulting in major amputation. It was estimated that foot ulcer patients have a decreased survival compared to control patients

Therefore, appropriate treatments with suitable antioxidant and radical scavenging drugs such as raxofelast may be useful in healing or halting the progression of foot ulcers,

which are known to cause much discomfort in many diabetic patients [29].

In a Phase II clinical trial conducted at the Centre for Cardiovascular Biology of London, UK, ten normotensive, normocholesterolaemic men with Type II diabetes and ten controls (age matched healthy men) received raxofelast as oral treatment (600 mg twice daily) for one week. At the end of the treatment was evidenced a pronounced reduction in oxidative stress and a significant improve in endothelial function in Type II diabetic patients [30]. In contrast, vitamin E (α-tocopherol) treatment did not improve vascular endothelial dysfunction in the same clinical model [31].

SAFETY PROFILE

Raxofelast was positively evaluated in a complete battery of toxicity studies (up to six months) as well as in genetic toxicology and reproduction toxicology tests. The antioxidant activity of raxofelast and its deacetylated active metabolite IRFI 005 has been described in previous *in vitro* and *in vivo* studies. In addition, IRFI 005 has been shown to be a scavenger of superoxide anion, with a linear dose-response curve starting from 5 μmol/l. After systemic administration of raxofelast to rats, dogs, and humans, the plasma concentrations of the parent compound were very low, whereas high levels of IRFI 005 were found in plasma and tissue [32, 33]. Dose-range studies in healthy volunteers indicated a good tolerability. Furthermore a pharmacodynamic study conducted in patients with NIDDM showed that oral treatment with raxofelast reduces oxidative stress and effectively augmented endothelium-dependent vasodilation to metacholine in forearm resistance vessels of diabetic patients, an effect that may be linked to the counteraction of nitric oxide inactivation by oxygen free radicals [30].

CONCLUSION

Raxofelast in the several experimental models significantly limited the extension and severity of lipid peroxidation and restored the endogenous antioxidant defenses. Ischemia-reperfusion injury was also significantly counteracted by raxofelast in different experimental models.

The compound was also shown to exert multiple protective effects in carrageenan-induced acute inflammation and to attenuate severe damage in acute pancreatitis in rats [34].

The systemic administration of raxofelast significantly improved the impaired wound healing in genetically diabetic mice and in mice subjected to thermal injury through the stimulation of angiogenesis, re-epithelialization, and synthesis and maturation of extracellular matrix.

Thus, it could be speculated that the pharmacological effects of raxofelast may be dependent upon a combination of: a scavenging action toward oxygen radicals, which results in the prevention of peroxynitrite formation with consequent protection against the development of peroxynitrite-induced cellular energetic failure, a reduced neutrophil recruitment into the inflammatory site, which may represent an important additional mechanism for the observed anti-inflammatory action, an indirect inhibition of NF-kB and the relative inflammatory cascade, the stimulation of angiogenesis through an augmented VEGF expression.

These encouraging findings are strengthened by the positive results obtained in type II diabetic patients, but still need to be confirmed by other studies in order to better understand all the potential clinical applications of this new antioxidant.

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Received: 02 September, 2006 Revised: 08 December, 2006 Accepted: 27 December, 2006