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CICLO XXVIII°

**Biological activity of some natural and synthetic
N⁶-substituted adenosine derivatives (cytokinin ribosides)**

BIO/12

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A.A. 2014/2015

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ABSTRACT

In this Ph.D project, some natural N⁶ – substituted adenosine derivatives, cytokinin ribosides (CKRs) have been investigated with the aim to draw a profile of their biological activity. CKRs belong to a class of plant hormones playing various roles in many aspects of plant development. We chose the most representative among natural cytokinin ribosides, namely N⁶ – isopentenyl adenosine (iPAdo), kinetin riboside (KR), N⁶-benzyl adenosine (BA) and its hydroxylated derivative, ρ -topolin riboside (p-TR).

In the first part of the thesis, the platelet anti aggregation activity CKRs has been evaluated *in vitro* as inhibitors of platelet aggregation. The activity has been interpreted by *in silico* docking experiments as due to interaction of CKRs with P2Y₁₂ receptor. ρ -Topolin riboside showed the best platelet anti aggregation activity and *in silico* interaction with P2Y₁₂ receptor, followed by N⁶ – benzyladenosine.

Some synthetic N⁶ – substituted adenosine derivatives have been synthesized and investigated as antagonists toward the human adenosine receptors A₁, A_{2A}, A_{2B}, A₃. p-TR again was the best antagonist of A_{2A} and A_{2B} adenosine receptors, both involved in the platelet aggregation mechanism. Synthetic N⁶ – substituted adenosine derivatives were antagonists of A₃ adenosine receptor much stronger than natural CKRs.

In a structure-activity study, the cytotoxic activity of natural CKRs and the synthetic analogues of p-TR were evaluated on 661W and Neuro2A cell lines trough Trypan blue and Tunel assays. Synthetic N⁶ – substituted adenosine derivatives showed a cytotoxic activity stronger than p-TR itself, that, in turn, exhibited the best apoptotic property.

Many biological activities shown by the CKRs examined in this thesis could be related to an effect of these compounds on the cellular oxidative stress. Thus, as a part of the PhD project, the antioxidant profile of natural and synthetic CKRs has been investigated using the most common antioxidant tests *in vitro*. The heterogeneity of the results suggests in some instance a possible structure – activity relationship. However, since not all the compounds are active in every antioxidant assay, further characterization of the antioxidant profile of CKRs seems desirable, including suitable cellular assays.

1. INTRODUCTION

1.1 BIOLOGICAL ROLE AND FUNCTION IN PLANTS OF N⁶-SUBSTITUTED ADENINE DERIVATIVES (CYTOKININS)

Sixty years ago the first cytokinin (CK), N⁶-furfuryladenine (kinetin, K) was isolated in autoclaved products of herring sperm DNA and was demonstrated to be able to promote cell division in plants [1]. Since that discovery, a number of compounds with CK activity have been identified and subsequent studies have clarified many structural requirements for CK activity.

In general, the term cytokinin identifies N⁶-substituted adenines with plant hormone activity that are able to promote cell division and differentiation, playing various roles in many aspects of plant and development [2]. Naturally occurring CKs are adenine derivatives carrying either an isoprene-derived [2] or an aromatic side chain [3] at the N⁶-terminus (Fig. 1.1). Conventionally, these families are called isoprenoid CKs and aromatic CKs, respectively.

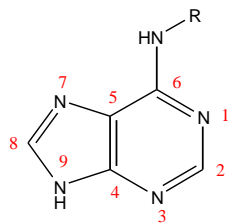
Despite the wealth of information concerning cytokinin chemistry and physiology, the transition from descriptive studies to molecular biology has been relatively slow compared with other plant hormones, such as ethylene or gibberellins. For instance, only a few years ago, candidates for cytokinin receptors begun to emerge [4, 5] while it is still uncertain how cytokinins are synthesized in plants.

Both isoprenoid and aromatic CKs are naturally occurring, with the former more frequently found in plants and in greater abundance than the latter. Common natural isoprenoid CKs are N⁶-(Δ^2 -isopentenyl)adenine, *trans*-zeatin (t-Z) and the related *cis*-isomer (c-Z) (Fig. 1.1).

Among the aromatic CKs so far discovered, N⁶-furfuryladenine (kinetin, K), the first cytokinin discovered in 1955 [1], N⁶-benzyladenine (B) and the three isomeric hydroxybenzyl adenines (topolins, T) (Fig. 1.1) were identified in several plant species including poplar and *Arabidopsis* [3, 6, 7].

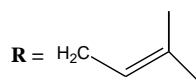
Although the general differences in biological activity of aromatic cytokinins and isoprenoid cytokinins in plants have not been fully characterized and understood, there are some emerging trends that are noteworthy [8-11]. It appears that aromatic cytokinins are implicated in the developmental and morphogenetic events, whereas isoprenoid cytokinins play a greater role in growth processes including the control of plant cell cycling.

Usually, for all natural CK nucleobases the corresponding nucleosides, nucleotides, and glycosides (Fig. 1.2) have been isolated [12]. Glycosylation of CK has been observed at

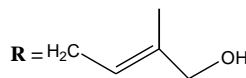


general structure of a cytokine CK

Isoprenoid CKs

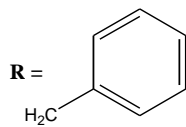


N^6 -isopentenyl adenine

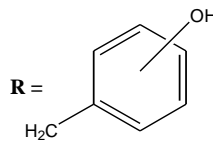


trans-Zeatin (tZ)

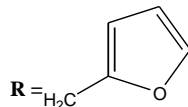
Aromatic CKs



N^6 -benzyl adenine (BA)



N^6 -hydroxybenzyl adenine (topolin, T)



kinetin (K)

Figure 1.1 Structure of naturally occurring cytokinins CKs.

the N^3 , N^7 , and N^9 -position of the purine moiety as *N*-glycosides, and at the hydroxyl group of the side chains of t-Z, c-Z, and dihydrozeatin as *O*-glucosides or *O*-xylosides (Fig. 1.2). *O*-glycosylation is reversible and the de-glycosylation is catalyzed by a β -glucosidase. On the contrary, *N*-glycoconjugates are not efficiently cleaved by β -glucosidase [13] and *N*-glycosylation results in a practically irreversible process. The physiological consequences of the differences in stability of *N*-glycosides and *O*-glycosides are not fully understood to date. However, it has been suggested that the readily cleaved *O*-glycosides represent inactive, stable storage forms of CKs [12].

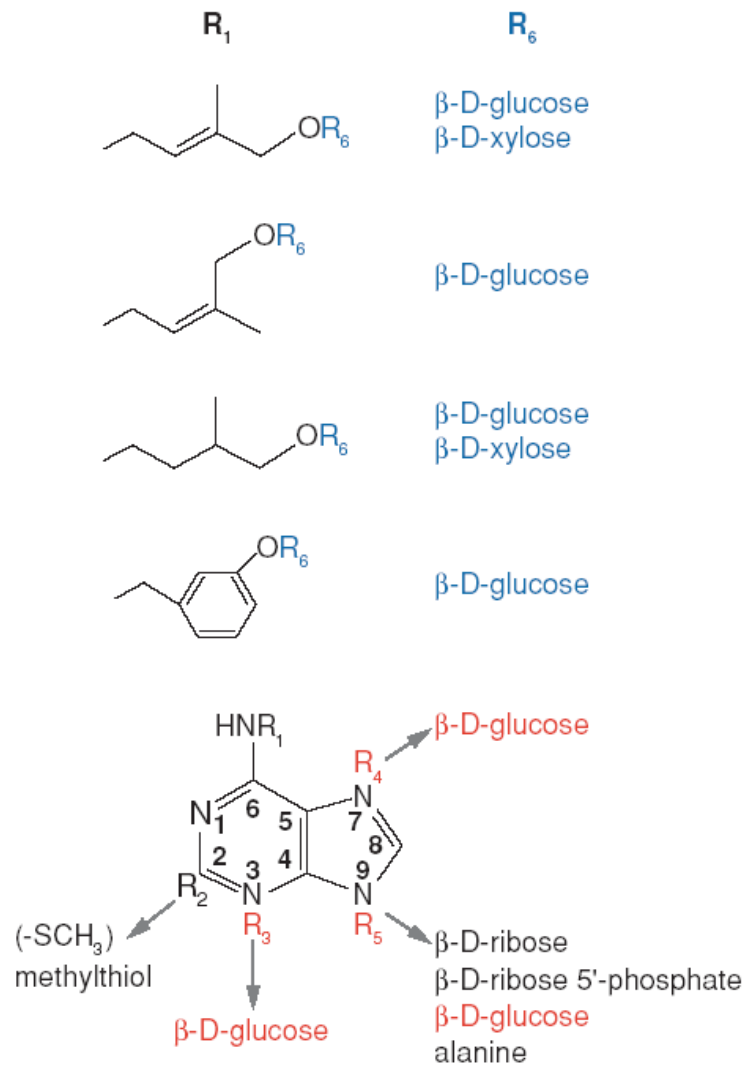


Figure 2

Cytokinin (CK) conjugates with sugars, sugar phosphates, and others. *O*-Glycosylation of side chain (colored in *blue*) is catalyzed by zeatin *O*-glucosyltransferase or *O*-xylosyltransferase. *N*-glycosylation of adenine moiety (colored in *red*) is catalyzed by cytokinin *N*-glucosyltransferase.

Figure 1.2 CK conjugates with sugars, sugar phosphates and others. *O*-Glycosylation of side chains colored in *blue* and *N*-glycosylation of adenine moiety in *red* (reported from ref. [5]).

1.2 BIOLOGICAL ROLE AND FUNCTION IN PLANTS OF CYTOKININ RIBOSIDES

The structures of the β -ribosides of previously shown CKs, cytokinin ribosides (CKRs), are shown in Figure 1.3.

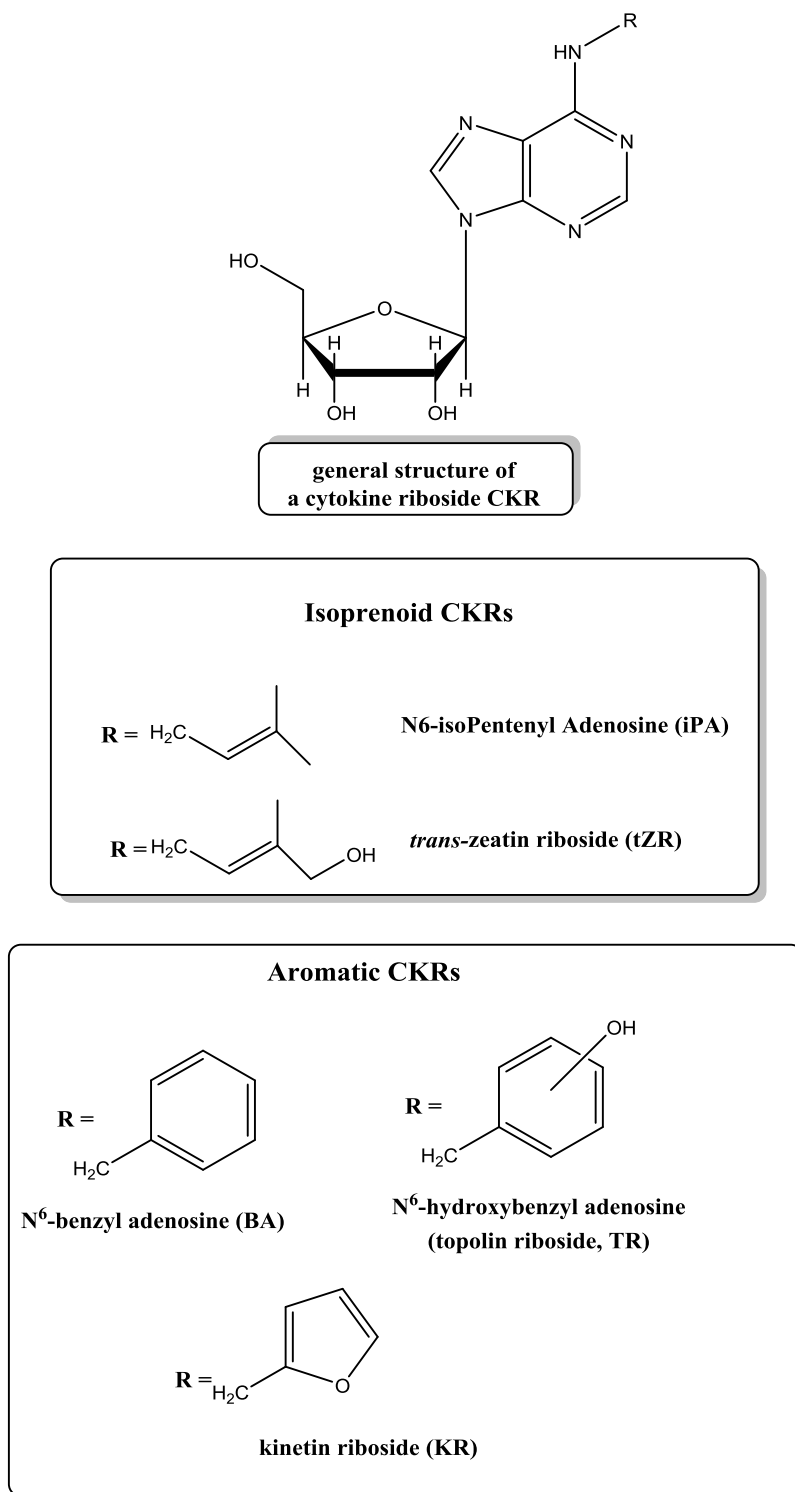


Figure 1.3 Structure of naturally occurring cytokinin β - ribosides CKRs.

CKRs and their 5'-phosphates (nucleotides) predominantly represent the primary products of CK biosynthesis and their occurrence in plant tissues suggests that important metabolic steps are shared with the purine metabolic pathway, i.e., salvage pathway [2].

Thus, the metabolic flow from CK nucleotides to the active nucleobases is probably not unidirectional but circular (Fig. 1.4).

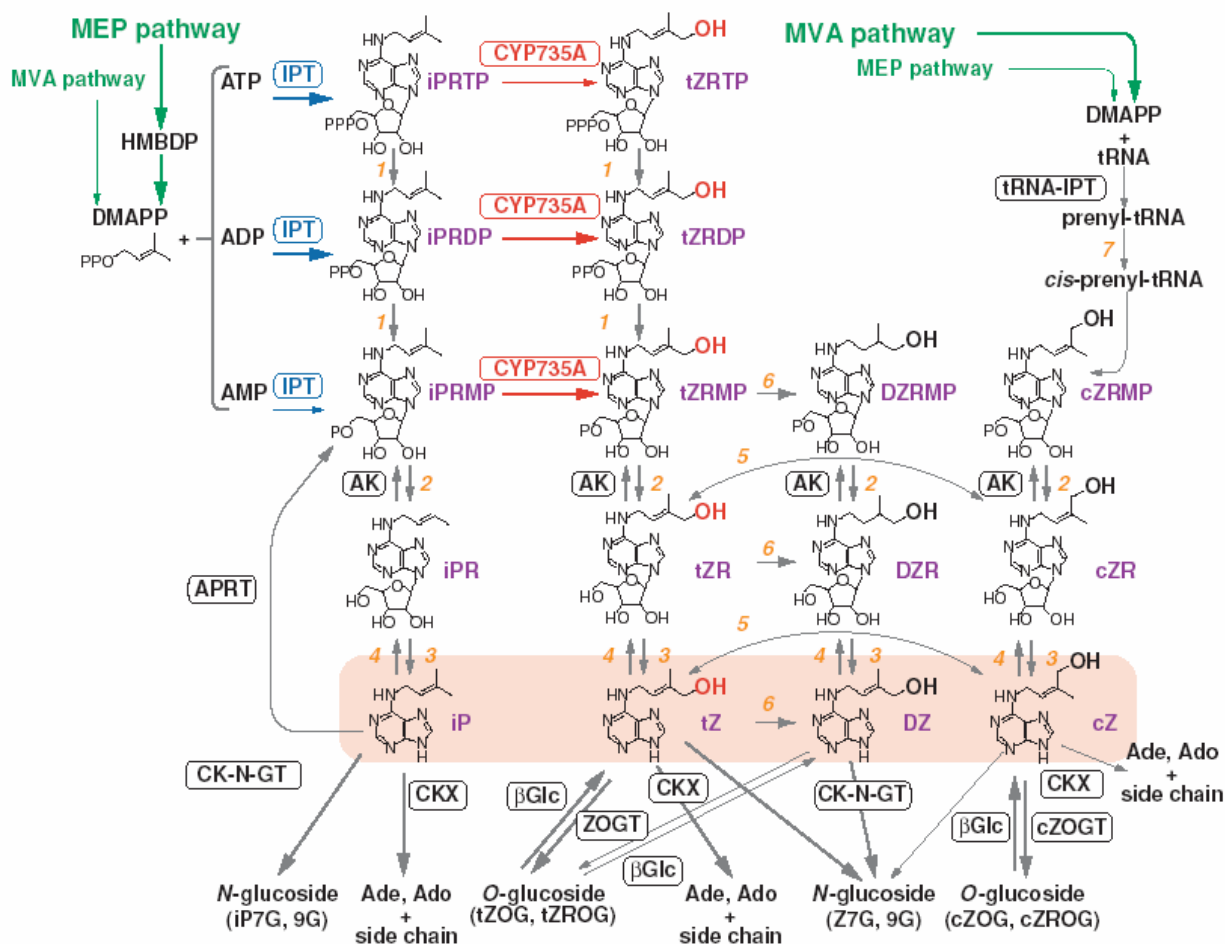


Figure 1.4 Current model of isoprenoid CK biosynthesis pathway in *Arabidopsis*. Methylerythritolphosphate (MEP) and mevalonate (MVA) pathways as isoprenoid side chain sources of cytokinins (reported from ref. [5]).

Isoprenoid CKs may be formed by *N*-prenylation of adenosine 5'-phosphates (AMP, ADP, or ATP) at the *N*6-terminus with dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyl diphosphate (HMBDP); catalyzed by adenosine phosphate-isopentenyltransferase (IPT; EC 2.5.1.27) [14] (Fig. 1.5). Further biosynthetic steps involve enzyme-catalyzed hydrolysis of nucleotides to nucleoside CKRs and to CKs.

Alternatively, CKs may derive from tRNA prenylation and successive degradation [15-17]. In addition to the biosynthetic relevance of CKRs as precursors or deriving from of CKs, the ribosides can be considered the major translocation form of CKs in plants. The CK

transport is achieved by the translocation systems that also mediate the transport of purine derivatives and nucleosides [12].

Among aromatic CKRs, ortho-toplin riboside (o-TR), is present at micro-molar concentrations in poplar leaves after daybreak [18], kinetin riboside (KR) and its free base (K) have been recently detected in the endosperm liquid of fresh young coconut fruits at concentrations of 0.31 and 0.33 nM, respectively [19].

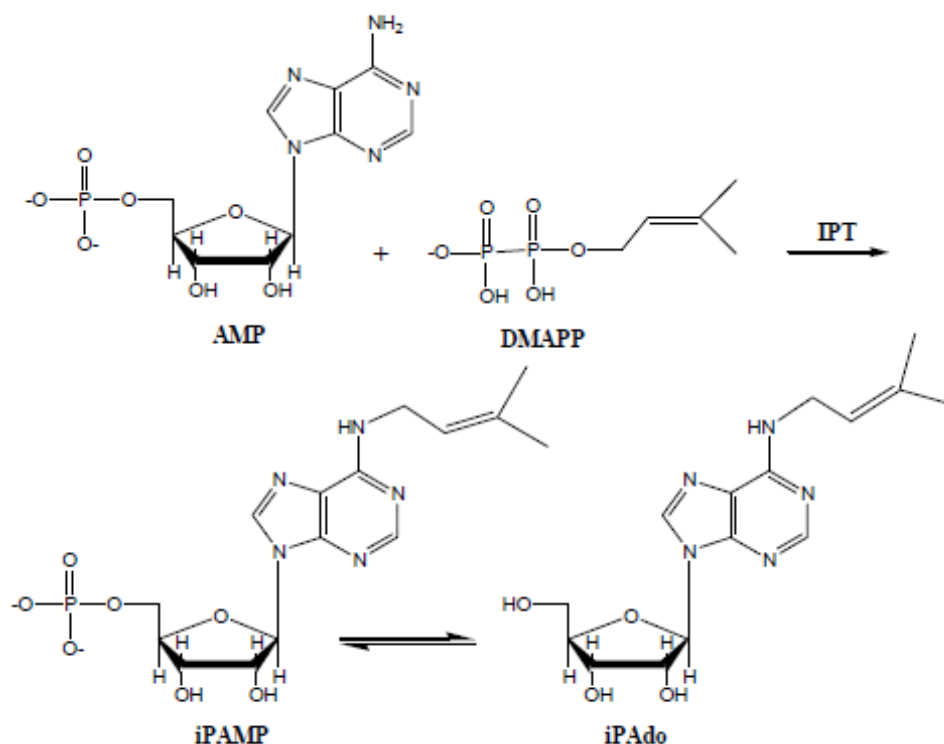


Figure 1.5 Biosynthesis of N⁶-isoPentenyl Adenosine in plants. AMP and DMAPP (dimethylallylpyrophosphate) are converted in (iPAMP) isoPentenyl Adenosine-5'-monophosphate and iPAdo. IPT (isopentenyl transferase) is the key regulatory enzyme of the biosynthesis (reported from ref. [36]).

As a result of this bulk of research carried out during the last two decades, it has been established that CKs play a key role in the hormonal regulation of plant growth and differentiation, but can also induce cell death in plant cell cultures after intracellular conversion to their monophosphates [20].

In plants, most of the studies on the biological activity of cytokinins have been concentrated on cytokinin bases (CKs) that, although occurring at low concentration in plant-tissue extracts [2,12] are generally more abundant than the corresponding ribosides CKRs.

1.3 BIOLOGICAL ACTIVITY OF CYTOKININ RIBOSIDES IN MAMMALIAN CELLS

The effects observed on plant cells have led to the hypothesis that CKs could also affect growth and differentiation in animals. Consequently, these natural compounds could have potentiality for treating human diseases related to dysfunctional cell proliferation and/or differentiation. Abundant experimental evidences supporting these hypotheses have subsequently been obtained. The ability of CKs to induce or promote the differentiation of human cells has been demonstrated in both keratinocytes [21] and a few leukemia cell lines, including HL-60 and K-562 [22].

However, while CKs induce differentiation at relatively high concentrations (25–100 μM), it was soon observed that their ribosides CKRs cause rapid apoptosis of leukemia cell lines at lower micro-molar concentrations than CKs [22, 23]. As in plant cells, cell death in HL-60 is preceded by depletion of adenosine triphosphate, activation of caspases and mitochondrial depolarization [23, 24].

Interestingly, the anticancer activity of CKRs requires the intracellular conversion of CKRs to their respective monophosphates [25].

CKRs have attracted further studies on their antitumor activity and it has recently been demonstrated that kinetin riboside (KR) is a potential drug for the treatment of multiple myelomas because in these tumor cells KR induces a rapid suppression of cyclin D1 and D2 transcription factors, followed by arrest of the cell-cycle and selective apoptosis [26].

Several authors have reported cytotoxic effects of N⁶-isopentenyladenosine (iPAdo), KR and N⁶-benzyladenosine (BA) on human cell lines derived from solid tumors [27-33]. Whether treatments resulted in cell cycle block and/or apoptosis, this was dependent on the cell line and the cytokinin used. The first report of the activity of o-TR has been published by Strnad *et al.* [34].

A general observation is that the concentrations of CKRs required to produce cytotoxic effects are higher than those found endogenously in plant tissues, but they do fall within the range used in plant bioassays [25, 35, 36].

Compared to the tested CKRs, the free bases CKs typically had much weaker effects on cell proliferation, with IC values either over the highest concentration used (>166 μM) or at least 50 times higher than the IC₅₀ values determined for their respective ribosides. Similar differences between the cytotoxic activity of cytokinin bases (K, iPAdo, and B) and their corresponding ribosides were reported by other authors [24, 25].

The observation that cytokinin bases and cytokinin glucosides showed limited activity, or none at all, supports the hypothesis that the presence of a ribose moiety at N9 of the purine ring is essential for potent anticancer activity in cytokinins.

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2. THE Ph.D PROJECT

The Ph.D project reported in the present thesis has been designed in view of exploring a few specific biological activities of CKRs. We selected the isoprenoid N⁶-(Δ^2 -isopentenyl) adenosine (iPA) and the aromatic N⁶-furfuryl adenosine (kinetin riboside, KR), N⁶-benzyladenosine (BA) and topolin riboside (p-TR) as the most representative natural CRKs (Fig. 2.1).

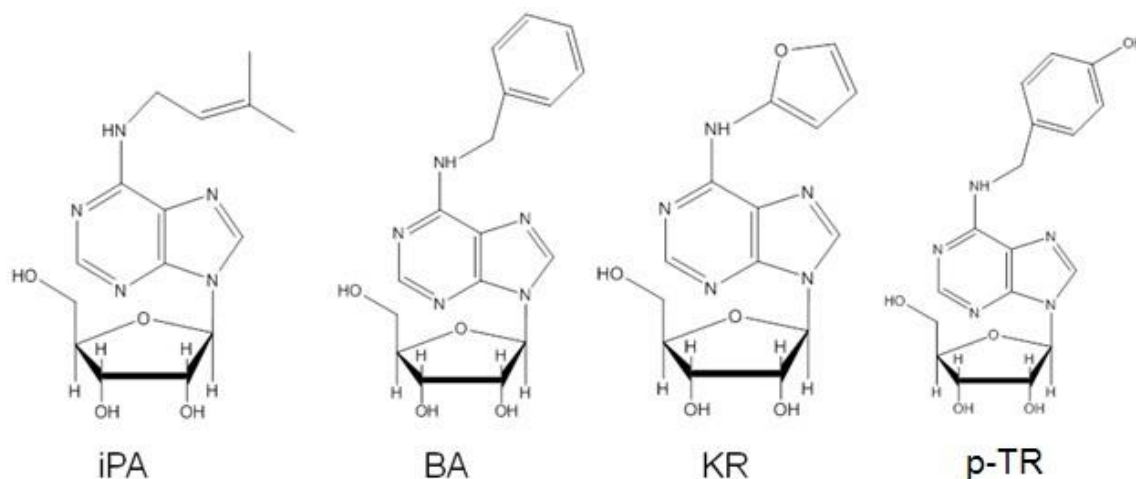


Figure 2.1 Structures of N⁶-(Δ^2 -isopentenyl) adenosine, kinetin riboside, N⁶-benzyladenosine and topolin riboside.

In the chapter 1, the wide spectrum of biological activities of CKs and CKRs has been reviewed. In this thesis we have mainly investigated:

1. the effect CKRs on platelet aggregation (in collaboration with the laboratory of hematology and thrombosis directed by Prof. Marco Cattaneo, Full Professor of Internal Medicine at Università degli Studi di Milano, Department of Health Sciences c/o San Paolo Hospital of Milan).
2. the *in silico* interaction between CKRs and P2Y₁₂ receptor, a chemoreceptor for adenosine diphosphate (ADP) that has been recently recognized to have a key role in platelet activation and thrombogenesis. This part of the thesis has been developed in collaboration with Prof. Giulio Vistoli, Associated Professor of Pharmaceutical Chemistry at Department of Pharmaceutical Sciences, Università degli Studi di Milano).
3. the effect of CKRs and some synthetic N⁶ substituted adenosines on A₁, A_{2A}, A_{2B}, A₃ adenosine receptors. The synthesis of adenosine derivatives was performed

during six months of the Erasmus placement fellowship spent in Portugal under the supervision of Prof. Fernanda Borges (Department of Chemistry and Biochemistry, University of Porto, Portugal). CKRs were evaluated for their activity as antagonists of adenosine receptors also under the supervision of Prof. Borges and in collaboration with Prof. Karl N. Klotz of the Institute of Pharmacology and Toxicology of the University of Würzburg, Germany.

4. Other biological activities of natural and non-natural CKRs: under the supervision of Prof. Borges, CKRs were tested as inhibitors of acetylcholinesterase and of monoamine oxidase B. In collaboration with Dott. Alberto Scarafoni, we evaluated the capacity of Kinetin and Kinetin riboside to inhibit the oxidation/glycooxidation of bovine serum albumin (BSA)
5. the *in vitro* antioxidant activity of CKs, CKRs and synthetic N⁶-substituted adenosines using various antioxidant tests. The antioxidant activity was carried out in collaboration with Prof. Marina Carini, Full Professor of Pharmaceutical Chemistry at Department of Pharmaceutical Sciences, Università degli Studi of Milano.
6. the effect of topolin riboside (p-TR) and its synthetic analogs on 661W cell line.
7. the anticancer activity of topolin riboside (p-TR) and its synthetic derivatives analogs on Neuro2A cell line.

The first four points of this Ph.D project were developed under the supervision of Prof. Enzo Santaniello, Full Professor of Medical Chemistry (BIO/10) (Università degli Studi of Milan, Department of Health Sciences c/o San Paolo Hospital of Milan).

The last part of the project was performed under the guide of Prof. Riccardo Ghidoni, Full Professor of Biochemistry (Università degli Studi of Milan, Department of Health Sciences c/o San Paolo Hospital of Milan) after the official retirement of Prof. E. Santaniello on November 1st, 2014.

3. *IN VITRO* ACTIVITY OF CYTOKININ RIBOSIDES AS PLATELET AGGREGATION INHIBITORS AND *IN SILICO* EVALUATION OF THEIR INTERACTION WITH THE P2Y₁₂ RECEPTOR

3.1 PLATELET AGGREGATION MECHANISM

3.1.1 Platelet adhesion

Platelets are cytoplasm fragments that originate from megakaryocytes in the bone marrow and circulate to maintain the integrity of the vascular system [1]. Platelets are found only in mammals while in others animals, such as birds or reptiles, thrombocytes circulate as intact cells. They don't interact with the inner surface of vessels but adhere promptly where the endothelium is altered or extracellular matrix substrates are exposed [2]. This is a critical step in hemostasis and thrombosis mechanisms, as in inflammatory and immunopathogenic responses [3-5]. The functions of mammalian platelets are conserved throughout evolution and reflect those of nucleated thrombocytes in the other vertebrates [6-9]. After the adhering to damaged vessel, platelets can rapidly recruit to the site of injury another platelets, which are necessary to achieve hemostasis, or different cell types of immune system, which set off host defense responses.

Membrane receptors operate independently of cellular activation mediate the initial binding of platelets to the injured vessel wall. This facilitates rapid interactions to overcome the constraints on bond formation inducted by blood flow, leading to a monolayer of activated platelets being firmly adherent to the area of injury. Depending on the depth of the lesion, platelets come into contact with different extracellular matrix components that may be associated with other molecules in response to injury or derived from plasma, as in the case of collagen with blood glycoproteins. Proteoglycans, collagen type IV, entactin, laminin and fibulin are the main basement membrane constituents, exposed to circulating blood after a superficial breaking of endothelium integrity. Proteoglycans play a key role in the coagulation modulation, but may not be directly involved in platelet adhesion [10]. Collagen type IV, similar to many other types [11], can induce platelet responses but might be less effective than other collagen types, found in the vascular wall, especially types I, III and VI. Two platelet membrane glycoproteins (GPs), integrin- $\alpha_2\beta_1$ and GPIIb/IIIa, interact directly with collagen. Other GPs are supposed collagen receptors, some selective for specific types, but their identification and functional properties have not yet been established clearly [12]. Platelets seem to have a mechanism with which to inhibit the

response to collagen and avoid the risk of unregulated thrombus formation and vascular occlusion [13]. Collagen receptors can't start and propagate thrombus formation in conditions of high blood flow unless platelets are initially bound to the surface through the interaction between GPIb α and Von Willebrand factor (vWF) glycoprotein to collagen [14,15]. Platelets express laminin receptors and have the potential to join to this substrate through $\alpha_2\beta_1$ receptor, but it's uncertain if the interaction has pathophysiological relevance with regard to hemostasis and thrombosis. Fibulins can combine with fibronectin [16] and fibrinogen [17] and possibly regulate the thrombogenic activity of these substrates. In particular, fibrinogen bound to fibulin may exhibit high capacity to promote platelet adhesion and thrombus formation [18]. Several types of fibulin, present in circulating blood, may be localized at sites of vascular injury, potentially influencing platelet interaction with fibrinogen and fibrin. Fibrinogen and fibrin aren't normal components of the vessel wall. Fibrinogen may become immobilized on the exposed extracellular matrix, and insoluble fibrin strands are generated as a consequence of the local induction of coagulation. Integrin- $\alpha_{IIb}\beta_3$ can selectively regulate platelet adhesion to both fibrinogen and fibrin, showing a greater specificity on unstimulated platelets than after activation [14]. Additional matrix constituents that become exposed to platelets include fibronectin. Fibronectins [19] are modular macromolecules that support platelet adhesion and interact with $\alpha_5\beta_1$ and activated $\alpha_{IIb}\beta_3$ [20]. Optimal platelet adhesion to fibronectin may require vWF and its GPIb α receptor [21], and the synergistic interactions between these adhesive substrates may contribute to the initiation of thrombus formation.

3.1.2 Platelet activation and procoagulant activity

A stimulation originated from the initial adhesive interactions and from the agonists released or generated at a site of vascular lesion act through signaling networks the adhesive and procoagulant properties of the platelets binding to a injury or circulating in close proximity [22] (Fig. 3.1a). The activation starts with the binding of adhesive ligands and excitatory agonists to specific receptors on the platelet membrane, and is propagated by intracellular signaling reactions, involving enzymes, substrates and co-factors engaged in specific protein–protein and protein–lipid interactions. Activation is under tight negative control, especially through the action of inhibitory substances, such as prostacyclin and nitric oxide, that contribute to limit the thrombus formation within the boundaries of a lesion in the vessel wall.

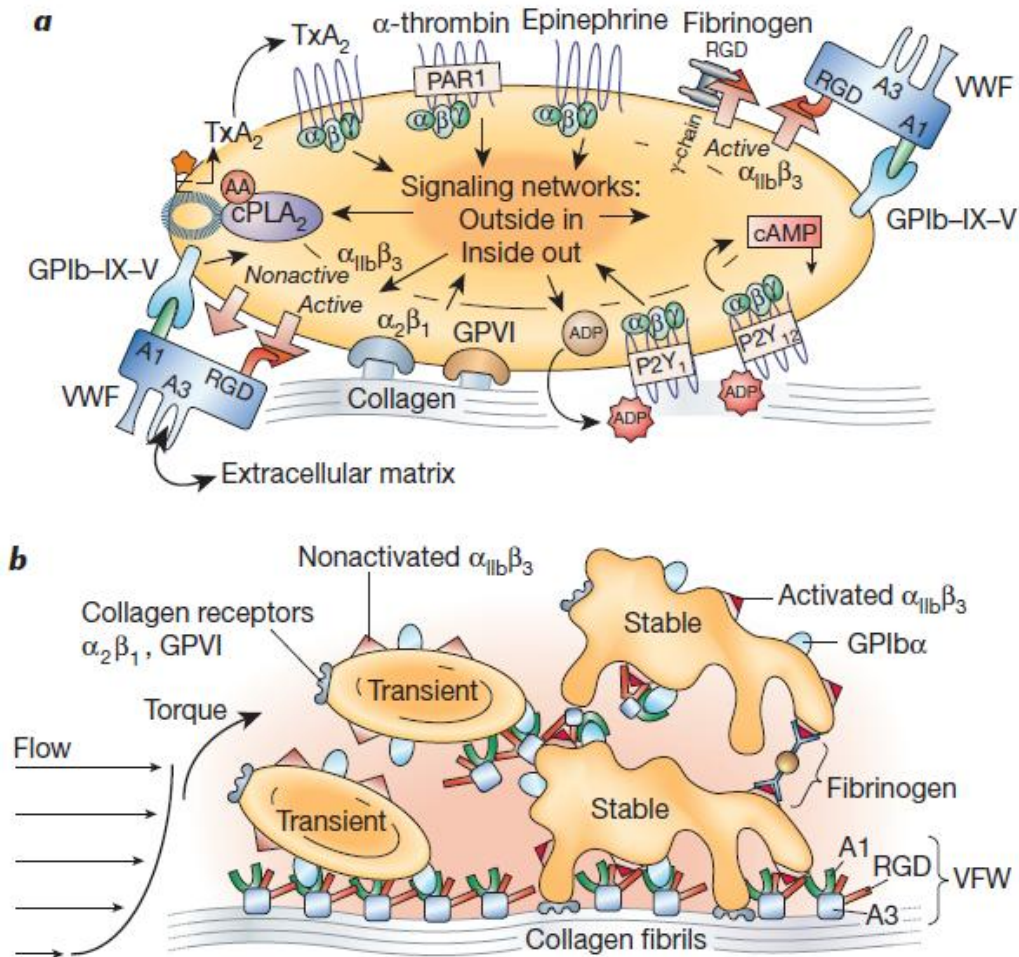


Figure 3.1 Platelet adhesion (a), activation and aggregation (b) (from ref. [1]).

Among the adhesive substrates, the main inducers of platelet activation are collagen and vWF, α-thrombin generated on the membrane of stimulated platelets, ADP released from vascular cells and stimulated platelets, epinephrine, a hormone involved in response to stress and thromboxane A₂. The serine protease α-thrombin activates platelets through G protein-linked protease-activated receptors (PARs) that convert an extracellular proteolytic cleavage event into an intracellular signal [23, 24]. ADP is a weak agonist that directly induces only a form change and reversible platelet aggregation, whereas the consequent secretion and secondary aggregation are caused by the ADP-induced synthesis of thromboxane A₂. On the other hand, ADP is essential in platelet function because, after its secretion from the platelet-dense granules where it is stored, ADP amplifies the responses induced by other agonists. ADP interacts with two specific receptors on platelets surface. The transduction of its signal involves both a transient rise in free cytoplasmic calcium, mediated by the G_q-coupled P2Y₁ receptor, and inhibition of adenylyl cyclase, mediated by the G_i-coupled P2Y₁₂ receptor. The activation of both the G_q and G_i pathways is necessary to induce normal ADP-induced aggregation [25]. Adrenaline and thromboxane

A₂ also activate platelets through specific G protein–coupled transmembrane domain receptors. Thus, the procoagulant activity of activated platelets leads to the generation of thrombin and facilitates the deposition of fibrin within the aggregates to contribute to thrombus stability.

3.1.3 Platelet aggregation

Aggregation is the amplification step that leads to the accumulation of platelets into the hemostatic thrombus. It is mediated by adhesive substrates bound to the membranes of activated platelets. The main effect of activation is a change in the ligand-binding function of integrin $\alpha_{IIb}\beta_3$ [26]. Activation of this receptor contributes to stable adhesion and mediates the immobilization of soluble adhesive proteins, vWF, fibrinogen and fibronectin, on the surface of adherent platelets. This is the substrate where more platelets are recruited. The multiplicity of $\alpha_{IIb}\beta_3$ ligands may explain the residual, but limited, ability to generate thrombi after concurrent obliteration of the fibrinogen and vWF genes [27], in which case fibronectin may support aggregation. Stability of the aggregates is crucial as the rate of growth in determining whether a thrombus will occlude an artery, and recent findings have shown that CD40 ligand, expressed on the membrane of activated platelets, is involved in this process by binding to $\alpha_{IIb}\beta_3$ [28]. CD40 ligand is also involved in the pathogenesis of atherosclerosis, and might represent a connection between platelets and the development of atherosclerotic plaques [29].

3.2 PLATELET ANTI AGGREGATION ACTIVITY OF CYTOKININ RIBOSIDES

3.2.1 Platelet role in thrombosis diseases

Intravascular thrombosis is one of the main causes of a wide variety of cardiovascular diseases. Initiation of a thrombosis is believed to involve platelet adherence and aggregation. In normal conditions, platelets can't aggregate by themselves. However, when a blood vessel is injured, platelets adhere to the disrupted surface, and release some biologically active constituents and aggregates [30]. Platelet thrombi can occlude the coronary arteries of the heart. This event is precipitated by unstable atherosclerotic plaques or altered vascular surfaces after coronary angioplasty.

The extension to which platelet thrombi contribute to cause disturbances in the arterial circulation of the limbs is also not fully clear. In cases not directly resulting from embolism, which is usually cardiac in origin, there is a generally opinion that the cause is thrombosis, triggered by underlying atherosclerotic lesions, and thus that the pathogenesis is similar to that of coronary artery disease [31]. The essential aspects of thrombus formation are probably to be the same whether it is in response to hemorrhage or to a pathological lesion in the arterial wall. However, it's reasonable to assume that differentiating factors may exist, and their identification would be important to find new approaches to the treatment of thrombosis that don't interfere excessively with normal hemostasis. The composition of an atherosclerotic plaque differs from that of the normal arterial wall, but not all lesions are thrombogenic in the same way. The property of inducing a platelet response may be an attribute of unstable or vulnerable plaques, probably in connection with breaking. Local dysfunction of endothelial cells, potentially associated with inflammatory responses, might be important in increasing thrombogenicity, as is the exposure of tissue factor and highly reactive collagens [32].

Platelets react to a variety of activating and inhibitory stimulations that may distinctly influence how an occluding thrombus will form. This variety of responses to environmental conditions indicates that the consequences of inhibiting specific platelet agonists may differ in normal hemostasis and pathological thrombosis, depending on the vessel involved. For example, blood-borne tissue factor localizes at the site of a developing thrombus, where it may contribute substantially to platelet activation through the generation of thrombin [33]. The nature and extent of a vascular lesion may influence the deposition and activity of this tissue factor and the rate of thrombus growth. All aspects of

platelet response to vascular injury may be controlled by genetic variations in the many proteins involved in adhesion, activation and aggregation. The search for polymorphisms in the relevant genes and the evaluation of their correlation to the risk of thrombotic events might prove useful in the identification of suitable targets for pharmacological modulation [34, 35].

3.2.2 Platelet anti aggregation agents

Thrombotic diseases and their consequences may have severe effects. Platelets play a key role in thrombosis, and anti-platelet therapies may prevent as well the thrombotic diseases. Therefore, anti-platelet drugs that can inhibit platelet adhesion, aggregation, release, and activation need to be developed (Fig. 3.2). The anti platelet drugs can be classified on the base of the action mechanism [36].

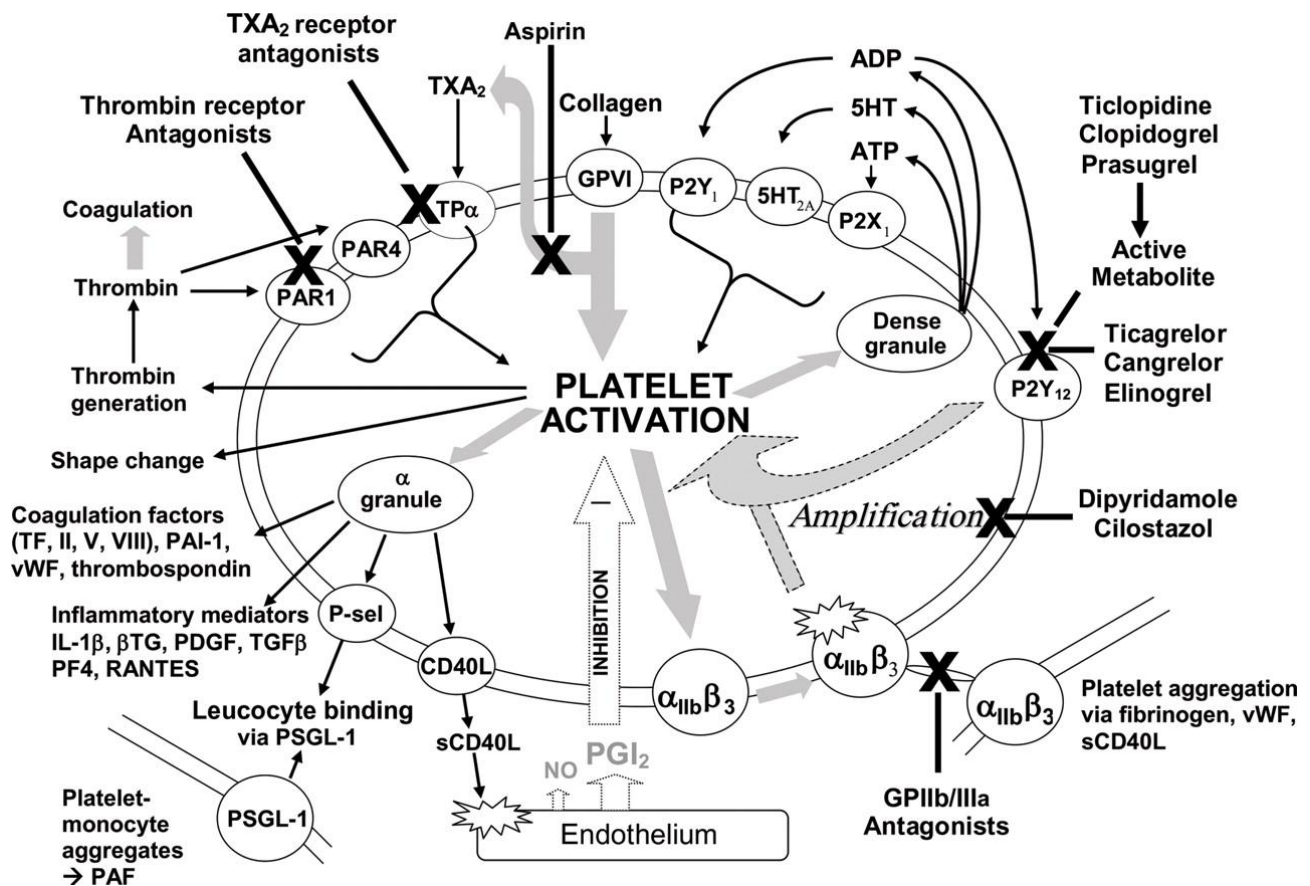


Figure 3.2 Different drugs for anti platelet therapy (from ref. [62]).

It's possible to block platelet aggregation inhibiting GP IIb/IIIa receptors. After the platelet activation, GP IIb/IIIa receptors on the surface of platelets transform into their active states, which can combine with fibrinogen and the von Willebrand factor (vWF). The GP IIb/IIIa receptor operates in the final pathway of platelet aggregation. Blocking the GP

IIb/IIIa receptor, it is possible to inhibit platelet aggregation induced by activating factors. Once platelet aggregation is blocked, platelet thrombi can't form. The development of GP IIb/IIIa antagonists, such as the recently approved abciximab, eptifibatide, and tirofiban, is crucial in anti-platelet therapy. Pharmacodynamic studies on these three agents have revealed their capabilities of establishing and maintaining more than 80% inhibition of platelet aggregation [36, 37].

Platelet aggregation can be also inhibited by the blocking of membrane receptors or interaction with intracellular signaling pathways. cAMP and cyclic guanosine 3'-5'-monophosphate (cGMP) are two important intracellular second messengers for platelet function. Phosphodiesterase (PDE), which is obtained by catalyzing the hydrolysis of cAMP and cGMP, limits the intracellular levels of cyclic nucleotides to regulate platelet function. Therefore, the inhibition of PDEs might confer a strong inhibitory effect on platelets: cilostazol was developed in this way. Unlike aspirin, cilostazol is a reversible platelet inhibitor that can prevent both primary and secondary aggregation. A combination of aspirin and cilostazol might be a good treatment option for these patients [36, 38-39].

Between many natural and synthetic agents available in literature [40-44], only two papers reported results of cytokinins on platelet aggregation. Specifically, only the N⁶-modified nucleobase kinetin was investigated [30, 45].

3.2.3 Platelet anti aggregation activity of N⁶-furfuryladenine (kinetin)

Sheu investigated the platelet anti aggregation activity of kinetin, a cytokinin characterized by the presence of an aromatic furane ring at the N⁶-substitution. Results showed kinetin the concentration-dependent (50-150 μM) inhibition of platelet aggregation in human platelets by kinetin. The inhibitory effect was stimulated by agonist such as collagen (1μg/ml) and arachidonic acid (60 μM). Kinetin inhibited intracellular Ca²⁺ mobilization and phosphoinositidine breakdown in platelets stimulated by collagen in a concentration-dependent manner. In addition, kinetin inhibited thromboxane A₂ formation stimulated by collagen and arachidonic acid and increased the formation of cyclic AMP. The anti platelet activity of kinetin may be involved in the following pathways: kinetin's effect may initially be due to inhibition of the activation of phospholipase C and Na⁺/H⁺ exchanger. This leads to lower intracellular Ca²⁺ mobilization, followed by inhibition of TxA₂ formation and then increased cyclic AMP formation, followed by a further inhibition of the Na⁺/H⁺ exchanger, ultimately resulting in markedly decreased intracellular Ca²⁺ mobilization and

phosphorylation of P47, a platelet protein that is a marker of protein kinase C activation [30].

Based on these results, the authors believe that kinetin could be a potential therapeutic agent for arterial thrombosis, once its toxicity was further assessed [45].

Relying on these results, the first part of this Ph.D project has been focused to investigate the platelet anti-aggregation of the natural kinetin riboside (KR) and other natural CKRs.

3.3 PLATELET ANTI AGGREGATION EXPERIMENTAL SECTION

3.3.1 MATERIALS

N⁶-(Δ^2 -isopentenyl) adenosine (iPA) and the aromatic kinetin riboside (KR), N⁶-benzyladenosine (BA) and topoline riboside (p-TR) were obtained from OIChemIm Ltd. (Olomouc, Czech Republic).

All reagents were purchased from Sigma–Aldrich Italy and were of analytical grade.

3.3.2 METHODS

3.3.2.1 Platelet anti aggregation test

Washed platelets were prepared from autologous platelet-poor plasma according to Mustard *et al.* [46] and counted. In this study, human volunteers gave informed consent. Blood was collected from healthy human volunteers who had taken no medicine during the preceding two weeks. Washed platelets were pre-warmed in the presence of a 0.5% DMSO solution of the CKRs at study and then collagen (2 μ g/ml) was added. The inhibitory activity of CKRs was evaluated using a 25–750 μ M range of concentration and was expressed as percentage of platelet aggregation. The aggregation response was recorded after 3 min and monitored by a light transmission aggregometer (Chrono-Log 560, Havertown, PA, USA). All the measurements were performed in quadruplicate.

For each compound, the concentration of 50% platelet aggregation inhibition (IC₅₀ value) was extrapolated from the related curve using GraphPaD 6 software.

3.4 RESULTS AND DISCUSSION

3.4.1 Platelet anti aggregation activity of cytokinin ribosides

The inhibitory activities of kinetin and of the corresponding riboside KR were evaluated within a 25–750 μM concentration range and the results are reported in Figure 3.3.

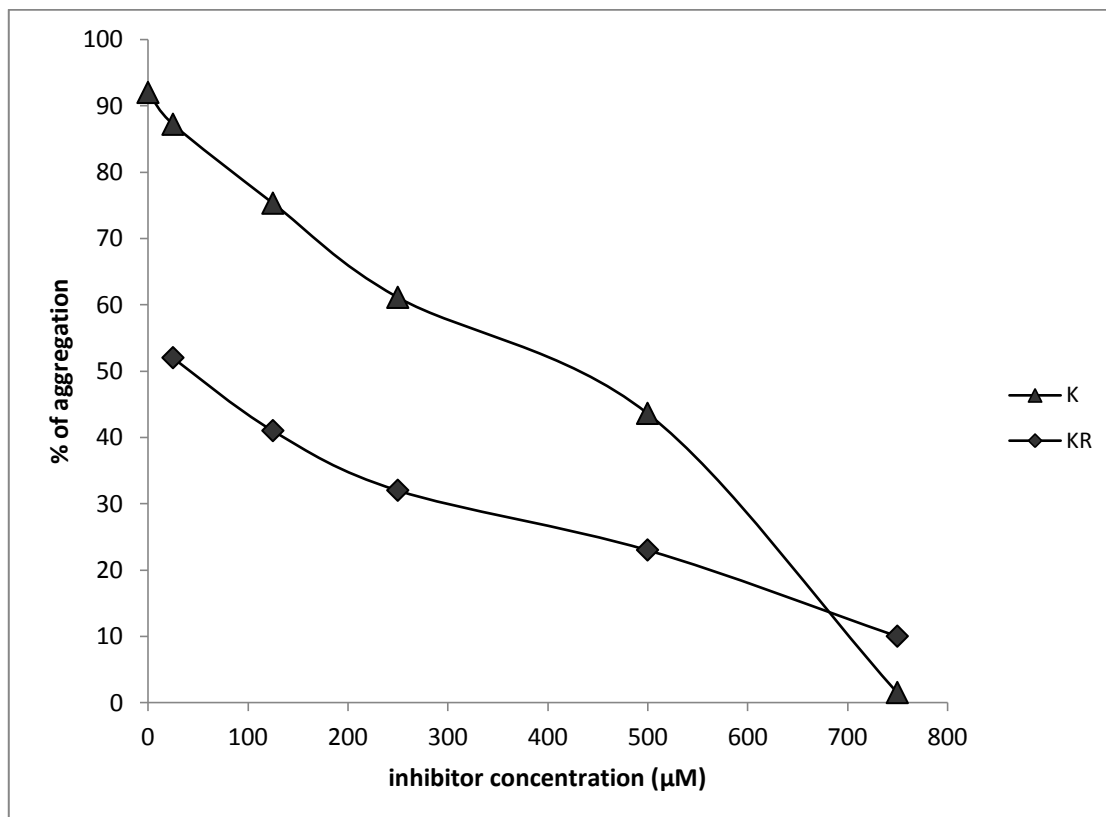


Figure 3.3 Activity of kinetin (K) and kinetin riboside (KR) on collagen-induced aggregation in washed human platelets.

As shown in the graphic, kinetin riboside has been found to be more effective than the corresponding kinetin at tested concentrations. These data are in line with other reported observations, confirming that CKRs are often more active than the corresponding CKs [47-50].

We then evaluated the *in vitro* inhibition of platelet aggregation by The results of this study are shown in Figure 3.4, while the calculated values of IC_{50} for the CKRs are collected in Table 3.1.

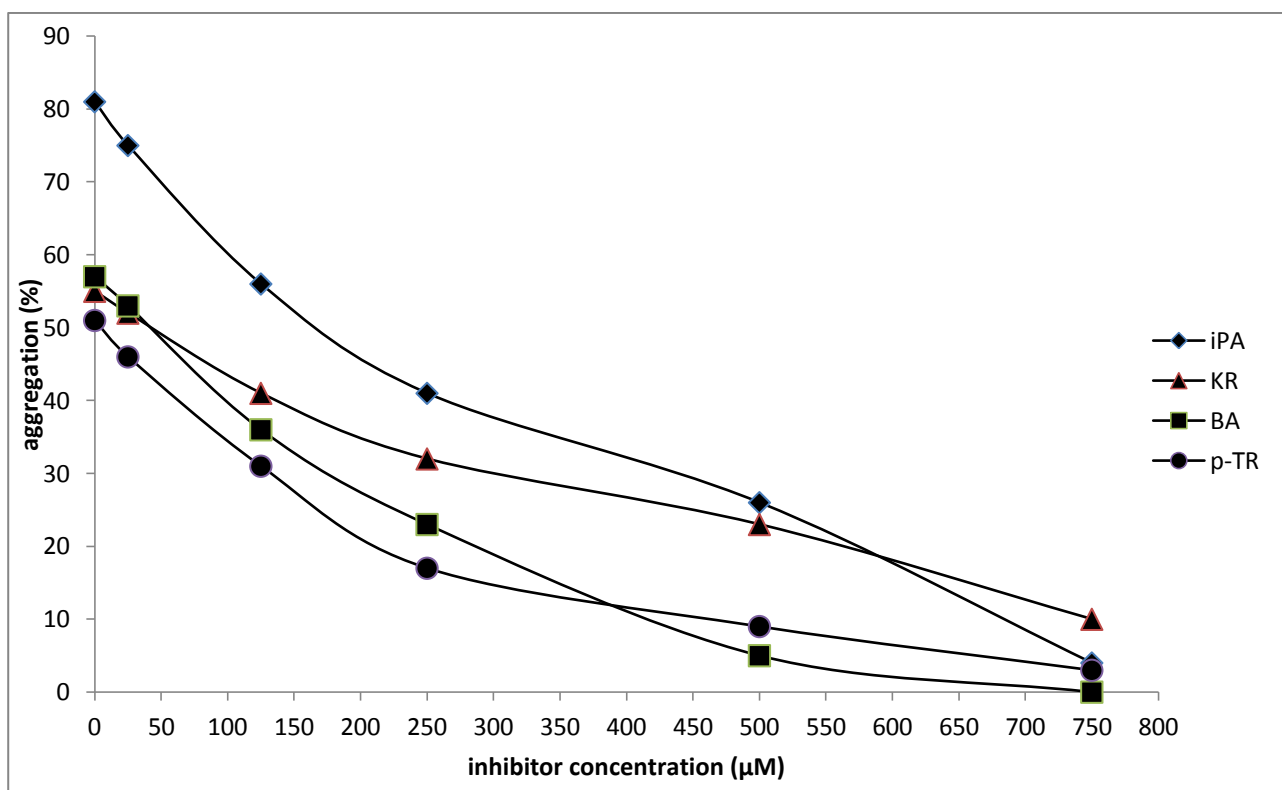


Figure 3.4 Inhibition of collagen-induced platelet aggregation in washed human platelets by CKRs.

The N⁶-4-hydroxybenzyl substituted adenosine (p-topolin riboside) seems the most effective compound in the adopted *in vitro* model of platelet aggregation. The activity of p-topolin riboside is higher than that of kinetin riboside and N⁶-benzyl adenosine, that, in

Table 3.1 IC₅₀ of CKRs against *in vitro* platelet aggregation.

Compounds	IC ₅₀ (µM)
IPAdo	141.87 ± 4.3
KR	41.16 ± 1.8
BA	37.12 ± 1.4
TR	6.77 ± 0.31

^a IC₅₀ value is defined as the concentration of 50% platelet aggregation inhibition and expressed as mean ±SD (n = 3).

turn, show similar activity. The non-aromatic N⁶-substituted adenosine, isopentenyl adenosine is less efficient as inhibitor.

3.5 *IN SILICO* INTERACTION OF CYTOKININ RIBOSIDES WITH THE P2Y₁₂ RECEPTOR

The structures of CKRs have in common a structural characteristic at the N⁶-position that makes them similar to other N⁶-substituted adenosine derivatives that have been investigated for their interaction with the four adenosine receptors A₁, A_{2A}, A_{2B} and A₃. [51-53]. Although, recent binding studies showed that the N⁶-substitution is particularly detrimental for the A₂ recognition [54], the involvement of other receptors can be proposed for explaining the anti-aggregation properties of CKRs. For instance, N⁶-substituted adenosine derivatives, 2-methylthio and 2-ethylthio-6-phenethyl aminoadenosine (BF061 and BF066, Figure 3.5) have been recently studied as anti-platelet and antithrombotic agents.

Their activity has been explained through a dual action on a phosphodiesterase and the P2Y₁₂ receptor [55, 56].

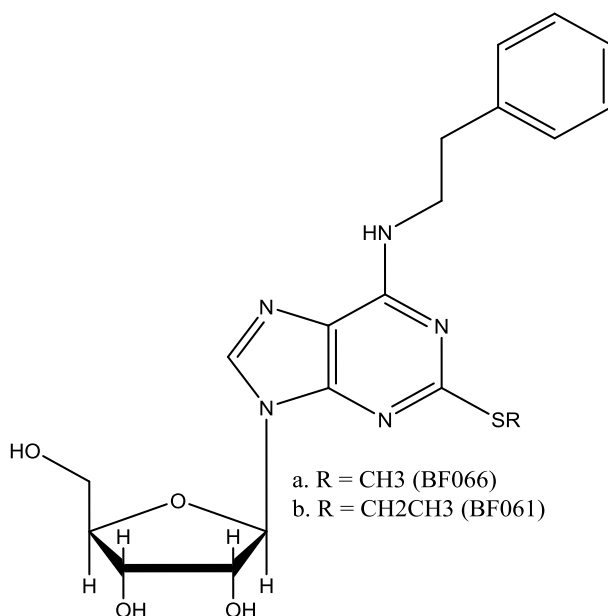


Figure 3.5 Structure of the P2Y₁₂ antagonist compounds BF066 e BF061.

3.5.1 The P2Y₁₂ Receptor

P2Y₁₂ belongs to the G_i class of a group of G protein coupled (GPCR) purinergic receptors (Fig. 3.6) and it is a chemoreceptor for adenosine diphosphate (ADP) [57, 58].

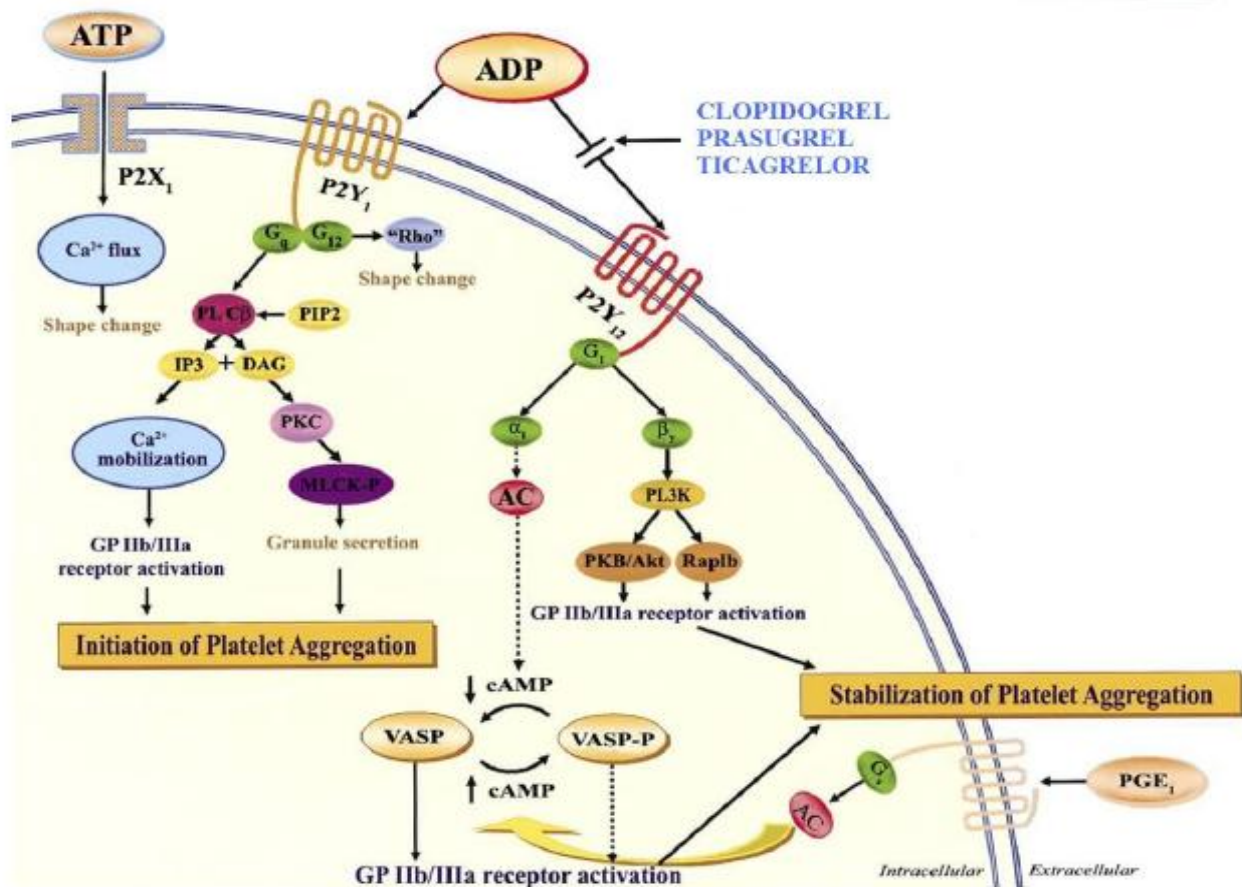


Figure 3.6 Scheme of P2 platelet receptors (ref. [60]).

The overall fold of the P2Y₁₂ receptor structure consists of a seven transmembrane (7TM) bundle of α -helices and a carboxy-terminal helix VIII that is parallel to the membrane bilayer. Several loops, especially ECL2, appear to be flexible and result in a total of 24 unmodelled loop residues (88–91 in ECL1, 133–135 intracellular loop 2 (ICL2), 163–178 ECL2 and 230 in ICL3). Only one disulphide bond is clearly observed in the structure, connecting the amino terminus (C17) with helix VII (C2707.25). Two receptor molecules from adjacent unit cells form receptor–receptor interactions in a parallel orientation mediated by helix V. Two cholesterol molecules are observed bound to each receptor: one is at the interface of helices III and V, stabilizing the receptor–receptor interaction, and the other is at the interface of helices I and VII and does not participate in crystal contacts [59, 60].

However, the importance of P2Y₁₂ lies in its capacity to amplify and sustain platelet activation responses to not only ADP, but also other platelet agonists, including TxA₂, thrombin, and collagen, since activation of platelets by these agonists inevitably lead to release of ADP from dense granules [61].

During the platelet activation, ADP is released from the dense granules, following platelet activation in response to several agonists and stimulates the P2Y receptors on the source,

and near platelets. As a result, this nucleotide, when bound to the P2Y₁₂ receptor, begins the G_i signaling pathway, and brings about irreversible GPIIb/IIIa activation and complete aggregation in response to any platelet agonist [62]. Synergistic activation of the G_q, G₁₃, GPVI, or GPIb-IX pathways, in addition to G_i stimulation, is crucial for these effects [63-66]. Thus, in the absence of P2Y₁₂ activation, platelet aggregation is readily reversible, with GPIIb-IIIa reverting to its inactive form [65, 67].

P2Y₁₂ potentiates these activation responses by sustaining elevated Ca²⁺ levels within the platelet, which would otherwise diminish [61]. This is thought to be mediated by inhibition of adenylyl cyclase (AC) activity and stimulation of PI3K [68]. Induced via endothelial PG_{I2}, adenylyl cyclase regulates platelet activity *in vivo* by the production of cyclic adenosine monophosphate (cAMP), which inhibits intracellular Ca²⁺ release [69]. Consequently, P2Y₁₂ inhibition of AC removes the inhibitory effects of cAMP on Ca²⁺ mobilization; however, Hardy *et al.* demonstrated this was only partially responsible for the calcium levels [68]. P2Y₁₂-mediated activation of PI3K was also shown to contribute the sustained Ca²⁺ levels via activation of Phospholipase C (PLC) [68, 70]. In summary, the P2Y₁₂ receptor is extremely influential in platelet activation, by potentiating platelet secretion and aggregation [62]. Furthermore, P2Y₁₂ activation also promotes procoagulant responses by improving surface membrane phosphatidylserine (PS) expression and contributing to platelet microparticle formation, which are another source of PS and Transferrin. As a result, P2Y₁₂ activity plays an important role in thrombosis. [62, 71, 72].

P2Y₁₂ is an attractive target for anti-aggregation drug discovery [73, 74], due to its essential role in potentiating platelet responses initiated by other important activators such as thrombin and thromboxane, thus representing a critical regulator of hemostasis and thrombosis.

P2Y₁₂ receptor antagonists are anti-thrombotic agents that inhibit platelet function by blocking the ADP at P2Y₁₂ receptor sites. Adenine nucleotides act on platelets via three distinct P2 receptors, namely, two G protein-coupled ADP receptors, P2Y₁ and P2Y₁₂, as well as a P2X₁ receptor ligand-gated cation channel activated by adenosine triphosphate (ATP). The P2Y₁ receptor initiates platelet aggregation, but is not sufficient in response to ADP. On the other hand, the P2Y₁₂ receptor is responsible for the completion of aggregation in response to ADP. The P2Y₁₂ receptor is the molecular target of anti-thrombotic drugs such as clopidogrel, prasugrel, cangrelor, and ticagrelor. This receptor is responsible for most of the potentiating effects of ADP when platelets are activated by agonists such as collagen, thrombin [75, 76]. These platelet antagonists blocking the ADP-

receptor P2Y₁₂ decrease myocardial infarction, stroke, thrombosis and mortality in the patients with cardiovascular diseases [77].

In addition to all previously cited P2Y₁₂ antagonists, the recent report that N⁶-substituted adenosine derivatives, 2-methylthio and 2-ethylthio-6-phenethyl aminoadenosine (BF061 and BF066, Fig. 2.5) have shown an action on the P2Y₁₂ receptor [55, 58] prompted us to study *in silico* the interaction of CKRs with the P2Y₁₂ receptor through the docking simulations described below. This modeling approach was further fostered by recent resolution of the P2Y₁₂ receptor in complex with an antithrombotic drug (ethyl 6-{4-[(benzylsulfonyl) carbamoyl]piperidin-1-yl}-5-cyano-2-methylpiperidin-3-carboxylate, AZJ) [60]. We investigated the *in silico* interaction between CKRs and P2Y₁₂ receptor in collaboration with Prof. Giulio Vistoli, Associated Professor of Pharmaceutical Chemistry at Department of Pharmaceutical Sciences, Università degli Studi of Milan.

3.6 MODELLING EXPERIMENTAL SECTION

3.6.1 Modelling experiment

The recently resolved structure of the P2Y₁₂ receptor in complex with an antithrombotic drug was retrieved from PDB. To remain compatible with physiological pH value, the side-chains of Arg, Lys, Glu, and Asp were ionized, while His and Cys residues were considered neutral by default. The complete structure was carefully checked and then underwent a minimization with backbone fixed until RMS = 0.01 kcal mol⁻¹ Å⁻¹ to preserve the resolved folding. The conformational behavior of adenosine, the two known P2Y₁₂ inhibitors (BF066 e BF061) and the four analyzed ribosides was investigated by a Monte Carlo procedure (as implemented in the VEGA suite of programs) which generated 1000 conformers by randomly rotating the rotors. For each ligand, the so obtained lowest energy structure was then exploited in the following docking simulations which were performed by using the PLANTS software, which finds plausible ligand poses through ant colony optimization algorithms (ACO) as reported by Korb *et al.* [78].

For all docking simulations, PLANTS was used with default settings and without geometric constraints. The search was focused on an 8.0 Å radius sphere around the co-crystallized ligand thus encompassing the entire binding cavity. The simulations were carried out using ChemPlp as score function with speed equal to 1 and 10 pose were generated for each ligand. The so obtained best complexes were minimized keeping fixed all atoms outside a 10 Å radius sphere around the bound ligand to favor the mutual adaptability between

ligand and receptor. The optimized complexes were then used to re-calculate the reported ChemPlp scores as well as the shared volumes.

3.6.2 Results of *docking simulation of cytokinin ribosides with P2Y₁₂ receptor*

Results obtained from docking simulations show that cytokinin ribosides and 2-methylthio and 2-ethylthio-6-phenethyl aminoadenosine (BF061 and BF066) assume a binding mode rather similar to that of the co-crystallized inhibitor AZJ and are engaged in clear contacts within the P2Y₁₂ binding cavity. All simulated ligands show a common interaction pattern which can be summarized as follows: (i) the sugar ring stabilizes reinforced H-bonds with Lys280 and Arg256; (ii) the purine base elicits an extended network of π - π stacking interactions with Tyr105, Tyr109, and Phe252, (iii) the N⁶ amino group (ANH) generates a H-bond with Cys194, while the N⁶-linked unsaturated moiety is engaged in π - π stacking with Tyr105 and Tyr109. The above mentioned interactions are clearly documented in Figure 3.7 which shows the putative complex between the P2Y₁₂ receptor model and the

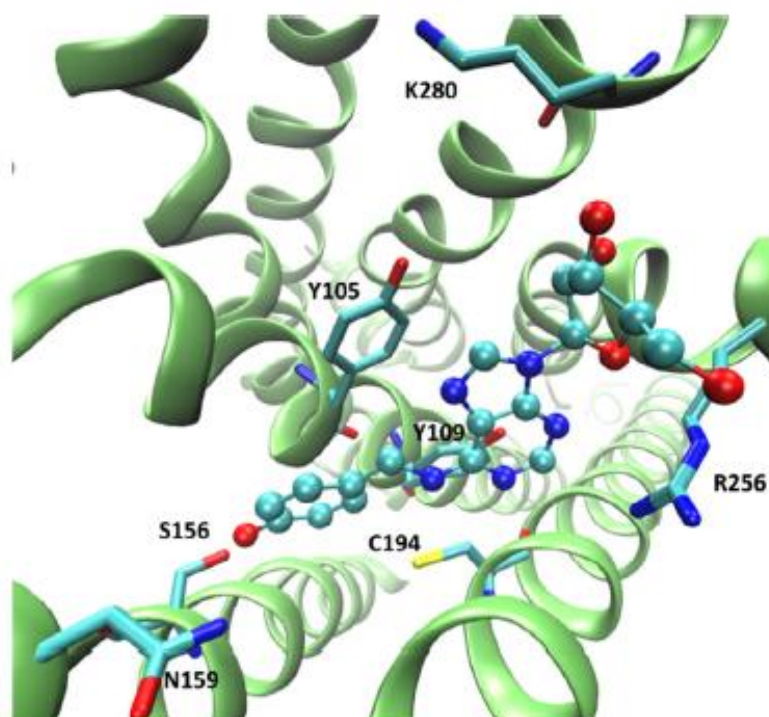


Figure 3.7 Main interactions stabilizing the putative complexes between P2Y₁₂ and topolin riboside.

topolin riboside. whose anti platelet activity is the highest among the CKRs tested by us, as suggested by the IC₅₀ values (Table 3.1). Besides the common contacts, the phenolic hydroxyl group elicits H-bonds with Ser156 and Asn159. Overall, such an interaction

pattern can explain the beneficial role of an aromatic ring connected to the N⁶ atom which can stabilize π - π stacking contacts with the above mentioned aromatic residues. Also the iso-pentenyl moiety of the CKR iso-pentenyl adenosine can elicit stacking interactions through its double bond although their role is less relevant if compared to aromatic CKRs. Table 3.2 reports the ChemPlp docking scores as computed for the minimized complexes. Except for adenosine (Ado), all compounds show docking scores which are comparable with (or at most a little worse than) that of AZJ. The successful poses of the tested

Table 3.2 Plp95 docking scores (kcal/mol) and shared volumes as computed for the simulated P2Y₁₂ complexes.

Compound	ChemPlp score (Kcal/mol)	Shared Volume with AZJ (Å ³)
AZJ	-99,93	----
Ado	-72,61	139,4
iPAdo	-83,07	187,9
KR	-93,08	205,4
BA	-95,11	205,2
TR	-85,57	183,4

compounds within the P2Y₁₂ binding site are further witnessed by the significant shared volumes with the co-crystallized antagonist, AZJ. These results overall emphasize the capacity of N⁶-substituted adenosine derivatives CKRs to interact with P2Y₁₂ assuming a binding mode in line with that of ligands. Notably, the analyzed ribosides show score values markedly better than that of adenosine, which is indeed unable to stably bind P2Y₁₂. This emphasizes the markedly beneficial effect of N⁶ substitution on the P2Y₁₂ affinity.

Of note, the reported scores are in line with the observed *in vitro* anti-aggregation activity values thus indicating that the measured biological activity may be roughly ascribed to the interaction with the P2Y₁₂ receptor. In particular, docking scores emphasize the beneficial role played by the phenolic function thus justifying the highest activity of p-topolin riboside, as expressed by its IC₅₀ value.

3.7 CONCLUSIONS

In conclusion, we have shown that naturally occurring cytokinin ribosides are inhibitors of *in vitro* platelet aggregation (IC_{50} 6.77–141 μ M). The docking study herein reported suggests a putative affinity of CKRs for the ADP receptor $P2Y_{12}$, an attractive target for drug discovery of platelet anti-aggregation agents. The results obtained from the coagulation assay could be caused by a number of different events, including blockade of target related signaling present in platelets [72, 73]. Further investigations are required in order to clarify this and other aspects of the anti-aggregation effect of cytokinin ribosides activity herein described.

In this context, we have carried out preliminary experiments aimed to evaluate the platelet anti aggregation activity of previously tested CKRs on human washed platelets from autologous platelet-poor blood. This is a simplified model for the absence of many components of the blood. Consequently, the true efficacy of a compound as anticoagulant should be evaluated using tests carried out on the whole blood. We used only a single concentration of 50 μ M for each compound. The procedure was the same described above, except that we used in this case the blood with all its components. The results are reported in Figure 3.8. We found that only aromatic CKRs (BA, KR and p-TR) were active as anti platelet aggregation agents. Similarly to the previous results obtained from washed platelets from plasma, p-TR shows the highest value of inhibition (26%), while the activity of BA and KR were similar, but lower than TR (8% and 9%, respectively). The activity of p-TR might be explained as due to the phenolic group in N^6 -position, a functional group

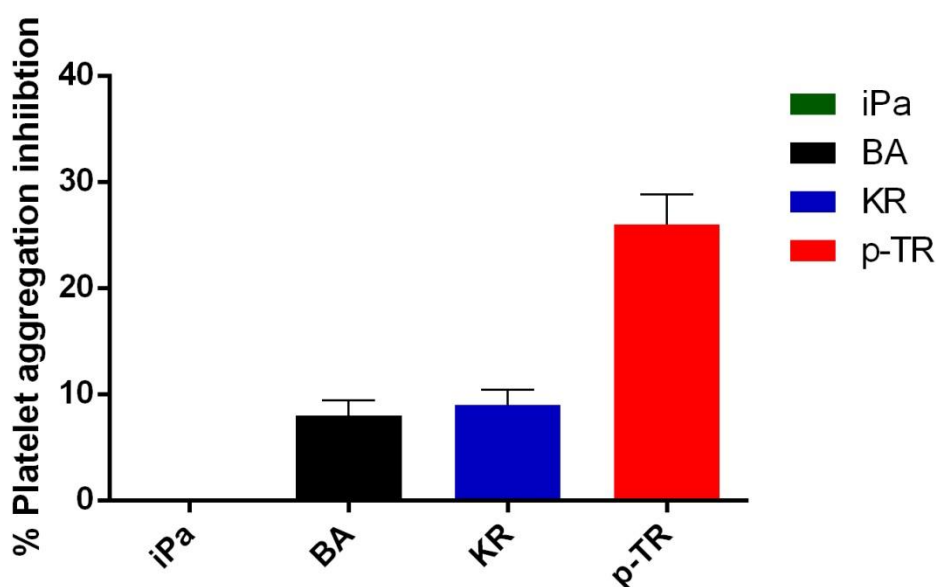


Figure 3.8 Inhibition of platelet aggregation of CKRs in whole blood.

present in many other natural anti platelet aggregation compounds such as hydroxytyrosol, resveratrol [40-41] and *per se* endowed with antioxidant activity.

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4. SYNTHESIS OF NATURAL AND NON-NATURAL N⁶ – SUBSTITUTED ADENOSINE DERIVATIVES

Two main procedures can be followed for the preparation of CKRs and we have experimentally applied both synthetic approaches to the preparation of N⁶ – isopentenyladenosine (iPA). The general scheme is reported in Figure 4.1.

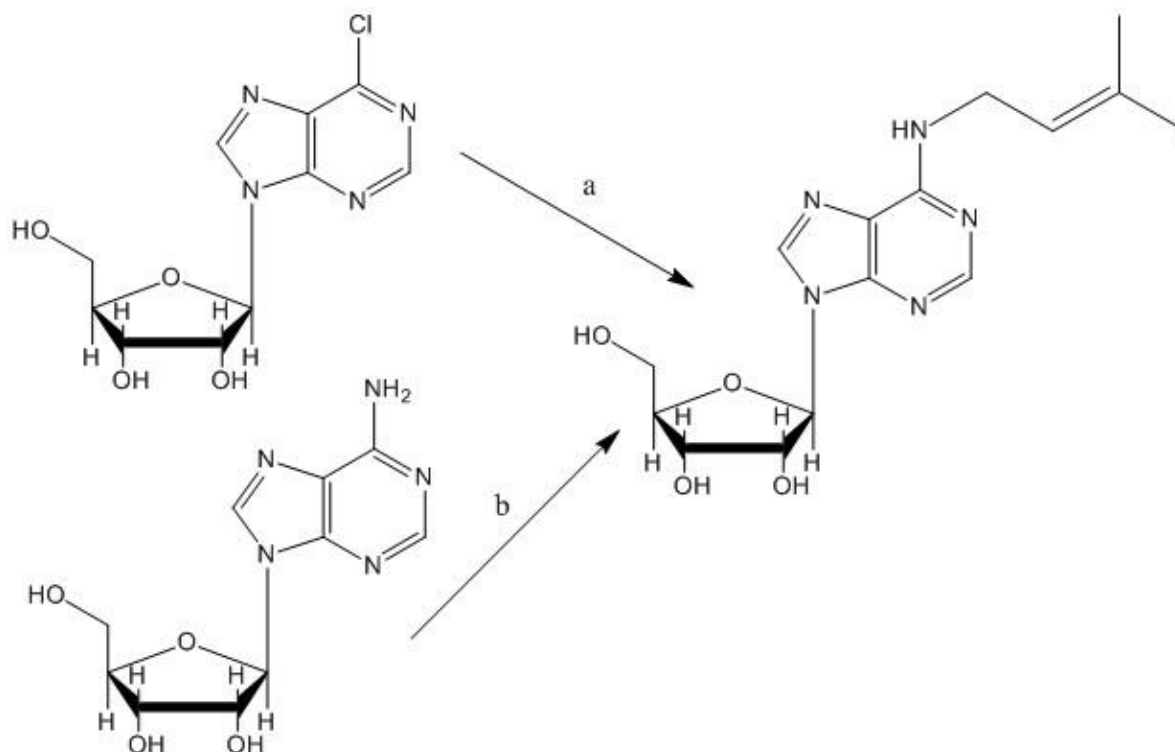


Figure 4.1: Synthesis of iPA. Reagent and conditions: (a) 3,3-dimethylallylamine, K₂CO₃, DMF, 120°C, 3h; (b), isopentenylbromide, BaCO₃, DMF, NH₄OH, reflux, 5h

In the first way (path a), iPA was prepared following the general procedure for chlorine substitution starting from 6-chloropurine riboside and 3,3 – dimethylallylamine. Thus, K₂CO₃ (4.5 mmol) and 3,3 – dimethylallylamine (4.5 mmol) were added to a solution of 6-chloropurine riboside (1.5 mmol) in DMF (20 ml). The mixture was heated at 120- 130°C for 3h, cooled to room temperature, filtered on celite pad and the solvent was removed under vacuum.

In the second path (b), the alkylation of adenosine with isopentenylbromide was performed, followed by the alkaline rearrangement called Dimroth rearrangement. The Dimroth rearrangement is an isomerization of the purine nitrogen atoms that consists in a translocation of endo- or exocyclic heteroatoms through a ring-opening/ring-closure sequence. It can be catalyzed by acids, bases, heat or light [1-3].

Taking into account all the described procedures in literature [4-9], we have found that a combination of the two was more efficient than the previously described protocols [5, 9]. More specifically, we have used ammonium hydroxide for the basic Dimroth rearrangement. The reagent is less expensive and easy-to-use than a solution of gaseous NH₃ whose preparation requires a specific chemical apparatus. When performing the conversion, we operated some simplifying changes, e.g. by avoiding laborious filtration of reaction mixture after N1-alkylation, evaporation of toxic DMF, co-evaporation of solvent [6], that, however, did not significantly influenced the yield.

Finally, we have later observed that the reaction can be performed N,N-dimethylacetamide (DMA) rather than in N,N-dimethylformamide (DMF). This can be advantageous because the boiling point of DMA is lower than of DMF and DMA seems to be less toxic than DMF.

4.1 EXPERIMENTAL SECTION

4.1.1 MATERIALS

6-chloropurinosides and adenosine were obtained from OlChemIm Ltd. (Olomouc, Czech Republic).

All the other reagents and solvents were purchased by Sigma-Aldrich and were of analytical grade.

4.1.2 INSTRUMENTS

Melting points were determined with a Stuart Scientific SMP3 melting point apparatus and left uncorrected.

¹H-NMR spectra of FEA and 2HFEA were registered in Portugal on a Bruker AVANCE III HD spectrometer equipped with a 5 mm broadband reverse probe with field z-gradient operating at 400 MHz while ¹H-NMR spectra of 2FEA was registered on a Bruker AVANCE 500 equipped with a 5 mm broadband reverse probe with field z-gradient operating at 500 MHz. ¹³C-NMR spectra were also registered on the Bruker AVANCE 500 spectrometer with field z-gradient operating at 125.76 MHz.

All NMR spectra were recorded at 298 in DMSO-*d*₆ (isotopic enrichment 99.95%) solution and the chemical shifts were reported on a δ (ppm) scale and coupling constants (*J*) are given in Hertz.

Mass spectra were obtained from a Bruker ICR-FTMS APEX II mass spectrometer using an ESI (Electrospray Ionization) source.

4.1.3 SYNTHESIS OF NATURAL CITOKININ RIBOSIDES

4.1.3.1 N⁶-isopentenyladenosine (iPa)

To a solution of adenosine (0.075 mmol) in DMF (1.5 ml), BaCO₃ (0.128 mmol) and 3,3-dimethylallylbromide (0.128 mmol) were added. The mixture was stirred at room temperature for 37 h, while protected from light and humidity. TLC indicated that N1-alkylation was about 90% complete. To the heterogeneous reaction medium water (1.5 ml) was added and the pH was adjusted to 10.0 with ammonium hydroxide and the solution was refluxed for 5.0 hr. The pH of the solution was maintained at 10.0 by periodic additions of ammonium hydroxide. The solution was cooled to room temperature and was extracted with three 5-ml portions of ethyl acetate. Chromatographic analysis (MeOH:CH₂Cl₂ 10:90) showed complete extraction of iPA_{do} into the ethyl acetate while adenosine remained in the aqueous phase. The ethyl acetate solution was dried over sodium sulfate, evaporated to dryness *in vacuo*, and the residue was, crystallized from 0.8 ml of acetonitrile-ethanol (3:1), furnishing 18.0 mg crystals. On recrystallization from 1 ml of acetonitrile-ethanol (3:1) pure crystalline iPA_{do} (14.8 mg, 59% yield) was obtained, which showed identical physico-chemical properties with the previously described compound [5, 8, 9] and with commercial sample.

4.1.3.2 p-Topolin Riboside (p-TR)

Topolin ribosides was obtained according to the general procedure described by Tarkowská *et al.* [10] with slight modification. Briefly, 858 mg (3 mmol) of 6-chloropurino riboside were heated with 494 mg (4 mmol) of 4-hydroxybenzylamine and triethylamine (695 µl, 5 mmol) in 15 ml of 2-propanol to 82 °C for 7 hours. The reaction was monitoring by TLC (CHCl₃/MeOH/NH₄OH 70:30:3). After cooling, the precipitated product was collected by filtration, washed with cold water and 2-propanol, and crystallized from ethanol or DMF. The obtained product showed identical physico-chemical properties with the commercial sample.

4.1.4 SYNTHESIS OF NON-NATURAL CITOKININ RIBOSIDES

4.1.4.1 p-hydroxyphenylethyladenosine (FEA)

Title compound was prepared according to Doležal *et al.* [11], with some modifications, from the reaction between 6-chloropurino riboside and tyramine. In a two neck balloon, 143 mg (0.5 mmol) of 6-chloropurino riboside and 434 mg (2.5 mmol) of tyramine

monochloride salt were suspended to 25 ml of ethanol. Then, 2 ml of diisopropylamine (12mmol) were added to the mixture under stirring. The reaction was refluxed for 6 hours, monitoring with TLC (CH₂Cl₂:MeOH 80:20), cooled overnight, filtered on filter paper and concentrated with the rotavapor. The solid residue was extracted two times with ethyl acetate and the organic phase was dried with Na₂SO₄, filtered and concentrated with rotavapor. The raw compound was crystallized with methanol. Compound: white/yellow solid (100 mg, 50% yield), mp: 194 °C ; ESI-MS *m/z* 388,16243 (M+1); ¹H-NMR (DMSO-d₆ 400 MHz): 9.15 (s, 1H, OH), 8.34 (s, 1H, CH), 8.23 (s, 1H, CH), 7.04 (s, 2H, CH), 6.67 (s, 2H, CH), 5.89 (s, 1H, CH), 5.4 (s, 1H, NH), 5.18 (s, 1H, CH), 4.62 (s, 1H, OH), 4.61 (s, 1H, CH), 4.16 (s, 1H, OH), 3.97 (s, 1H, OH), 3.78 (s, 2H, CH₂), 3.57 (s, 2H, CH₂), 2.81 (s, 2H, CH₂). ¹³C-NMR (DMSO-d₆, 125,76 MHz): 148.68 (C-6), 146.62 (N6, C-2), 139.07 (C-2), 129.81 (C-4), 124.95 (C-8), 115.62 (N6, C-5), 88.02 (N6, C-4), 86.13 (N6, C-6), 74.54 (N6, C-3), 70.82 (N6, C-1), 61.82 (C-1'), 58.84 (C-4'), 40.55 (C-2'), 40.38 (C-3'), 40.21 (C-5'), 25.96 (CH₂), 8.47 (CH₂).

4.1.3.4 3,4-dihydroxyphenylethyladenosine (2HFEA)

Title compound was synthesized with a similar procedure to that of FEA. Briefly, 500 mg (1.75 mmol) of 6-chloropurino riboside and 445 mg (2.35 mmol) of dopamine monochloride salt were suspended in 20 ml of 2-propanol. Then, 405 µl of triethylamine were added to the mixture under stirring. The reaction was refluxed for 4 hours, monitoring with TLC (CH₂Cl₂:MeOH 80:20), cooled overnight and concentrated with the rotavapor. The raw compound was purified on a silica column using CH₂Cl₂:MeOH 80:20 as mobile phase. The product was dried with Na₂SO₄, filtered and concentrated with rotavapor. The compound was crystallized with hexane and methanol. Compound: white/yellow solid (568 mg, 83% yield), mp: 196 °C; ESI-MS *m/z* 404.1384 (M+1); ¹H NMR (DMSO-d₆ 400 MHz): 8.94 (s, 2H, OH), 8.33 (s, 1H, CH), 8.23 (s, 1H, CH), 6.64 (s, 1H, CH), 6.62 (s, 1H, CH), 6.49 (s, 1H, CH), 5.90 (s, 1H, CH), 4.61 (s, 1H, CH), 4.60 (s, 1H, CH), 4.14 (s, 1H, OH), 3.97 (s, 1H, CH), 3.96 (s, 1H₃., OH), 3.95 (s, 1H, OH), 3.57 (s, 2H, CH₂), 2.74 (s, 2H, CH₂), 2.08 (s, 2H, CH₂). ¹³C-NMR (DMSO-d₆, 125.76 MHz): 155.0 (C-6), 152.9 (C-2), 145.5 (C-4), 144.0 (N6, C-6), 140.0 (N6, C-1), 130.7 (C-8), 119.7 (N6, C-4), 116.5 (N6, C-2), 116.0 (N6, C-5), 88.4 (N6, C-4), 86.4 (C-5), 74.0 (C-1'), 71.1 (C-4'), 62.2 (C-2'), 46.2 (C-3'), 42.1 (C-5'), 39.5 (CH₂), 34.9 (CH₂).

4.1.3.5 3,4-dihydroxyphenyladenosine (2FEA)

Title compound was synthesized with a similar procedure to that of 2HFEA. Briefly, 250 mg (0.77 mmol) of 6-chloropurino riboside and 575 mg (2.60 mmol) of 3,4-dihydroxybenzilamine monochloride salt were suspended in 20 ml of 2-propanol. Then, 405 μ l of triethylamine were added to the mixture under stirring. The reaction was refluxed for 4 hours, monitoring with TLC (CH_2Cl_2 :MeOH 80:20), cooled overnight and concentrated with the rotavapor. The raw compound was purified on a silica column using CH_2Cl_2 :MeOH 80:20 as mobile phase. The product was dried with Na_2SO_4 , filtered and concentrated with rotavapor. The compound was crystallized with ethanol and DMF. Compound: white/yellow solid (149.8 mg, 50% yield), mp: 186 °C; ESI-MS m/z 390.1420 (M+1); ^1H NMR (DMSO- d_6 500 MHz): 9.71 (s, 2H, OH), 8.36 (s, 1H, CH), 8.18 (s, 1H, CH), 8.18 (s, 1H, CH), 6.94 (s, 1H, CH), 6.65 (s, 1H, CH), 6.64 (s, 1H, CH), 6.60 (s, 1H, CH), 6.06 (s, 1H, CH), 5.89 (s, 1H, NH) 4.61 (s, 1H, CH), 4.60 (s, 1H, CH), 4.21 (s, 1H, OH), 4.01 (s, 1H, CH), 4.00 (s, 1H, OH), 3.70 (s, 1H, OH), 3.59 (s, 1H, OH), 3.59 (s, 1H, CH₂), 3.57 (s, 1H, CH₂). ^{13}C -NMR (DMSO- d_6 , 125.76 MHz): 152.8 (C-6), 152.2 (C-2), 152.1 (C-4), 149.7 (C-8), 146.2 (N6, C-6), 145.4 (N6, C-1), 131.8 (N6, C-4), 118.7 (N6, C-3), 118.6 (C-5), 115.8 (N6, C-2), 115.5 (N6, C-5), 88.6 (C-1'), 88.4 (C-4'), 74.4 (C-2'), 70.6 (C-3'), 61.5 (C-5'), 45.9 (CH₂).

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5. INTERACTION OF NATURAL AND SYNTHETIC CYTOKININ RIBOSIDES WITH THE ADENOSINE RECEPTORS A₁, A_{2A}, A_{2B}, A₃

5.1 ADENOSINE RECEPTORS

The adenosine receptors, also called P1 receptors, are a class of purinergic G protein-coupled receptors with adenosine as endogenous ligand [1, 2]. They are metabotropic type monomers, composed by a single peptide chain crossing seven times the cell membrane and interacting with a G protein (Fig. 5.1).

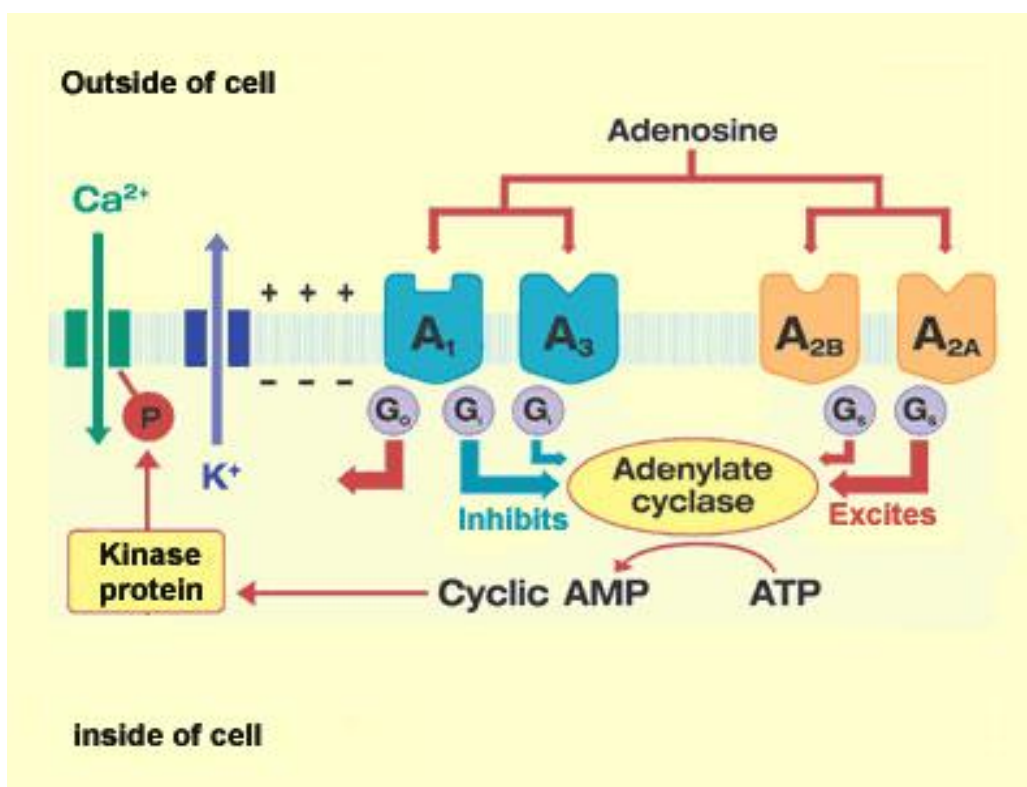


Figure 5.1 Schematic representation of Adenosine receptors.

In 1978 it was proposed the existence of at least two types of receptors for purines, named P1 and P2. P1 identified the receptor family more sensible to adenosine whereas P2 receptors are mainly activated by ATP and ADP [3]. The functional antagonism between ATP and adenosine is very interesting, because the ATP present in the synaptic and parasynaptic terminals activates the production of adenosine. ATP induces rapid and excitatory activities while adenosine produces slow and inhibitor effects. Therefore, adenosine rules both the ATP excitatory activities and those of neurotransmitters with ATP release through a mechanism of feedback inhibition. This complex regulation suggests the

amount of released ATP is directly proportional to the inhibitor effect of adenosine because ATP induces the production of adenosine [4]. Another differentiation between P1 and P2 receptors is based on sensitivity to different xanthine-type antagonists: in fact the P1 receptors are competitively inhibited by compounds such as xanthine, caffeine, theophylline and theobromine, which are inactive on P2 receptors. Both the receptor families include several receptor subtypes, differentiated on the base of pharmacology profile, translation mechanism and molecular structure [2].

Adenosine interacts with receptor subtypes (P1), distributed on the cytoplasmic membrane (A_1 , A_{2A} , A_{2B} , A_3) and present in the principal tissues of the human body (central nervous system, cardiovascular, renal, respiratory, immune and gastroenteric apparatus) where regulate the biological functions [5]. Each subtype is coupled to a G-protein that can have a stimulator (Gs) or inhibitor (Gi) effect. These receptors can activate or inhibit adenylate cyclase, respectively. In some tissue, A_1 and A_3 receptors can regulate the activity of phospholipase C activity and, in the case of A_1 receptor, that of Ca^{2+} and K^+ ionic channels [2].

5.1.1 A_1 Receptor

A_1 receptor (Fig. 5.2) is distributed throughout the entire body but it's mainly localized in central nervous system, cardiac apparatus and adipose tissue [6]. This receptor is coupled to G_i protein and its activation leads to a decrease of intracellular cAMP levels trough the inhibition of Adenylate cyclase (AC). This in turn regulates the activity of cAMP-dependent protein kinase, which phosphorylates different protein targets. A_1 coupling to AC has been described in several tissues including brain, adipose tissue. In addition to direct modulation of signaling pathways downstream to cAMP, inhibition of AC via A_1 receptors blocks the effects of other agents which operate by stimulating AC activity in cells. Further studies showed different pathways associated with the activity of this receptor including the activation of K channels and the inactivation of those of Ca^{2+} . Another effects include the activation of phospholipase C and the adjustment of several mitogenic kinases [2, 5].

A_1 receptors are involved in sleep promotion by inhibiting wake-promoting cholinergic neurons in the basal forebrain. In sleep deprived subjects, some authors found an increase of the apparent equilibrium total distribution volume in a region-specific pattern in all examined brain regions with a maximum increase of A_1 receptor in the orbitofrontal cortex. The regional distribution of adenosine receptors is a crucial aspect related to the local control of sleep–wake organization. Another important discovery is the increase

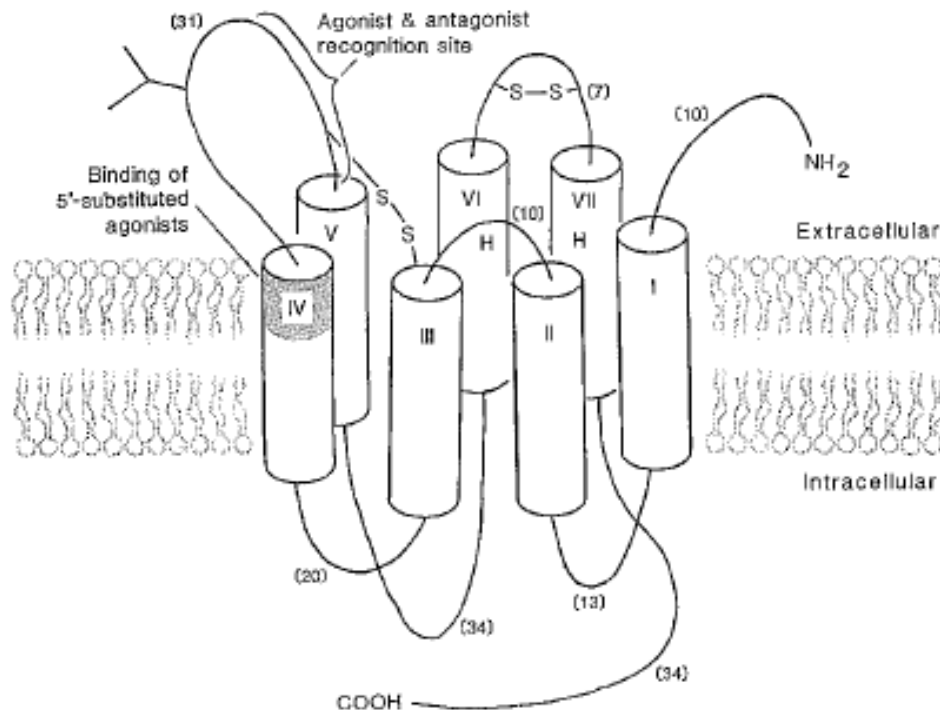


Figure 5.2 Schematic representation of A₁ receptor (from ref. [5]).

of A₁ density occurs all over the brain, which is consistent with a global effect, connected to basic cell functions. In man, the cerebral A₁ exhibits highest densities in the thalamus and the neocortex, both of which are important structures in the promotion and maintenance of slow-wave sleep [7].

A₁ receptors are also present in smooth muscle throughout the vascular system [8]. A₁ receptors mediate cardiac depression through negative chronotropic, dromotropic, and inotropic effects. Slowing of the heart rate occurs via A₁ receptors on sinoatrial and atrioventricular nodes, leading to bradycardia and heart block, respectively, while the inotropic effects include a decrease in atrial contractility and action potential duration. This aspect of A₁ receptor mediated effects has found application in the medical use of adenosine to treat supraventricular tachycardia, and in the use of adenosine receptor antagonists in the treatment of bradyarrhythmias. Direct effects on blood vessel tone via adenosine action on A₁ receptors are rare. A more significant role of A₁ receptors with regard to regulation of blood vessel tone appears to be prejunctional modulation of neurotransmitter release [5].

5.1.2 A_{2A} Receptor

A_{2A} receptor (Fig. 5.3) is a member of the G protein-coupled receptor (GPCR) family which possesses seven transmembrane alpha helices. The crystallographic structure of the adenosine A_{2A} receptor shows a ligand binding pocket different from that of other structurally determined GPCRs [9]. The gene encodes a protein that is one of several receptor subtypes for adenosine. The activity of the encoded protein, a G protein-coupled receptor family member, is modulated by G proteins which activate AC and induce synthesis of intracellular cAMP. The encoded protein is abundant in basal ganglia, vasculature, T lymphocytes and platelets and it is the main target of caffeine, a competitive antagonist of this protein [10].

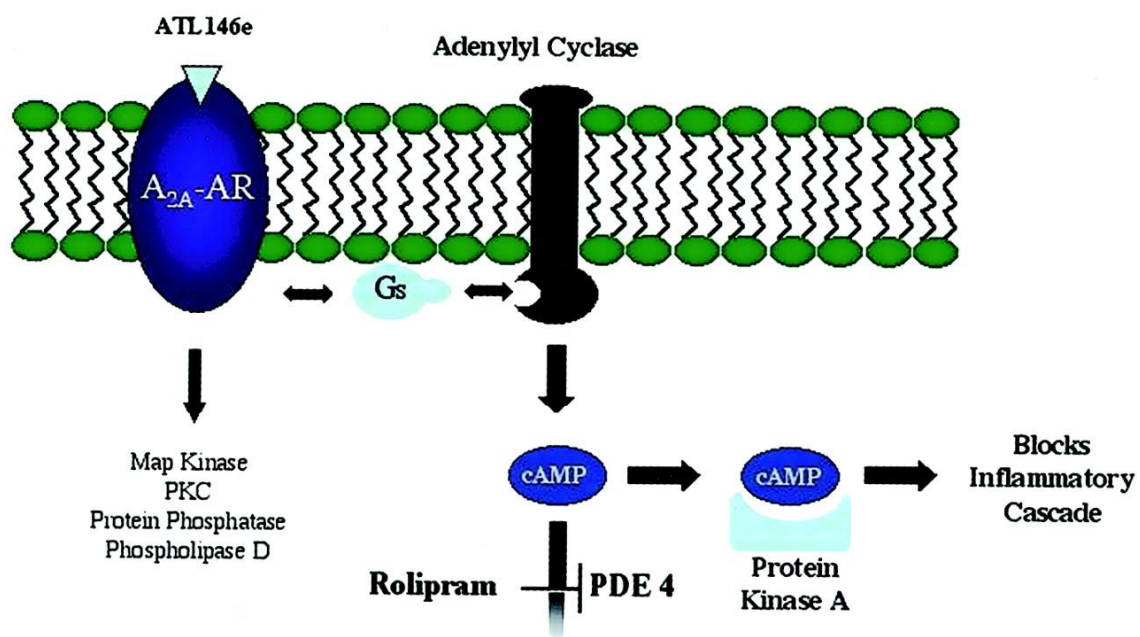


Figure 5.3 Scheme of A_{2A} receptor.

A_{2A} receptors have a wide-ranging but restricted distribution, including immune tissues, platelets, the CNS, and vascular smooth muscle and endothelium.

As the A₁ receptor, the A_{2A} receptors are believed to play a key role in regulating myocardial oxygen consumption and coronary blood flow. The A_{2A} receptor is responsible for regulation of myocardial blood flow through a vasodilation of the coronary arteries, which increases blood flow to the myocardium, but may lead to hypotension. In the vasculature, A_{2A} receptors have been described on both the smooth muscle and endothelium, where they are associated with vasodilatation. This seems to be a considerable variation in A_{2A} receptor expression between blood vessels, although it is possible that vessels unresponsive to A_{2A}-selective agonists don't express the receptor but at very low levels, or that the receptor is not coupled to a functional response [2].

Platelets express only one adenosine receptor subtype, the A_{2A} receptor. Activation of this receptor in platelets causes an increase in cAMP accumulation and a decrease of platelet aggregation. In a recent study, with A_{2A} receptor– knockout mice, it was reported the increasing of platelet aggregation, indicating the importance of this receptor subtype in platelet function [11]. Biaggioni *et al.* found that a repeated dosing regimen with caffeine in human volunteers produces a significant change in the functional response of platelets to the adenosine receptor agonist 59-Nethylcarboxamidoadenosine (NECA) while Varani *et al.* investigated the changes in the density and affinity of A_{2A} adenosine receptors in human platelet membranes of control (before caffeine somministration) and caffeine-treated subjects. Caffeine is one of antagonist of this receptor and this might explain the functional change in platelet responsiveness to activation of A_{2A} receptors. The results of this study support the hypothesis that chronic caffeine consumption results in sensitization and/or upregulation of endogenous adenosine receptors in sane subjects. The upregulation of adenosine A_{2A} receptors caused by chronic intake of caffeine could be interpreted as indicator of influence of endogenous adenosine on human platelets, and the presence of the antagonist is counterbalanced by the upregulation of A_{2A} receptors. [12, 13].

A_{2A} receptors in the CNS and especially in the peripheral nervous system (PNS) generally facilitate neurotransmitter release and important roles in the modulation of glutamate and dopamine release. The negative interactions, observed between A_{2A} and dopamine D_2 receptors, involve a reduced affinity of agonist binding to dopamine D_2 receptors upon stimulation of A_{2A} receptors in rat striatal membranes. This raises the possibility of using A_{2A} receptor antagonists as a novel therapeutic approach in the treatment of Parkinson's disease, to reduce the profound disabling effects arising from degeneration of dopaminergic nigrostriatal neurons of the basal ganglia in this disease, making it a potential therapeutic target for the treatment of conditions such as insomnia, pain, depression, drug addiction and Parkinson's disease [2, 14-16].

5.1.3 A_{2B} Receptor

A_{2B} receptors (fig. 5.4) are found practically in every cell in most species. On the other hand, the number of receptors is small and relatively high concentrations of adenosine are generally needed to generate a response [2].

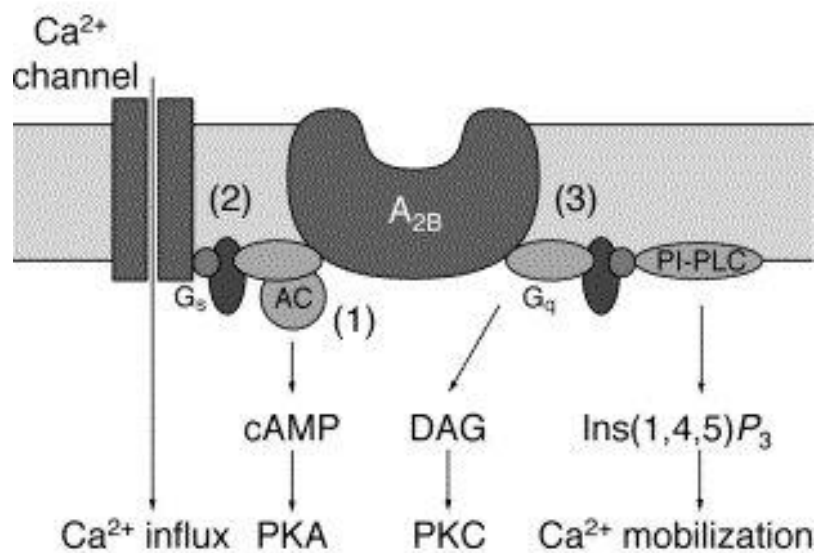


Figure 5.4 A_{2B} Adenosine receptor.

Expression of adenosine A_{2B} receptors has been found in bronchial epithelium in cultured human smooth muscle, in human mast cells, monocytes and fibroblasts [17]. Signal transduction occurs through a G_s protein that induces an increase in cAMP levels and a subsequent activation of protein kinase [18]. The A_{2B} receptor has also the capacity to increase the levels of IP₃, suggesting a coupling with a G_q protein type and determining the release of calcium ions from cell stores [19].

In human lung fibroblasts activation of A_{2B} adenosine receptor increases the release of IL-6 and induces differentiation into myofibroblasts thus suggesting that adenosine, via A_{2B} receptors, participates in the remodelling process occurring in chronic inflammatory lung diseases. Adenosine, via A_{2B} receptors, increases the release of IL-6 and monocyte chemotactic protein-1 from bronchial smooth muscle cells. Recently, the pro-inflammatory role of adenosine A_{2B} receptor has been confirmed by a study showing that activation of these receptors up-regulates Th2 cytokines (IL-3, IL-4, IL-8, IL-13) in mast cells and promotes IgE synthesis by lymphocytes B. The produced high level of IgE, as compared with B lymphocytes co-cultured with non-stimulated mast cells, suggests a more specific role for these receptors in the allergic inflammation occurring in asthma. Taken together these evidences suggest that adenosine A_{2B} receptors are deeply involved in the mechanisms underlying mediators release by mast cells, the major mechanism by which adenosine induces bronchoconstriction and airway inflammation in asthma [17].

A recent study confirmed that A_{2B}AR mRNA is expressed in human platelets at similar levels to A_{2A}AR mRNA. This study also employed pharmacologic ligands to detect an active A_{2B}AR on human platelets [20]. Ravid *et al.* determined whether A_{2B}AR ablation or

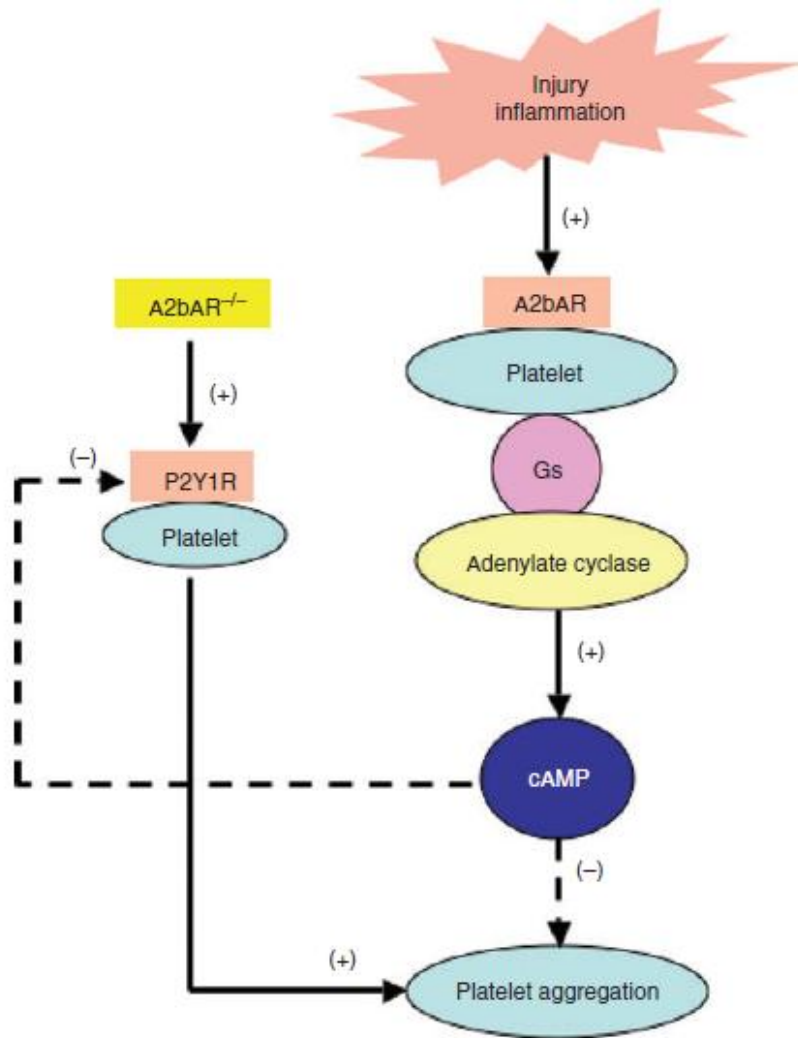


Figure 5.5 Schematic representation of the regulatory mechanism of platelet aggregation described by Ravid *et al.* (from ref. [21]).

activation is associated with changes in ADP receptor expression in mice. Their results we showed that a mild elevation in P2Y₁R mRNA in MKs and platelets *in vivo* leads to increased platelet aggregation and confirmed that elevated cAMP downregulates P2Y₁R expression. It is, then, conceivable that the lifelong lower basal cAMP level in A_{2B}AR KO platelets could contribute to greater basal expression of ADP receptors, which would induce higher aggregation activity. They investigated the induction of the platelet A_{2B}AR under stress, and to its role in inhibiting platelet aggregation, associated with changes in cAMP level and a newly identified regulation of the ADP receptor by A_{2B}AR-mediated changes in cAMP level (Fig. 5.5) [21].

5.1.4 A₃ Receptor

A₃AR is widely expressed in the testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon, and eye of rats, sheep, and humans [22].

A₃ARs have been shown to couple to classic or G protein–dependent second messenger pathways through activation of both Gi and Gq family G proteins. This receptor subtype has a transduction mechanism similar to that of A₁ receptors, where the protein is G-type inhibitor with the ability to block the activity of adenylate cyclase by reducing levels of cAMP. However, this doesn't seem to be the only pathway, since it has been shown that increased levels of IP₃, resulting in activation of the calcium channels, through the activation of a Gq protein (fig. 5.6) [23].

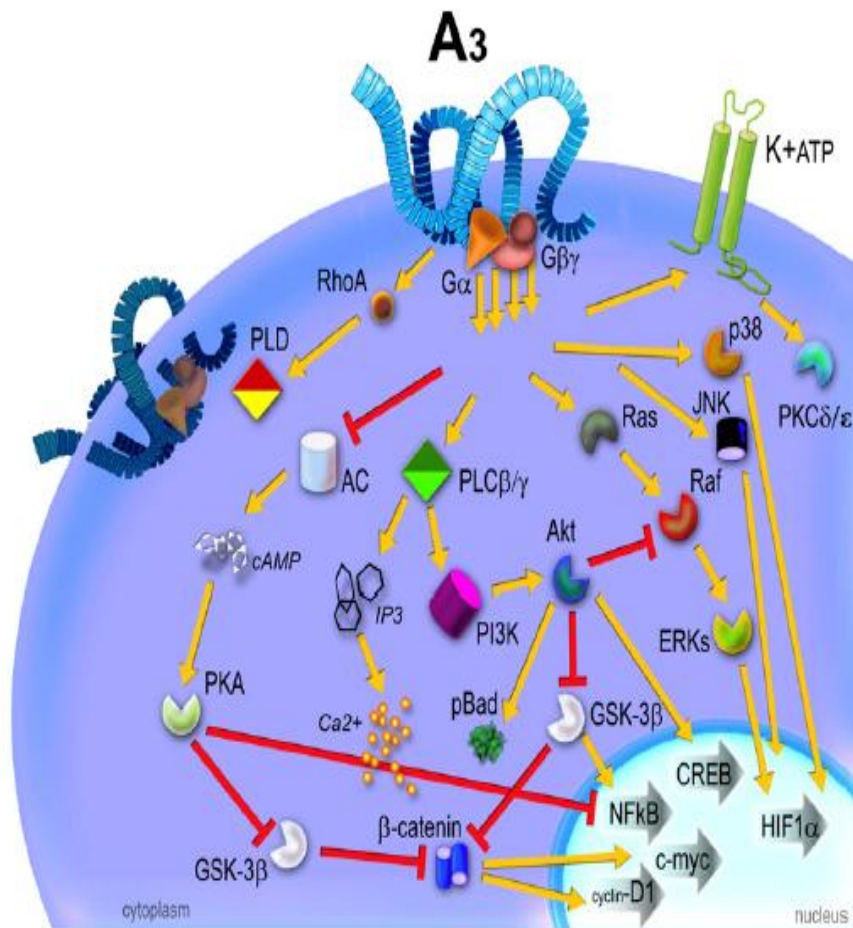


Figure 5.6 Schematic representation of second messengers and intracellular signaling pathways, downstream targets, mediated by A₃ARs stimulation (from ref. [22]).

A₃ARs are present in immune cells and are involved in the physiopathologic regulation of inflammatory and immune processes mediated by adenosine. The A₃ receptor on mast cells facilitates the release of allergic mediators including histamine, suggesting a role in inflammation [2, 22]. Neutrophil behavior is strongly affected by the A₃AR mediating inhibition of the oxidative burst and chemotaxis with anti-inflammatory activity. Accordingly, these effects have also been observed in a model of severe IR injury after lung transplantation. However, it was also reported that, together with P2Y₂, A₃AR guides

neutrophil chemotaxis after ATP release and also plays a role in neutrophil migration by positively affecting innate immune response. Especially, this adenosine subtype aggregates in immunomodulatory microdomains on human neutrophil membranes and promotes the formation of bacteria-tethering cytonemes, which are important for phagocytosis, thus suggesting a key role in innate immune response [22]. Adenosine is an endogenous regulator of monocyte macrophage functions, first producing high amounts of inflammatory mediators during the first stage of inflammation and later participating in the resolution of the inflammatory process. Another macrophage functions regulated by A₃AR include the reduction of the chemokine macrophage inflammatory protein (MIP) and the inhibition of interferon regulatory factor 1, inducible nitric oxide synthase, and CD36 gene expression in RAW264.7 murine and THP-1 human cells [22, 24]. Several studies indicate a role for A₃AR agonists as inhibitors of inflammation, a recent novel application of A₃AR antagonists was discovered for a novel series of truncated nucleosides that of inhibiting TGF-β1-induced collagen type I upregulation, making them appear as good therapeutic candidates for treating renal fibrosis [25]. More recently, A₃ARs were found to be overexpressed in different autoimmune disorders such as Crohn's disease and psoriasis. The upregulation observed in these pathologies could be attributed to adenosine, which, under conditions of stress, accumulates in the extracellular environment. Most transcription factors, such as NF-κB and CREB, have been revealed as promoting inflammation and being inversely associated with A₃AR upregulation. Accordingly, CF101 was tested in a phase II, multicenter, randomized, double-blind, dose-ranging, placebo-controlled trial in patients with moderate to severe chronic plaque-type psoriasis. In this study the drug was found to be safe and well tolerated, and the improvement was progressive and linear throughout the period examined [26, 27]. Overall, the data in the literature suggest that A₃AR activation can induce important anti-inflammatory effects in several cellular models. The results achieved thus far with A₃AR agonists in clinical studies on such major inflammatory conditions as arthritis and psoriasis are quite promising, with the possibility that they will be translated into treatments for other fibrosis-related pathologies [22].

A very interesting area of possible application for A₃AR ligands is in cancer therapy. Adenosine is present at high levels in cancer tissues and in the interstitial fluid of several tumors, in sufficient concentration to interact with adenosine receptors and they are present in several cancer cell types [22]. Several authors observed that both pro- and antiapoptotic as well as pro- and antiproliferative effects have been reported through

different mechanisms depending on the level of receptor activation with selected ligands [28-33]. At first, telomerase activity inhibition and cytostatic effects were observed in tumor cells and, then the intracellular pathway involved in A₃AR-mediated tumor growth inhibition was identified [34, 35]. In contrast, some studies showed that A₃AR agonist inhibition of cell proliferation was only obtained by micromolar concentrations [29, 36]. Other works reported that A₃ARs reduces the ability of prostate cancer cells to migrate *in vitro* and metastasize *in vivo*. Accordingly, in the same cells, N⁶-(3-iodobenzyl)adenosine-59-N-methyluronamide (IB-MECA) inhibits cell proliferation and induces G1 cell cycle arrest, apoptosis, and migration [22, 37]. Stimulation of A₃ARs exerts a cytotoxic and pro-apoptotic effect on malignant mesothelioma cells [33]. Interestingly, the antitumor effect of A₃ARs is potentiated by PEMFs in cultured neural cancer cells such as PC12 and U87MG glioblastoma cells, thus decreasing NF-κB activation and cell proliferation. Moreover, PEMF and A₃AR stimulation are able to significantly increase p53 levels, cytotoxicity, and apoptosis in tumor cells [22].

A₃AR agonists have also been investigated *in vivo* studies. In all the experimental models, given their stability and bioavailability profile, the drugs were administered orally. The studies included syngenic, xenograft, orthotopic, and metastatic experimental animal models utilizing IB-MECA and Cl-IB-MECA (2-chloro-N⁶-(3-iodobenzyl)-adenosine-59-N-methyluronamide) in melanoma, colon, prostate, and hepatocellular carcinomas. A₃AR agonists prevented the growth of primary B16-F10 murine melanoma tumors in syngenic models. In combination with some chemotherapeutic agents, IB-MECA and Cl-IB-MECA induced an additive antitumor effect on the development of B16-F10 melanoma lung metastatic foci and caused significant cytotoxicity on two melanoma cell lines through multiple mechanisms of cell death [38]. Several studies reported that the combined treatment with IB-MECA and 5-fluorouracil or taxol, respectively, resulted in an enhanced antitumor effect. IB-MECA prevented the growth of primary and liver metastases of CT-26 colon carcinoma cells inoculated in the spleen. Finally, Cl-IB-MECA treatment dose dependently inhibited hepatocellular tumor growth and reduced liver inflammation [39, 40]. Overall, these data suggest that A₃ARs might be a biologic tumor marker and that A₃AR modulation could be used to treat cancer.

5.2 CYTOKININS RIBOSIDES AND ADENOSINE RECEPTORS

In connection with the anti-aggregation activity of N⁶-substituted adenosine derivatives many studies have been carried out in order to establish the interaction of a big variety of N⁶-substituted adenosines with adenosine receptors, especially A_{2A} or A₃ subtypes [41-44]. However, available data about the interaction, as agonists and/or antagonists, between cytokinins and adenosine receptors are very poor.

Chao-Lee et al evaluated the interaction between a cytokinin riboside, zeatin riboside, and A_{2A} adenosine receptor. In this study the authors found that zeatin riboside can prevent pheochromocytoma (PC12) cells from serum deprivation-induced apoptosis by acting on the adenosine A_{2A} receptor which was blocked by an A_{2A} antagonist and a protein kinase A (PKA) inhibitor, demonstrating the functional ability of zeatin riboside by mediating through A_{2A} signaling event. Since the A_{2A} was implicated as a therapeutic target in treating Huntington's disease (HD), a cellular model of HD was applied by transfecting mutant huntingt in PC12 cells. On this data, zeatin riboside might have therapeutic potential as a novel neuroprotectant and a lead for treating neurodegenerative disorders [45].

Blad *et al.* evaluated the A₃ receptor antagonist activity of N⁶- isopentenyl adenosine (iPAdo) and *trans* zeatin riboside. In a functional assay in Chinese hamster ovary cells transfected with A₃ receptor, IPA and zeatin riboside inhibited forskolin-induced cAMP formation at micromolar concentration. They demonstrated strong and highly similar antiproliferative effects of IPA and on human and rat tumor cell lines LNCaP and N1S1. The antiproliferative effect of low concentrations of IPA on LNCaP cells could be fully blocked by the selective A₃R antagonist MRS1523 while higher concentrations of IPA appeared to inhibit cell growth by an A₃R-independent mechanism [46]. At our knowledge, no study has been performed yet on the interaction of aromatic CKRs and adenosine receptors. Therefore, one of the aim of this Ph.D project was the investigation on the interaction between the aromatic N⁶-furfuryladenosine, N⁶-benzyladenosine and topline riboside and the A₁, A_{2A}, A_{2B}, A₃ adenosine receptors. Due to the structural similarity of CKRs with adenosine, we tested the affinity of these compounds as antagonists. Then, we evaluated the affinity of two synthetic N⁶-substituted adenosines to confront them with natural CKRs in a structure-activity study. Due to the high price of zeatin riboside, the less expensive N⁶-substituted adenosine (N⁶-(Δ^2 -isopentenyl) adenosine was used as reference prenylated CKR.

5.3 EXPERIMENTAL SECTION

5.3.1 MATERIALS

Natural CKRs were obtained from OIChemIm Ltd. (Olomouc, Czech Republic). Synthesis of N⁶-substituted adenosines FEA and 2HFEA was described in Chapter 4. All reagents were purchased from Sigma–Aldrich and were of analytical grade.

5.3.2 METHODS

5.3.2.1 Evaluation of natural and non-natural cytokinin ribosides as antagonists toward A₁, A_{2A} and A_{2B} adenosine receptors

CKRs and synthetic N⁶-substituted adenosine derivatives FEA and 2HFEA were evaluated as antagonists with A₁, A_{2A}, A_{2B} and A₃ adenosine receptors. Tests with A₁, A_{2A}, A_{2B} receptors were performed according to Klotz *et al.* [47]. Dissociation constants of unlabeled compounds (K_i-values) were determined in radioligand competition experiments. All binding data were calculated by non-linear curve fitting with the program SCT-FIT. This protocol was adapted to a microplate format utilizing a 96-well microplate filtration system (Millipore MultiScreen MAFC). For saturation binding of the assay at A₁ adenosine receptor, increasing concentrations of the radioligands [³H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine) or [³H]CCPA (2-chloro-N⁶-cyclopentyladenosine) were incubated in a total volume of 200 ml containing 0.2 U/ml adenosine deaminase and 20 mg of membrane protein in 50 mM Tris/HCl pH 7.4. In competition experiments the wells contained 1 nM [³H]- DPCPX and the compound to be tested at different concentrations. Samples were incubated for 3 h at 25° C, filtered through the builtin filter at the bottom of the wells and washed three times with 200 ml of ice-cold binding buffer. After addition of 20 ml of scintillator to the dried filter plates samples were counted in a Wallac Micro- Beta counter. The conditions for A_{2A} adenosine receptor binding were essentially the same as for A₁ receptor binding. In competition experiments [³H]NECA (N-ethylcarboxamidoadenosine) at 30 nM was used as radioligand. Samples with a protein concentration of 50–80 mg were incubated for 3 h at 25° C and filtered individually as described for conventional radioligand binding [47].

5.3.2.2 Evaluation of natural and non-natural cytokinin ribosides as antagonists toward A₃ adenosine receptor

For the assay with A₃ adenosine receptor, the protocol was performed according to Klotz *et al.* [48]. Radioligand binding experiments with [³H]5'-N-ethylcarboxamidoadenosine were carried out at room temperature. Competition binding studies were done at a concentration of 1 nM, in saturation experiments a radioligand concentration in the range of 0.2–10 nM was used. In kinetic experiments, the tested compounds were added at several concentrations after 90 min of association to induce radioligand dissociation. Nonspecific binding was determined in the presence of 100 μM (R)-N⁶-phenylisopropyladenosine (R-PIA). For incubation and separation of bound from free ligand a 96-well microplate filtration system (Millipore Multiscreen MAFC) was used. K_i values were calculated from competition curves by nonlinear curve fitting with the program SCT-FIT [48].

5.4 RESULTS AND DISCUSSION

The results of the assays of CKRs and synthetic N⁶-substituted adenosine derivatives with A₁, A_{2A}, A_{2B} and A₃ adenosine receptors are reported in Table 5.1. Then K_i (inhibition constant) values were calculated from competition curves by nonlinear curve.

CKRs are good antagonists of A₁ receptor with a K_i range from 21.7 to 52.4 nM. The best

Table 5.1 K_i values of CKRs and synthetic N⁶-substituted adenosine derivatives with adenosine receptors.

Compound	A ₁			A _{2A}			A _{2B}			A ₃		
	K _i (nM)	95% confidence limits		K _i (nM)	95% confidence limits		K _i (nM)	95% confidence limits		K _i (nM)	95% confidence limits	
IPAdo	52,4	45,4	60,5	11.000	10.300	11.700	>20.000			146	114	185
BA	33,2	29,5	37,3	1.620	1.500	1.760	2.560	2.320	2.820	114	101	130
KR	34,9	32,2	37,9	2.880	2.500	3.330	3.720	3.210	4.300	159	141	179
ρ-TR	21,7	20	23,6	583	444	764	952	770	1.180	124	101	152
FEA	10,8	6,2	18,9	1.260	1.150	1.370	1.640	1.250	2.160	4,51	4,30	4,74
2-HFEA	27,1	22,3	33	458	405	517	1.280	949	1.720	6,96	5,81	8,33

value of CKRs is that of ρ- topolin riboside. A similar value has been observed for 2HFEA while FEA shows a lower K_i than ρ- topolin riboside.

The affinity of CKRs for A_{2A} was poor with the exception of ρ- topolin riboside that showed a K_i value of 583 nM. Only 2HFEA has a better value. Probably, the addition of an OH groups in the aromatic ring might improve the activity of the compounds as antagonist of A_{2A} receptor, involved in the platelet aggregation mechanism. The good antagonist property of ρ- topolin riboside with respect to the receptor A_{2A} could partially explain the previously described activity of TR as inhibitor of platelet aggregation (Chapter 3). The same trend has been observed for A_{2B} receptor, also involved in platelet aggregation with a different mechanism, related to cAMP levels. The best value is that of ρ- topolin riboside with a K_i of 952 nM.

Similarly to what has been observed with A₁, CKRs are good antagonist with A₃ adenosine receptor with a K_i range from 114 to 146 nM. The best value was that of benzyl adenosine. The synthetic compounds FEA and 2HFEA were antagonist of A₃ receptor much stronger than natural CKRs. This affinity for this receptor may explain in part the anticancer activity of cytokinin ribosides, reported in several works.

5.5 CONCLUSIONS

We have shown that naturally occurring cytokinin ribosides are antagonists of adenosine receptors. The good interaction between A₂ receptors and topolin riboside confirmed its high anti platelet aggregation activity. Only 2HFEA showed a similar antagonist activity and only with A_{2A}AR. The synthetic N⁶-substituted adenosines were antagonists of A₃AR much stronger than natural CKRs but not with the other adenosine receptors. Further investigations and structure-activity studies are required in order to clarify the antagonist activity of cytokinin ribosides and other natural or synthetic N⁶-substituted adenosines toward the adenosine receptors herein described.

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6. PRELIMINARY RESULTS ON NEW BIOLOGICAL ACTIVITIES OF NATURAL AND NON-NATURAL CYTOKININ RIBOSIDES

In addition to the previously reported results, we evaluated other biological activities of natural and non-natural CKRs. During my stay in Portugal, the enzymatic inhibition activity of natural CKRs and synthetic N⁶-substituted adenosines toward acetylcholinesterase (AChE) and monoamine oxidase B (MAO-B) were investigated. Both these enzymes are in the field of interest of my Portuguese supervisor, Prof. Fernanda Borges [1-4].

6.1 Natural and syntethic N⁶ – substituted adenosine derivatives as Acetylcholinesterase inhibitors

Acetylcholinesterase (AChE) is a hydrolytic enzyme that catalyzes the break-down of acetylcholine (ACh) and several other choline esters that function as neurotransmitters. AChE is distributed mainly in neuromuscular junctions and in chemical synapses of the cholinergic type where its activity serves to terminate the synaptic transmission [5]. During the neurotransmission, acetylcholine is released from the nerve into the synaptic cleft and binds to the ACh receptors on the post-synaptic membrane, relaying the signal from the nerve. AChE, also located on the same membrane, terminates the signal transmission through the hydrolysis of ACh (Fig. 6.1).

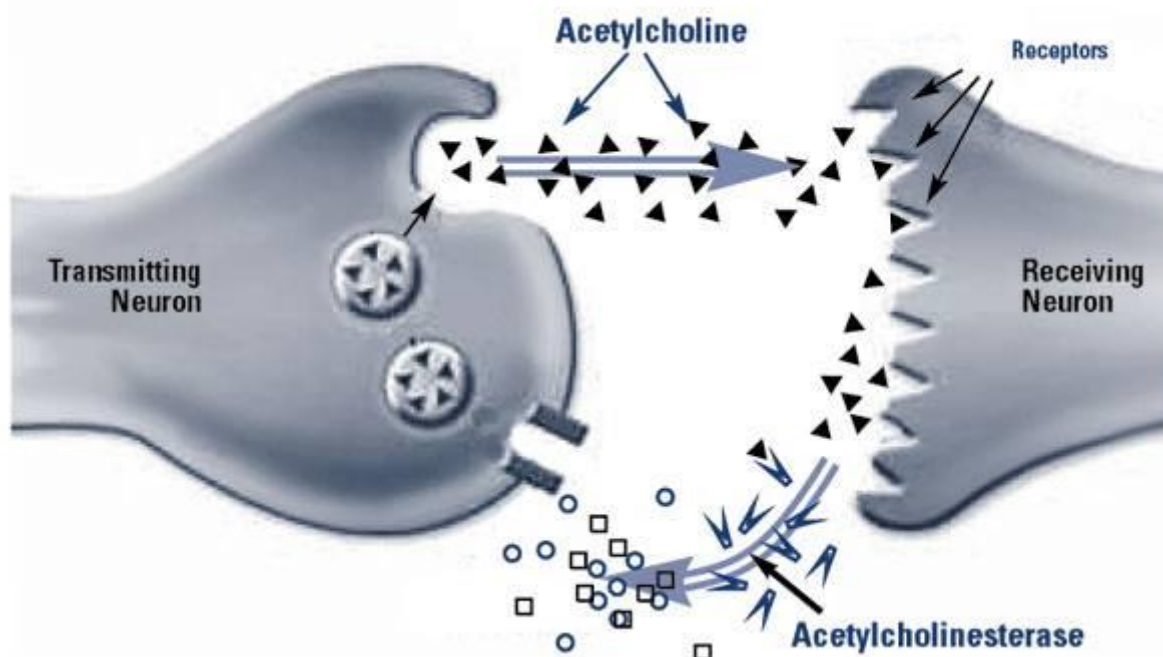


Figure 8.1 after signaling, ACh is released from receptors and broken down by AChE to be recycled in a continuous process.

The liberated choline is taken up again by the pre-synaptic nerve and ACh is synthesized by combining with acetyl coenzyme A through the action of choline acetyltransferase [6, 7].

If the AChE activity is hampered or blocked, the accumulation of acetylcholine at cholinergic receptor sites can generate an excessive stimulation of cholinergic receptor and cause a variety of clinical complications, such as paralysis, convulsions, bronchial constriction leading ultimately to the death by asphyxiation [8]. Organophosphates (OP) are esters of phosphoric acids that are irreversible inhibitors of AChE through a mechanism that starts from the AChE-catalyzed cleavage of OP. The slow hydrolysis (within days) leaves a phosphoryl group in the esterase site where it covalently binds to the enzyme in an irreversible mode [9].

Others irreversible AChE inhibitors are compounds, used as insecticides (e. g. malathion) [10], and herbicides such as MCPA, mecoprop, 2,4-D and dichlorprop [1]. These compounds can interact with DNA causing modifications such as adducts or strand breaks and, consequently, damaging the nucleic acid [11]. Reversible AChE inhibitors that are able to occupy the esterase site for a limited time (seconds to minutes) have attracted interest for applications in the treatment of the central nervous system diseases. Tetrahydroaminoacridine (THA) and donepezil are used to improve cognitive functions in Alzheimer's disease. Rivastigmine is also used to treat the Alzheimer's and Lewy body dementia and pyridostigmine is used to treat the myasthenia gravis. Alzheimer disease drugs donepezil, galantamine and rivastigmine are inhibitors of acetylcholinesterase as well [4, 12, 13].

In relation with the research interests of Prof. Borges in this area [1], natural and non-natural CKRs were investigated as AChE inhibitors. Acetylcholinesterase inhibition assay was based on Ellman's method [14] where the substrate acetylcholine was hydrolyzed by AChE to thiocholine that reacted with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to form 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, detected at 412 nm (Fig. 6.2).

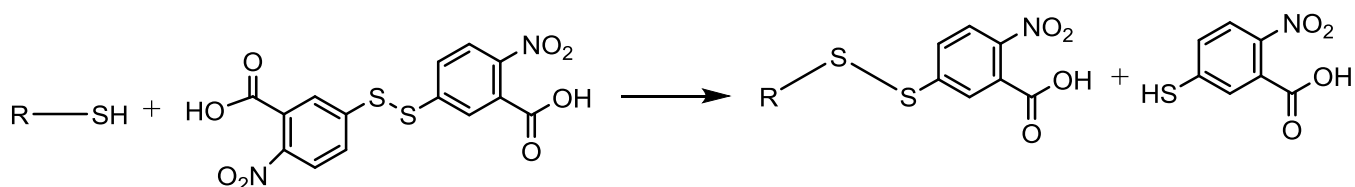


Figure 6.2 Reaction of DTNB of a compound with thiol groups.

The analysis was carried out according to Pereira *et al.* [15]. The percentage of inhibition was calculated by comparing the rates of the sample with the blank. Donepezil, one of the previously cited inhibitors of AChE was used as positive control. Natural and synthetic CKRs didn't show any acetylcholinesterase inhibition activity.

6.2 Natural and synthetic N⁶ – substituted adenosine derivatives as Monoamine oxidase B (MAO-B) inhibitors

L- Monoamine oxidases are a family of enzymes that are distributed on the outer membrane of mitochondria where they catalyze the oxidation of monoamines. In the mammals, there are two types of monoamine oxidase, MAO-A and MAO-B, both types involved in the oxidation of endogenous and exogenous monoamine neurotransmitters [16, 17]. MAO-A degrades amine neurotransmitters such as dopamine, norepinephrine and serotonin. As a result, it is a key regulator for normal brain function. This enzymes is highly expressed in neural and cardiac cells, liver, gastrointestinal tract and placenta. Its expression is regulated by the transcription factors SP1, GATA2 and TBP via the cAMP pathway in response to stress such as ischemia and inflammation [18]. MAO-B also degrades dopamine but it breaks mainly phenethylamine and benzylamine [18, 19]. MAO-B are mostly found in blood platelets [20]. Because of the vital role that MAOs play in the inactivation of neurotransmitters, MAO dysfunction is thought to be responsible for a number of psychiatric and neurological disorders. For example, unusually high or low levels of MAOs in the body have been associated with schizophrenia, depression, attention deficit disorders [21-23]. MAO-A inhibitors are one of the major classes of drug prescribed for the treatment of depression and anxiety, although they are often last-line treatment due to risk of the drug's interaction with diet or other drugs [24].

Alzheimer's and Parkinson's are both associated with elevated levels of MAO-B in the brain [25, 26]. The normal activity of MAO-B creates ROS that directly damage cells [27]. MAO-B levels have been found to increase with age, suggesting a role in natural age related cognitive decline and the increased likelihood of developing neurological diseases later in life [28]. MAO-B inhibitors are used alone or in combination with L-Dopa to treat Alzheimer's and Parkinson's diseases [17, 24].

Due to the interests of Prof. Borges in this area of research [3], natural and synthetic CKRs were investigated as inhibitors of Monoamine oxidase B, mostly found in blood platelets, using an Amplex[®] Red Monoamine Oxidase Kit assay (A12214). The analysis was carried out according to the manufacturer's instructions, supplied with the kit. This

assay was based on the detection of hydrogen peroxide in a horseradish peroxidase-coupled reaction with the Amplex Red reagent, a high sensitive probe for H₂O₂. Pargyline was used as positive control. Only 2HFEA was active as inhibitor of MAO-B with an I₅₀ value of 5.35 μM, similar to that of pargyline

6.3 Kinetin and Kinetin ribosides as inhibitors of protein oxidation and glycooxidation

Oxidized proteins are important factors in the ageing process as glycation and glycooxidation products accumulate in cells and tissues during ageing. Glycation results from the linking of sugars or of intermediate metabolic products to free amino groups of amino acids or nucleotides whereas glycooxidation is due to a sequential glycation and oxidation reaction [29]. The extracellular glycation/glycooxidation process is slow because it depends mainly on the concentration of glucose, which is the least reactive sugar. However, the intracellular process is much faster because it is due to an increase of the cytosolic concentration of more reactive glycation agents such as pentoses [30] and α-oxoaldehydes [31]. The end-result of this post-translational protein modification process is the formation of the so-called advanced glycation/glycooxidation end products (AGE) such as pentosidine [29].

In collaboration with dr. Alessio Scarafoni, (Department of Food, Environmental and Nutritional Sciences, Università degli Studi of Milano), we investigated the capacity of kinetin (K) and kinetin riboside (KR) to protect the bovine serum albumin (BSA) from the oxidative and glycooxidative damage caused by sugar through the inhibition of BSA-pentosidine formation. The glycation/glycooxidation inhibition activity of kinetin had been recently reported by Verbeke *et al.* [32]. The experiments with kinetin and kinetin were performed according to Verbeke *et al.* [32].

In contrast with reported results, in our hands K did not show the ability to decrease the glycosilation of BSA and similar negative results were obtained with the riboside KR. These controversial need further investigations.

6.4 CONCLUSIONS

In general, further investigations on biological activity of CKRs and their synthetic analogues seem desirable. These researches should include uptake of the compounds in the cell, their mechanisms of action and metabolic pathways. Taken altogether, the results would help to shed more light in the biological role of this class of compounds, structurally related to adenosine.

6.5 REFERENCES TO CHAPTER 6

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7. ANTIOXIDANT ACTIVITY OF N⁶-SUBSTITUTED ADENOSINE DERIVATIVES

7.1 OXIDATIVE STRESS

The oxidation of macromolecules such as DNA, proteins, lipids, is the unavoidable consequence of their existence under an oxygen-rich atmosphere, and it mainly occurs by a free-radical-mediated process called autoxidation. This condition concerns also man-made materials like plastics, pharmaceuticals, processed food, or cosmetics to biomolecules in a living organism, and the chemistry underlying the autoxidation of such different compounds, through different mechanisms, bears an amazing similarity [1].

Reactive oxygen species (ROS) are oxygen derived free radicals that involve a series of oxidants such as hydrogen peroxide (H₂O₂), lipid peroxides (LOOH), singlet oxygen (¹O₂), hydroxyl radical (•OH), peroxy radical (ROO•), peroxyxynitrite (˙OONO), superoxide anion (O₂²⁻) among which the radicals attract much attention because they can lead to carcinogenesis by damaging DNA and proteins and cause cardiovascular diseases by oxidizing LDL [2-4]. ROS can be produced through exogenous or endogenous way.

Exogenous ROS can be produced from pollutants, tobacco, smoke, drugs, xenobiotics, or radiation. Ionizing radiation can generate damaging intermediates through the interaction with water, a process termed radiolysis. Since the human body contains 55-60 % of water, the probability of radiolysis is quite high under the presence of ionizing radiation. In the process, water loses an electron and becomes highly reactive. Through a three-step chain reaction, water is sequentially converted to hydroxyl radical (•OH), hydrogen peroxide (H₂O₂), superoxide radical (•O₂²⁻) and ultimately oxygen (O₂) [1].

Endogenous ROS are produced through multiple mechanisms and depending on the cell and tissue types, in cell membranes, mitochondria, peroxisomes, and endoplasmic reticulum. Mitochondria convert energy for the cells into a usable form, adenosine triphosphate (ATP). This process, called oxidative phosphorylation, involves the transport of protons across the inner mitochondrial membrane by means of the electron transport chain. In the electron transport chain, electrons are passed through a series of proteins via oxidation-reduction reactions, with each acceptor protein along the chain having a greater reduction potential than the previous. The last acceptor for an electron along this chain is an oxygen molecule. In normal conditions, the oxygen is reduced to produce water; however, in about 0.1–2% of electrons passing through the oxygen is instead prematurely

and incompletely reduced to give the superoxide radical ($\cdot\text{O}_2^{2-}$), most well documented for Complex III (Fig. 7.1).

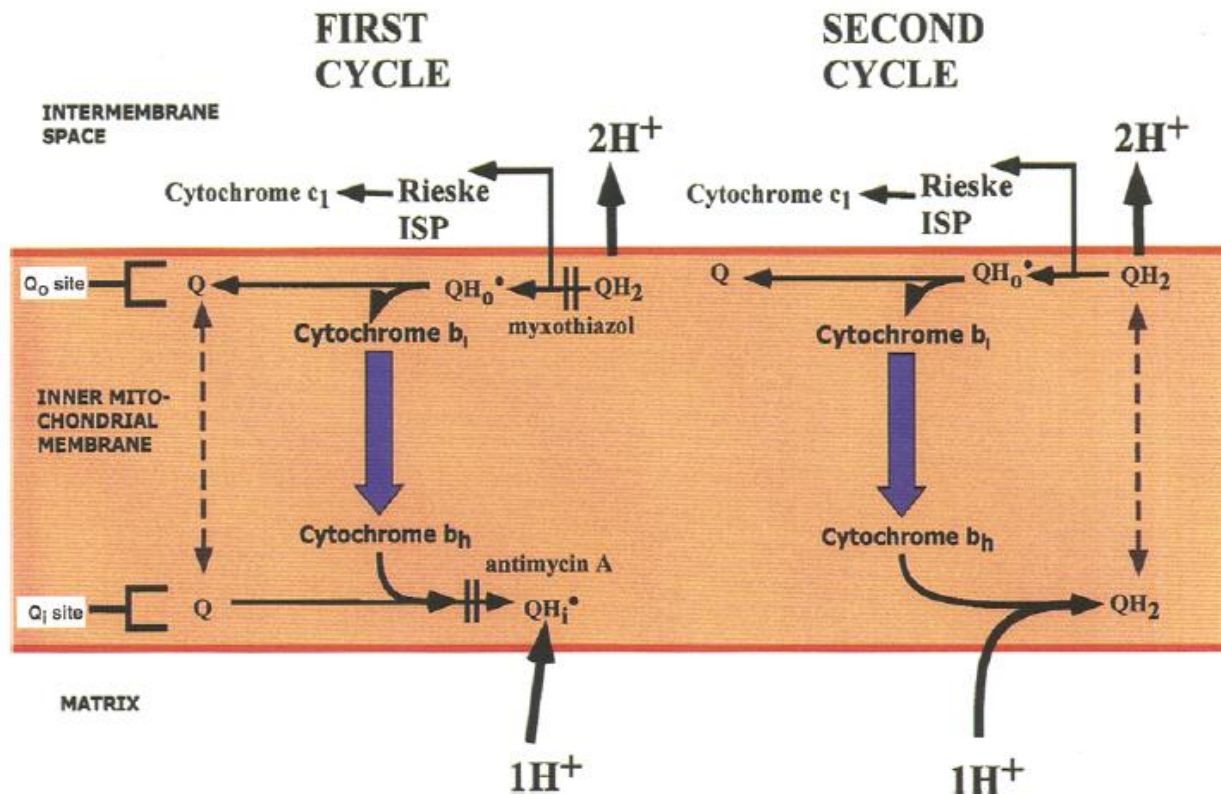


Figure 7.1 Scheme of electron transfer chain (from ref. [5]).

Superoxide is not particularly reactive by itself, but can inactivate specific enzymes or trigger lipid peroxidation in its protonated form, hydroperoxyl [5-7].

If too much damage is present in mitochondria, a cell undergoes apoptosis or programmed cell death. Bcl-2 proteins are layered on the surface of the mitochondria, detect damage, and activate a class of proteins called Bax, which punch holes in the mitochondrial membrane, causing cytochrome C to leak out. This cytochrome C binds to Apaf-1, or apoptotic protease activating factor-1, which is free-floating in the cell cytoplasm. Using energy from the ATPs in the mitochondrion, the Apaf-1 and cytochrome C bind together to form apoptosomes. The apoptosomes bind to and activate caspase-9, another free-floating protein. The caspase-9 then cleaves the proteins of the mitochondrial membrane, causing it to break down and start a chain reaction of protein denaturation and eventually phagocytosis of the cell [8].

Oxidizable organic molecules present a great structural variety and in biological systems, they are represented by lipids, proteins, and carbohydrates. Linoleic acid is the most

investigated and most representative oxidizable substrate, and its autoxidation can be taken as a model to classify antioxidants (Fig. 7.2) [1].

Autoxidation is occurred by some radical species $X \cdot$, able to react with the substrate RH (normally by H-atom abstraction), sometimes in presence of UV radiation or metal cations, to yield an alkyl radical $R \cdot$, which will react at a diffusion-controlled rate with oxygen to form a peroxy radical ($ROO \cdot$). The peroxy radical $ROO \cdot$ attacks another molecule of the substrate to give a hydroperoxide ROOH (the oxidized substrate) and another radical, therewith establishing the chain-reaction. The chain reaction proceeds for many cycles (propagation) and is ended by termination reactions in which free radicals collide and combine their odd electrons to form a new bond. [1].

During the propagation, short-chain alcohols, aldehydes and ketones are generated, with the production of bad smells and tastes.

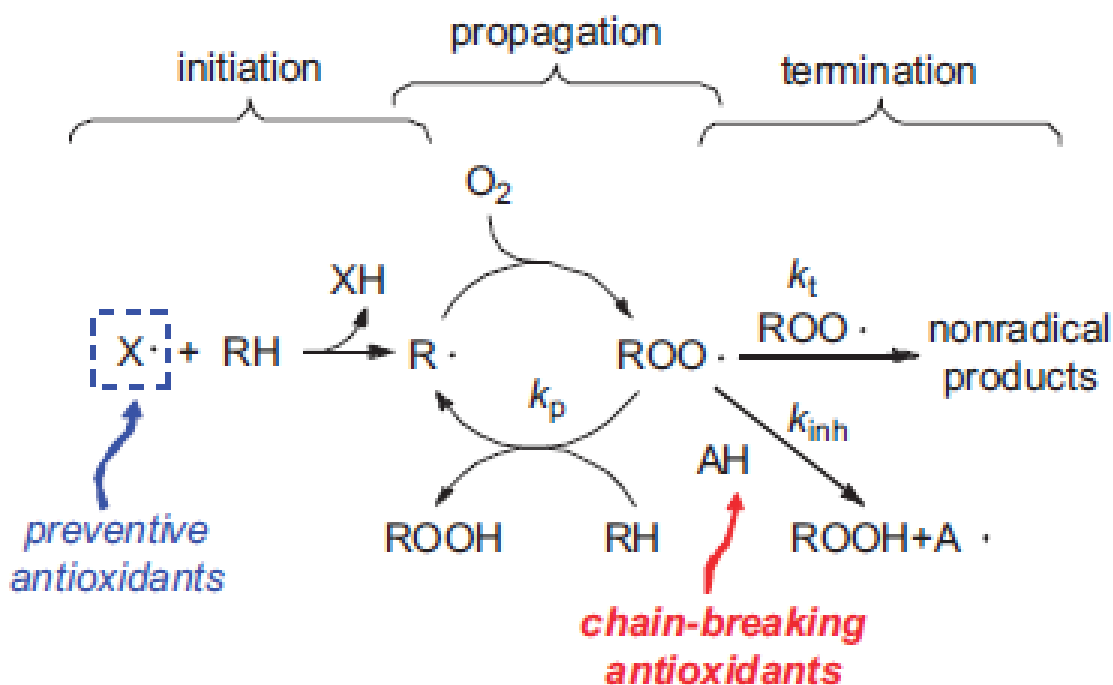


Figure 7.2 Simplified chain-reaction of autoxidation for a substrate RH and mode of interference by direct Antioxidants (from ref. [1]).

Some of these small molecules are used as marker of lipid oxidation such as malonaldehyde (MA) [9-11]. Its determination is still based on spectrophotometric assays and, among these, the one that relies on UV-evaluation of the adduct formed between malonaldehyde and thiobarbituric acid (TBA) is very popular and frequently used for oxidative stress evaluation [12]. More specific methods have been developed using HPLC techniques to detect MA derivatives. Among these, HPLC analysis of MA with the UV

detection can be realized using as a derivatizing reagent 2,4-dinitrophenylhydrazine (DNPH) [13].

In this respect we have recently reported that DAN (2,3-diaminonaphtalene) can be used as alternative to DNPH for the HPLC-based assay [13, 14]. This analytical method is based on the reaction between MA and DAN to afford a naphthodiazepinium ion that present a UV absorption optimum at 311 nm, useful for MA determination by HPLC with UV detector.

7.2 THE ROLE OF ANTIOXIDANTS

In nature, as in manufacture, the most effective and convenient approach to protect molecules from oxygen reactivity is to use antioxidants.

Antioxidants attract much attention since age-related and degenerative diseases are connected to the oxidation of biological macromolecules induced by reactive oxygen species (ROS). Trough recent studies, aging, cancer, atherosclerosis, and some other serious diseases have been confirmed to correlate with low density lipoprotein (LDL), cell membranes, and DNA exposed to oxidative stress [15, 16].

Compounds able to break the radical chain reaction are called primary antioxidants and are divided into two groups. Preventive antioxidants interfere with the initiation process blocking the formation of free radicals [17]. One of the most common sources of initiation is the Fenton reaction occurring between the reduced state of transition metal ions (Fe^{2+} , Cu^{2+}) and hydrogen peroxide (H_2O_2) or organic hydroperoxides (ROOH) [18]. Products of their decomposition are hydroxyl and alkoxy radicals which initiate the chain [17]. Several enzymes, such as glutathione peroxidase, phospholipid hydroperoxide, and catalase reduce hydroperoxide and hydrogen peroxide, blocking the formation of free radicals [17, 19, 20]. Proteins such as transferrin, ferritin, lactoferrin, and ceruloplasmin are known to prevent formation of free radicals by sequestering transition metal ion and they are also called iron-binding proteins. Carotenoids act as a quencher of singlet oxygen which oxidizes unsaturated lipids to give hydroperoxides initiating the lipid peroxidation. Some inhibitors of lipoxygenase that also oxidizes unsaturated lipids specifically could have a similar effect [21]. The chelation of transition metal is the another mechanism witch the primary antioxidants stop the formation of free radicals. By blocking redox-active metal ions in an oxidized form (Fe^{3+} , Cu^{3+}), metal chelators may prevent the occurrence of Fenton-type chemistry. Catalase (CAT) similarly blocks initiation by removing hydrogen peroxide, which is decomposed into non-radical species [22]. Phytic acid, present in edible

legumes, cereals and seeds, forms an iron chelate which greatly accelerates Fe^{2+} -mediated oxygen reduction yet blocks iron-driven hydroxyl radical generation and suppresses lipid peroxidation [23]. Another important metal chelator is lipoic acid. Lipoic acid can have antioxidant activity by chelating Fe^{2+} and Cu^{2+} , and DHLA (reduced form of lipoic acid) by chelating Cd^{2+} . Lipoic acid may provide antioxidant activity by chelation of iron. This conclusion is based on results in a hydroxyl radical scavenging assay in which deoxyribose was used as a detector molecule. Deoxyribose binds to iron, inducing site-specific degradation of deoxyribose. However, after addition of lipoic acid, it was concluded that lipoic acid displaces the deoxyribose from the deoxyribose-iron complex. Therefore, it can be deduced that lipoic acid chelates Fe^{2+} , thus diminishing the amount of OH^\cdot detectable by deoxyribose [24].

The second type of antioxidants is called chain-breaking antioxidants or radical-trapping antioxidants. They block the autoxidation by competing with the propagation reaction chain. They react with peroxy radicals more rapidly than they can attack the oxidizable substrate, and their products of reaction don't propagate the autoxidation chain reaction. The most important and effective small-molecule antioxidants are chain-breaking. Polyphenols and phenols are the prototypical examples: they are able to trap several peroxy radicals per molecule of antioxidant, depending on the stoichiometry behind the antioxidant activity [1].

The third class of antioxidants is that of indirect antioxidants as they can only operate by increasing the activity of true endogenous antioxidants. Many dietary antioxidants don't show relevant antioxidant behavior, for example in the protection of linoleic acid in model systems. Nonetheless, they increase the antioxidant defenses in living systems, for example by inducing the expression of antioxidant enzymes such as glutathione reductase and glutathione peroxidase [25].

The distinction between antioxidants and pro-oxidants should not be seen as a dichotomy, since it often depends on the system conditions, and known antioxidants in a model chemical system not infrequently can act as pro-oxidants in living cells or a different model system [25, 26]. Compounds that are able to increase the rate of autoxidation in a system or the oxidative damage in a living system by depleting antioxidants or increasing the radical generation. A typical mechanism by which antioxidants can act as prooxidants is the reduction of the transition metals such as Fe^{3+} and Cu^{3+} . By their reduction, antioxidants might make these reduced forms available to reduce hydrogen peroxide or organic hydroperoxides in a Fenton-type reaction, increasing the rate of initiation [26]. The

mixture of iron salts and ascorbic acid is a well-known pro-oxidant system (in the presence of H₂O₂) that turns into an antioxidant at a high concentration of ascorbic acid [27]. On the other hand, some antioxidants like flavonoids can simultaneously operate with different mechanisms, preventive, chain-breaking, and indirect, with relative contributions that depend on experimental conditions. These aspects are actually a major source of controversy in antioxidant testing capacity.

7.3 ANTIOXIDANT ACTIVITY OF CYTOKININS AND CYTOKININ RIBOSIDES

In addition to the role of plant hormones, several evidences indicate that cytokinins may act as antioxidant or regulator of antioxidant. Kinetin has been shown to have a direct effect on superoxide dismutase, prevent oxidation of unsaturated acids in plant membrane, stimulate the biosynthesis of isoprenoid and tocopherols to alleviate the salt stress in the plants [31-33]. Similar activity is that of N⁶-benzyladenine: exogenous 6-BA also significantly reduces the production rate of superoxide anion and malondialdehyde content. N⁶-benzyladenine can markedly increase the activity of antioxidant enzymes superoxide dismutase and peroxidase, the antioxidant metabolites ascorbate and reduced glutathione (GSH), and proline in both genotypes under salt stress [34]. Kinetin may act as antioxidant: at the concentration which kinetin inhibits *in vitro* platelet aggregation, the electron spin resonance (ESR) signal intensity of hydroxyl radical has been reduced in a concentration dependent manner [35]. Hydroxyl radical and the other free radicals are involved in several pathological events related to inflammatory processes. This could be extended to pathologies related to altered platelet functions.

Although the few papers here reported can be related to an antioxidant activity of cytokinins, the characterization of the antioxidant profile of cytokinin is at present lacking. One of the purposes of this PhD project was the evaluation of the antioxidant capacity of natural and non-natural CKRs by a panel of antioxidant assays. The study considered also the antioxidant activity of the bases CKs that are devoid of the ribose moiety. By this approach the influence of ribose on the antioxidant capacity of CKRs could be eventually be assessed.

7.4 ANTIOXIDANT CAPACITY ASSAYS

The antioxidant methods in foods, cosmetics and biological systems are mainly classified into two groups. In the assays that evaluate lipid peroxidation using lipid or lipoprotein substrate under standard conditions, the rate of inhibition of oxidation is measured and tests that investigate free radical scavenging ability necessarily to measure the rate of scavenging of the preformed free radicals [28]. Normally, two different approaches have been considered. The first are inhibition assays, for which the extent of the scavenging of a free radical by hydrogen atom or electron donation is the marker of antioxidant activity. These methods are an indirect measure of total antioxidant power [29]. The second approach include assays involving the presence of antioxidant systems during the generation of the radical, for which the activity is measured and compared with the rate of oxidation of a target molecule in presence and absence (blank) of the antioxidant [30]. In both approaches, a reference compound for each antioxidant assay should be used to check the correct procedure of the test. In this section, only the assays used to characterize the antioxidant profile of CKs and CKRs, will be described.

7.4.1 Hydroxyl radical

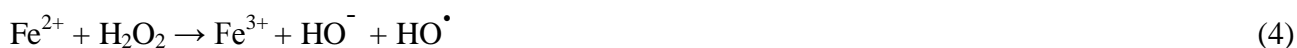
Among ROS, hydroxyl radicals (HO^\bullet) are the most instable and aggressive radicals known in biology [36]. Hydroxyl radical is generated under various chemical conditions, essentially consisting of a mono-electronic reduction of hydrogen peroxide (H_2O_2) (equation 1) or through a Haber-Weiss reaction which generates hydroxyl radicals from hydrogen peroxide and superoxide radicals (equation 2):



However, it has been pointed out that this reaction cannot take place under physiological conditions [37], whereas $\text{O}_2^{\bullet -}$ can *in vivo* reduce ferric ions (Fe^{3+}) present in iron storage proteins. Therefore, Fe^{2+} ions are liberated in solution according to the equation 3:



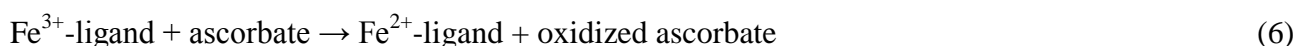
Ultimately, the mono-electronic reduction of H_2O_2 can take place with Fe^{2+} ions to generate HO^\bullet according to the well known Fenton reaction (equation 4) [17]:



In most of the chemical assays of the hydroxyl radicals (HO^\bullet), the reducing specie (Fe^{2+}) is bound to a variety of ligands (equation 5) and the formed oxidized specie Fe^{3+} is in situ reduced in order to regenerate the Fe^{2+} specie.



For this specific purpose, ascorbic acid can be efficiently used as a reductant agent (equation 6) [38]:



Among various methods currently available for the *in vitro* quantitative detection of ROS in various aqueous environments [28, 39], the 2-deoxyribose (2-DR) degradation assay is commonly used for the evaluation of HO^\bullet scavenging activity of a given compound and a great number of relevant applications has been reported up to now [40]. The method was introduced in 1981 [41, 42] and has been used as a suitable approach to determine the HO^\bullet scavenging activity of a great number of compounds [43].

The 2-DR assay is conceptually simple, although the generation of the hydroxyl radicals itself is not a simple process so that many variables of the experimental protocol might influence the final outcome of the assay. The assay relies on the reaction of hydroxyl radicals with 2-DR and the formation of malondialdehyde (MA) [44] and other carbonyl reacting species generally referred to as MA-like products arising from abstraction of hydrogen radical H^\bullet from 2-DR. The amount of MA-like products depends on the 2-DR concentration and/or the HO^\bullet supply dose and ranges from less than 2% for MA to variable yields for other carbonyl compounds [45].

MA and MA-like products react with thiobarbituric acid (TBA) and for this reason are called TBA-reactive substances (TBARS, Fig. 7.3) that can be evaluated spectrophotometrically at 532 nm. The required pink color for the spectrophotometric determination can be developed through the reaction between TBA and TBARS. It should be noted that this reaction requires strongly acidic conditions and also has to be carried out at 90-95 °C and this is one of the limits of the method.

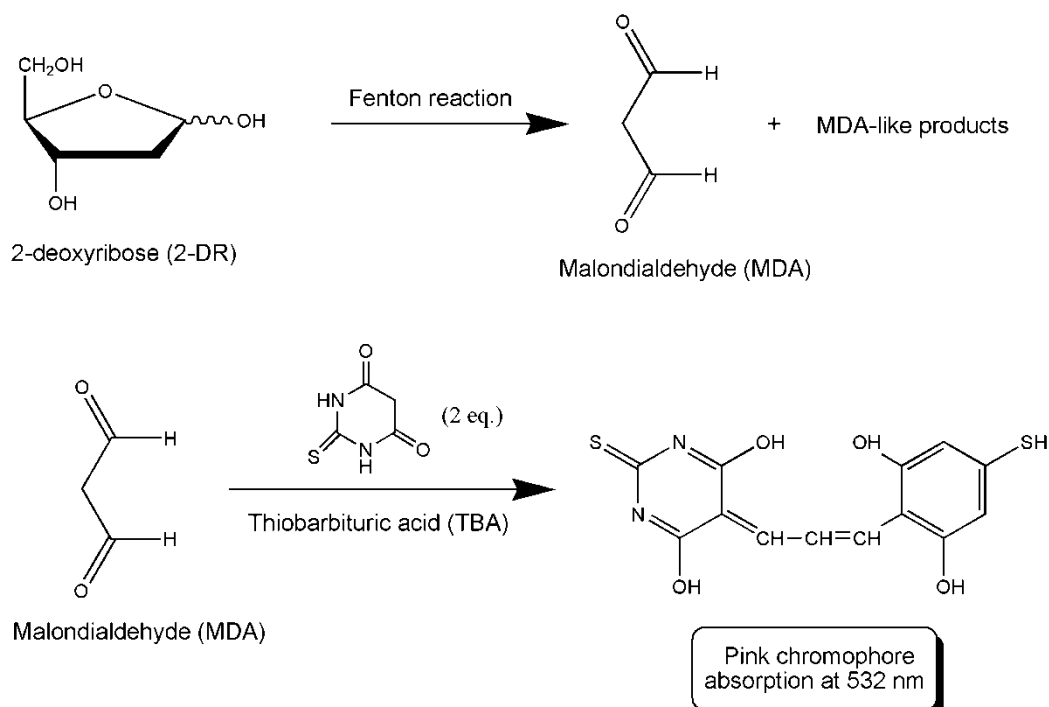


Figure 7.3 Scheme of the 2-deoxy ribose (2-DR) degradation assay.

Another very important point arises from the consideration that the range of reaction rates with the HO^\bullet radical is estimated to be 10^7 - $10^{10} \text{ M}^{-1} \text{ s}^{-1}$ for any organic compound [17] that nearly approaches the diffusion controlled limit [46]. As early as in 1990, Gutteridge and Halliwell had already raised other problems of the assay, suggesting that addition of metal ions, H_2O_2 , antioxidants and chelating agents can influence not only peroxidation in the incubation medium but also peroxide decomposition during the assay itself [47].

For all of the above reasons, there are still difficulties to standardize or validate the method as a general hydroxyl radical scavenging assay [48]. This issue has been critically examined in a recent paper [40] that discusses various parameters influencing the reliability of the assay and it was demonstrated that how a careful control of some variables provided a correction to the antioxidant capacity of compounds that were underestimated without this control.

7.4.2 Peroxyl radical

Hydroperoxyl radicals are generated through the transfer of a hydrogen atom to molecular oxygen, an oxygen atom to a hydroxyl radical or a proton to a superoxide anion [49]. Unlike the superoxide anion which mainly acts as reductant, peroxyl radical can act as oxidant in several important biological reactions such as the abstraction of hydrogen atoms

from α -tocopherol and polyunsaturated fatty acids [50] in the lipid peroxidation in the lipid bilayer of cell membrane.

Peroxy radicals are generated as intermediates in the enzymatic and in the non enzymatic induced LPO reactions (Fig. 7.4). But in the enzymatic reaction the generated LOOS radicals are transformed within the enzyme complex to LOOH molecules. On the other hand, in the non enzymatic reaction the LOOS radicals remove a hydrogen radical from any other activated C–H bond to form an LOOH molecule. As long as the reaction is carried out in an environment consisting of other polyunsaturated fatty acids (PUFA), the only available hydrogens are those of activated CH_2 groups of other PUFA molecules. Because of their high reactivity the LOOS radicals abstract the closest available hydrogen or react with any double bond in their environment by epoxidation. As a consequence, a great variety of secondary products and radicals is generated such as short-chain alcohols, aldehydes and ketones, leading finally to total destruction of biomolecules unless the chain reaction is blocked by antioxidants that act as radical scavengers. Tocopherols operate as peroxy radical scavengers blocking the propagation of free radicals by reacting with them to form the tocopheryl radical which will be reduced by a hydrogen donor such as the ascorbic acid and return to its reduced state [51].

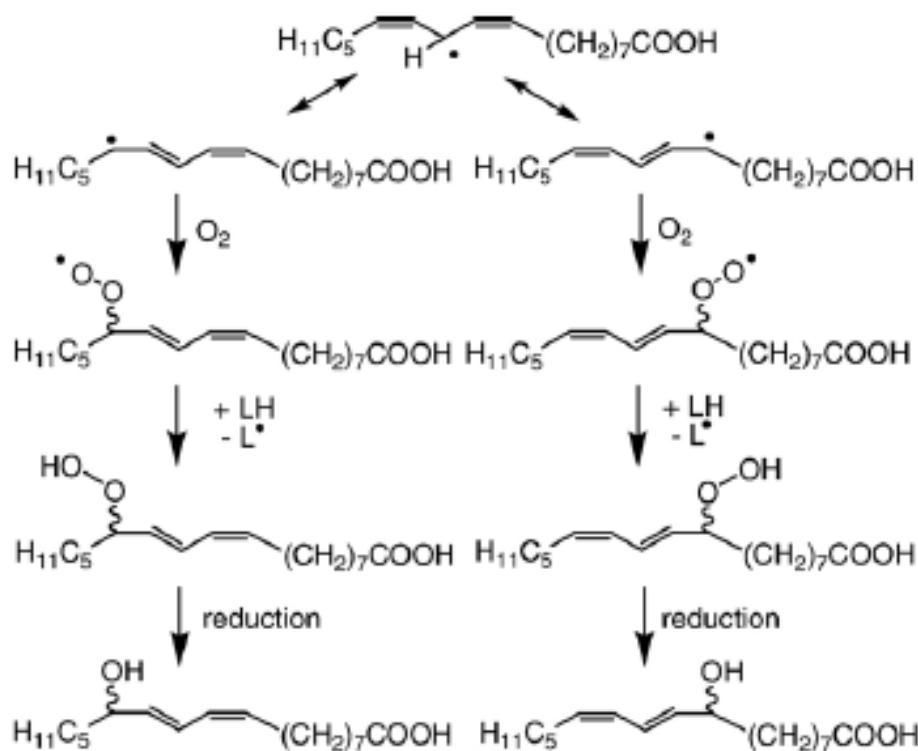


Figure 7.4 Hydrogen removal from a PUFA with generation of peroxy radicals.

The peroxy radical scavenging activity of compounds supposed to be antioxidants is determined by ORAC (Oxygen Radical Absorbance Capacity) assay. In this assay, antioxidants prevent the reaction between peroxy radicals and an oxidizable probe (Fig. 7.5), whose reaction can be easily detected by some spectroscopic techniques (UV-Vis, fluorescence, EPR) [1].

The antioxidant capacity is quantified by recording the fluorescence decay of β -phycoerythrin (β -PE) or fluorescein in the presence of antioxidants. Being a protein, the limit of β -phycoerythrin is due to its variable reactivity to peroxy radical and its short photostability during the exposition to excitation light [28]. To solve these problems, β -PE was replaced by fluorescein (FL) a synthetic phenolic derivative with a fluorescent emission at 520 nm on excitation at 480 nm [52]. FL is a synthetic non protein probe, created to overcome the limitations of β -PE. In addition, the reaction products of FL with peroxy radical have been characterized, and the product pattern was consistent with a classic HAT (Hydrogen Atom Transfer) reaction mechanism. The improved ORAC assay provides a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity versus peroxy radicals [53, 54]. AAPH (2,2'-azobis-(2-amidinopropane hydrochloride) as a peroxy radical generator (ORAC_{ROO·}) or Copper (II)-H₂O₂ as a hydroxyl radical generator (ORAC_{HO·}) are used in the method (Fig. 7.6).

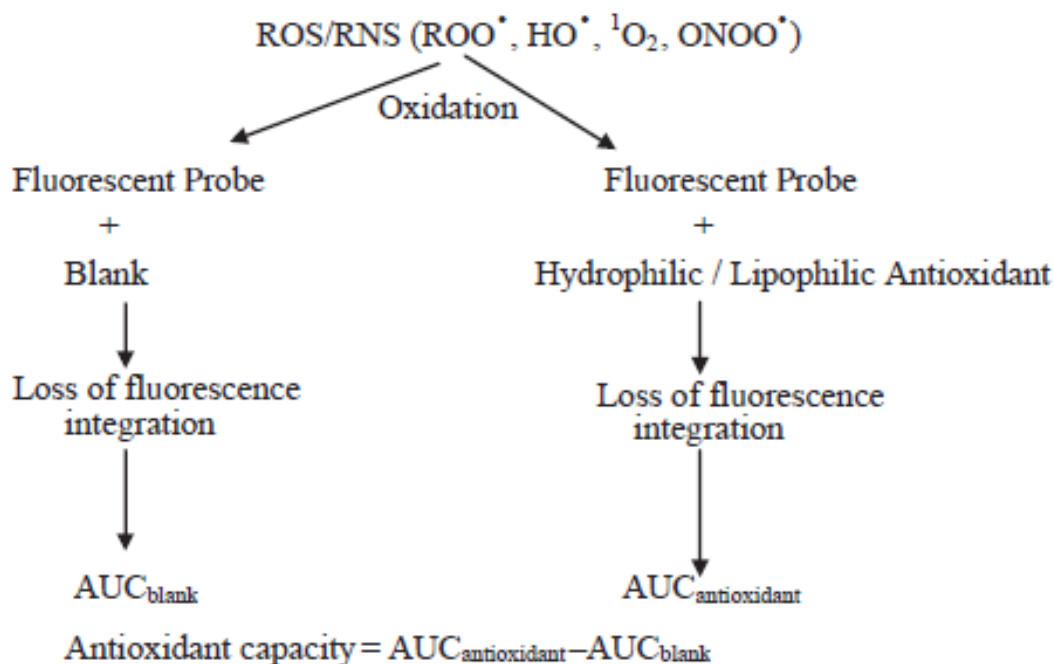


Figure 7.5 Principle of ORAC assay by using a fluorescent probe.

The ORAC combines both inhibition time and inhibition percentage of free radical action by antioxidants and expresses the results as Trolox equivalents [55]. Typically, samples,

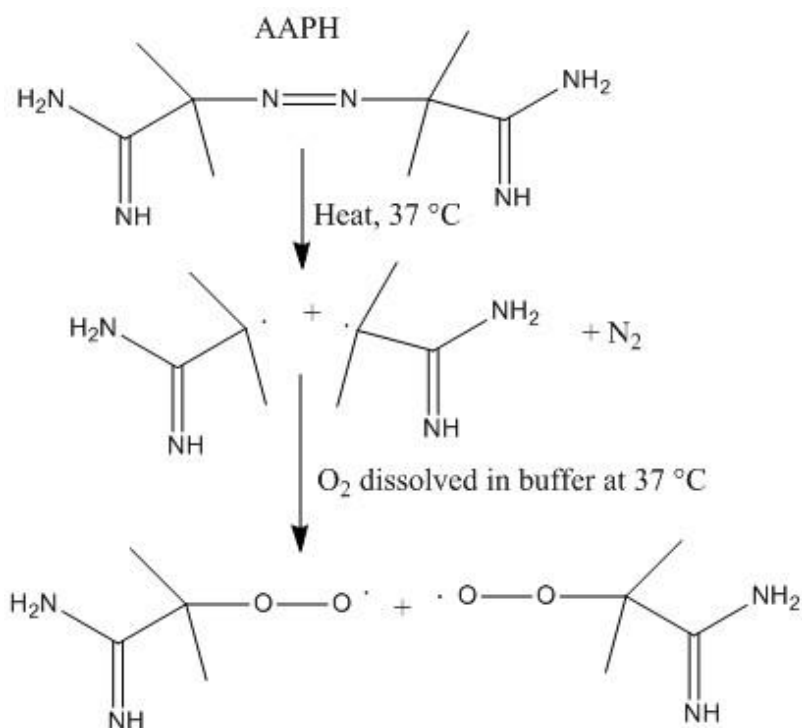


Figure 7.6 AAPH radical generator mechanism.

controls, and Trolox are mixed with fluorescein solution before AAPH solution is then added to initiate the reaction. The fluorescence intensity is measured at ambient conditions (pH 7.4, 37 °C). As the reaction progresses, fluorescein is consumed and FL intensity decreases. In the presence of antioxidant, the FL decay is inhibited [56]. Data reduction from the ORAC assay is achieved by calculating of the area under the kinetic curve (AUC) and net AUC ($\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}$), obtaining a standard curve by plotting the concentration of Trolox and the AUC and calculating the Trolox equivalents. The advantage of the AUC approach is that it applies equally well for both antioxidants that exhibit distinct lag phases and those samples that have no lag phases. There is a direct linear correlation of AUC and a broad range of sample types, including raw fruit and vegetable extracts, plasma, and pure phytochemicals [57].

7.4.3 Superoxide Anion

Superoxide anion is a one-electron (e^-) adduct of molecular oxygen (dioxygen, O_2) formed by the combination of O_2 and e^- . It is produced in response to environmental factors such as UV light, cigarette smoke, environmental pollutants or enzymes such as xanthine oxidase (equation 7) and NADPH oxidase (equation 8) [58, 59].



Through a Haber-Weiss reaction, superoxide radicals generate hydroxyl radicals reacting with hydrogen peroxide (equation 2). Although associated with oxidative stress, O_2^{2-} is an unusual species in that it can act as a reducing agent, donating its extra electron, to form ONOO^- with NO (equation 9) [60].



To determinate the superoxide scavenging activity of a compound, two approaches are available: the first is an enzymatic assay while the second is chemical. In the first case, superoxide anions are generated by a Xanthine \ Xanthine oxidase system to induce the reduction of nitro blue tetrazolium (NBT). In the presence of an antioxidant, the rate of NBT reduction decreases in respect to the blank [61]. In the second method, superoxide anions are generated by a non enzymatic (phenazine methosulfate - NADH) system [62].

The major advantage of this method is the use of physiologically relevant radical system and reaction pH. Other techniques such as DPPH and ABTS cation radical scavenging assays use free radical systems without resemblance to those involved in oxidative processes in biological systems. On the other hand, this assay presents several disadvantages. First, it is known that nitro blue tetrazolium (NBT) can be directly reduced by some antioxidants [63] with the risk to overestimate the antioxidant capacity using this method. Second, the enzymatic reaction of xanthine oxidase can generate another ROS, hydrogen peroxide and they can interfere with the assay [64]. Third, common to any antioxidant activity method using an enzymatic radical generating system, antioxidant capacity overestimation can result from test compounds directly interfering with the enzyme reaction. Fourth, the calculation of the results is based only on the kinetics of the antioxidant-radical reaction and doesn't considerer the thermodynamic properties. This may do hard to compare the results between complex matrix samples such as food extracts or cross laboratory analysis. To overtake these disadvantages, ESR has been used to generate on superoxide anions, compatible with hydrophilic and lipophilic extracts and compounds to be able to efficiently screen the antioxidant capacity of sample against this important radical [56].

7.4.4 DPPH Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical in methanol or ethanol solution and the assay is based on its reaction with a specific compound or extract to measure the scavenging activity of antioxidants [28, 66]. The odd electron of nitrogen atom in DPPH is reduced by a hydrogen atom to form antioxidants to the corresponding hydrazine (Fig. 7.7) [28].

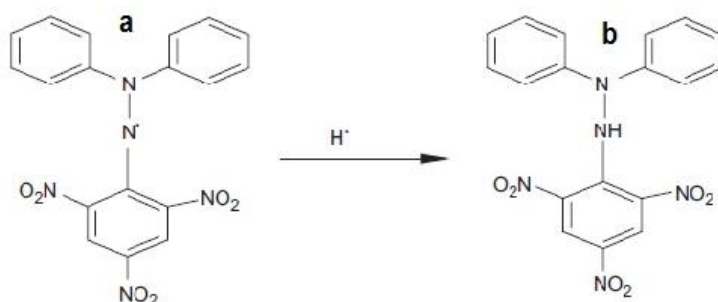


Figure 7.7 α,α Diphenyl- β -Picryl hydrazyl [DPPH] (a), α,α Diphenyl- β -Picryl hydrazine (b).

The DPPH assay was believed to involve hydrogen atom transfer reaction, but a recent study suggested another explanation. On the basis of the kinetic analysis of the reaction between phenols and DPPH, Foti *et al.* suggest that the reaction in fact behaves like an electron transfer (ET) reaction (Fig. 7.8).

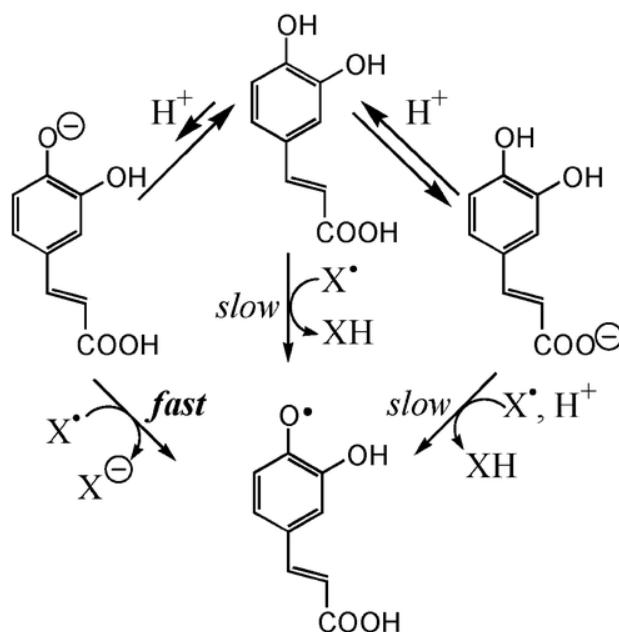


Figure 7.8 Electron transfer scheme in reaction between DPPH and phenols.

The authors found that the rate-determining step for this reaction consists of a fast ET process from the phenoxide anions to DPPH. The hydrogen atom abstraction from the neutral ArOH by DPPH becomes a secondary reaction path, because it occurs very slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol [67].

The reduction of DPPH[•] (equation 10-11) is following by monitoring the decrease of the



absorbance during the reaction. In its radical form, DPPH[•] absorbs at 515-517 nm [68], but upon reduction by an antioxidant (AH) or a radical species (R[•]), the absorption disappears in a concentration dependent manner [66]. The DPPH method is a valid, easy, accurate, sensitive, and economic method to evaluate scavenging activity of antioxidants, since the radical is stable and need not to be generated as in other scavenging assays. The results are highly reproducible and comparable to other scavenging methods such as ABTS [69]. The DPPH assay is technically simple, but some limitations reduce its applications. First, DPPH is a long-lived nitrogen radical, which bears no similarity to the highly reactive and transient peroxy radicals involved in lipid peroxidation. Many antioxidants that react quickly other radicals might react slowly or might even be inert to DPPH. Consequently, the antioxidant capacity is not properly rated and it's necessary to find an adequate incubation time to allow the reaction between antioxidants and radical. The reaction kinetics between DPPH and antioxidants are not linear to DPPH concentrations and it's rather arbitrary to express antioxidant capacity using EC₅₀ [28, 70]. Second, DPPH is sensitive to some Lewis bases and solvents [71]. However, the rate of reaction of DPPH depends strongly on the solvent. In dioxane or CCl₄, reactions obey second- or third-order kinetics. Recently, kinetic solvent effects on hydroxyl hydrogen abstraction from α-tocopherol or phenol were obtained in several solvents. The increased reactivity of DPPH in alcohols has been hypothesized due to the formation of an H-bond between DPPH nitrogen and the alcohol, decreasing radical delocalization and thus increasing its reactivity [72]. Third, DPPH is only soluble in organic solvents and interference of absorbance from sample compounds could be a problem for quantitative analysis. The method has a limitation in reflecting the partitioning of antioxidants in emulsion systems and is not useful for measuring the antioxidant activity of plasma as proteins are precipitated in the alcoholic medium [28].

7.4.5 Trolox Equivalence Antioxidant Capacity (TEAC)

The TEAC Test (Trolox Equivalent Antioxidant Capacity) is based on the reaction with the colored and relatively persistent 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) radical cation (or monoanion, if the two sulfonate groups are considered), which has a strong absorption band at 734 nm ($\epsilon_{734} = 0.015 \mu\text{M}^{-1} \text{cm}^{-1}$, where ϵ is the molar extinction coefficient) (Fig. 7.9).

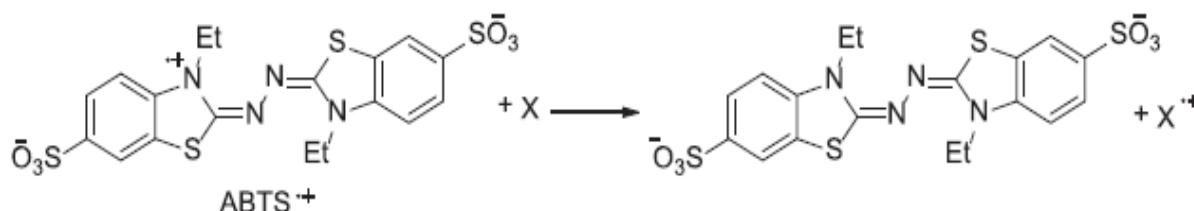


Figure 7.9 Reaction of ABTS cation with an antioxidant compound.

The radical is generated in buffered water just before the test, by reacting ABTS with an oxidizer such as potassium persulfate or MnO_2 . The antioxidant activity is defined as the amount of ABTS^{•+} quenched after a fixed time, and is compared with that produced by Trolox [73].

ABTS^{•+} radical cation can be dissolved in aqueous and acidified ethanol medium and thus can be used for determining the antioxidant capacity of both hydrophilic and lipophilic compounds, food products, extracts, and biological fluids. The method is rapid and easy to perform, avoids unwanted reactions and does not require drastic conditions to generate radicals, and antioxidant activity can be studied over a pH range [74]. However, the time interval should be taken into account, as different time intervals give different TEAC values because the time required to reach the stationary concentration, which inversely depends on the rate constant for the reaction between antioxidants and ABTS^{•+}, widely varies among antioxidants [75-77]. An important limitation of this assay is that ABTS^{•+} is a radical cation while the peroxy radical is neutral, so antioxidants react with ABTS^{•+} by an electron transfer mechanism, whereas with peroxy radicals they react by formal H-atom transfer [78].

7.5 EXPERIMENTAL SECTION

7.5.1 MATERIALS

The following CKs N⁶-(Δ^2 -isopentenyl) adenine (iPA_{do}), kinetin (K), N⁶-benzyladenine (B) and p-topoline (p-T) and their correspondent ribosides N⁶-(Δ^2 -isopentenyl) adenosine (iPA), kinetin riboside (KR), N⁶-benzyladenosine (BA) and p-topolin riboside (p-TR) were obtained from OlChemIm Ltd. (Olomouc, Czech Republic).

The synthesis of the N⁶-substituted adenosine derivatives FEA, 2HFEA and 2FEA has been described in Chapter 4.

All reagents were purchased from Sigma–Aldrich Italy and were of analytical grade.

7.5.2 METHODS

7.5.2.1 2-deoxyribose degradation assay (2-DRA)

7.5.2.1.1 Reagent preparation

Stock solutions of 2-deoxyribose (25 mM) and phosphate buffer 10 mM (pH 7.4) were prepared and kept in a cold room at 4-6 °C. Stock solution of EDTA (10 mM) was prepared at pH 7.0 according to the methods of Lopes *et al.* [79] and kept at room temperature. Fresh aqueous stock solutions of 10 mM FeCl₃, 2 mM H₂O₂ and 0.5 mM ascorbic acid were prepared daily. FeCl₃ was dissolved in 0.1 M HCl, as described by Lopes *et al.* [79]. 0.5 ml of 10 mM FeCl₃ was mixed with 9.5 ml of 10 mM EDTA to obtain Fe³⁺-EDTA complex (500 μ M Fe³⁺). Fresh solutions of the tested compounds were prepared daily. The cytokinins were dissolved in water by adding 1M HCl to obtain a clear solution of their salt. Then 1M NaOH was added to reach pH 7.4. CKRs were suspended in water and solubilised with ultrasound bath at the temperature of 50 °C for 15 minutes. Stock solution of 1% TBA (w/v) was prepared in 50 mM NaOH and used within 1 week.

7.5.2.1.2 Procedure

The hydroxyl radical scavenging activity of the cytokinins was evaluated as described by Aruoma [43] with some modifications. In a screw capped glass tube, 100 μ l of Fe³⁺-EDTA, 100 μ l of H₂O₂, 200 μ l of 2-deoxyribose and 200 μ l of 10 mM phosphate buffer (pH 7.4) were sequentially added.

To the reaction mixture, 200 μ l of a solution of the tested compound was added in order to reach the final concentrations from 10 μ M to 500 μ M (10, 20, 50, 100, 200, 300, and 500 μ M) At the end, 200 μ l of ascorbic acid was added to a final volume of 1 ml to start the reaction at 25 °C.

After 40 min the reaction was stopped by addition of 1 ml of 4% (v/v) phosphoric acid and then 1 ml of 1% (w/v) TBA was added. The tubes were placed in hot water (90–95 °C) to develop a pink color. After 15 min, the tubes were removed from the hot water, cooled at room temperature and the absorbance was recorded at 532 nm. The used apparatus was a spectrophotometer UV/VIS DU 640 (Beckman, Pasadena, USA). Each assay was performed in triplicate and the percentage of hydroxyl radical scavenging activity was calculated from the relationship:

$$\text{Scavenging activity (\%)} = [(A_0 - A_s) / A_b] \times 100$$

where A_0 is the absorbance of the blank and A_s is the absorbance of the sample. All the measurements were performed in triplicate. Results are expressed by reporting the percentage scavenging activities versus tested compound concentrations.

7.5.2.2 ORAC (Oxygen Radical Absorbance Capacity) assay

7.5.2.2.1 Reagent preparation

Stock solutions of 1mM 2,7-dichlorofluorescein and 56.67 mg/ml ABAP were prepared in phosphate buffer 75 mM (pH = 7.0) and distilled water respectively and kept at -30°C. For the assay, 1mM solution of 2,7-dichlorofluorescein was daily diluted with phosphate buffer to obtain a 500 nM solution. Trolox solution was prepared daily in a water : ethanol mixture 50:50 to obtain a 200 µM solution. Fresh solutions of the tested compounds were prepared daily, dissolving each cytokinin and cytokinin riboside in the same mixture used for Trolox. The concentration range of the cytokinins was from 5 to 50 µM.

7.5.2.2.2 Procedure

The assay was carried out at 37°C according to Cao *et al.* [80] with modifications. A 24-well plate was used for the analysis of the compounds. Briefly, in a plastic tube, a mixture containing 2 ml of 75 mM phosphate buffer pH = 7.0, 250 µl of 500 nM 2,7-dichlorofluorescein, 250 µl of Trolox 200 µM or of the sample at different concentrations was prepared. Then, the volume of every tube was divided in four rates of 475 µl and 25 µl of a solution of ABAP (2.83 mg/ml final concentration) was added to each aliquot to start the reaction. Before the addition, ABAP was activated in a dry bath for 10 minutes at 37 °C. The used apparatus was a spectrophotometric plate reader Victor² 1420 Multilabel counter (Wallac, Turku, Finland) with fluorescence filters. The analyzer was programmed to record the fluorescence of 2,7-dichlorofluorescein ($\lambda_{ex} = 485 \text{ nm} / \lambda_{em} = 535 \text{ nm}$) every 5 min after ABAP was added until the fluorescence disappeared (8 h). Experimental data

were calculated using the differences between areas under the blank 2,7-dichlorofluorescein decay curves and those obtained with sample or Trolox and expressed as Trolox equivalents ($\mu\text{mol/L}$). The data obtained by the ORAC assay were elaborated with GraphPad Prism version 6. The final results were presented by reporting Trolox equivalent versus tested compound concentrations.

7.5.2.3 Superoxide anion assay

7.5.2.3.1 Reagent preparation

Phosphate buffer 50 mM (pH = 7.4) was prepared and kept in a cold room at 4-6 °C. Aqueous solutions of 2.34 mM NADH, 300 μM phenazine methosulfate and 750 μM nitro blue tetrazolium (NBT) were prepared daily. Fresh solutions of CKs and CKRs were also prepared daily dissolving the compounds in DMSO. The concentration range of the CKs and CKRs was from 0.3 to 15 mM.

7.5.2.3.2 Procedure

The assay was carried out at room temperature according to Yen *et al.* [81] with modifications. In a plastic tube, 1,195 μl of phosphate buffer 50 mM (pH = 7.4), 5 μl of sample at different concentrations, 100 μl of 2.34 mM NADH, 100 μl of 300 μM phenazine methosulfate were mixed. Then 100 μl of 750 μM NBT were added to start the reaction. The reduction of NBT was monitored with a kinetic of 2 minutes. Caffeic acid was used as reference compound. At the end, the absorbance using a UV/VIS spectrophotometer Cary 50 Bio (Varian, Palo Alto, CA, USA). The percentage of inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = [(A_0 - A_s)/A_0]$$

where A_0 is the absorbance of the control and A_s is the absorbance of the sample or of the standard compound. All the measurements were performed in triplicate. Results are reported as percentage of inhibition of the superoxide anion versus tested compound concentrations.

7.5.2.4 Trolox Equivalence Antioxidant Capacity (TEAC) assay

7.5.2.4.1 Reagent preparation

ABTS was dissolved in water to give a 14 mM solution, according to Re *et al.* [73]. Potassium persulfate was dissolved in water to give a 4.9 mM solution. ABTS radical cation ($\text{ABTS}^{\bullet+}$) was produced by mixing same volumes of ABTS and potassium

persulfate stock solutions and allowing the mixture to stand in the dark at room temperature for 16 h before use. Tested compounds were prepared daily dissolving in ethanol in a concentration range from 5 to 100 μM . Trolox was used as positive control in the same concentration range.

7.5.2.4.2 Procedure

The antioxidant activity was assessed according with Re *et al.* [73] with slight modifications.

The ABTS^{•+} solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. After addition of 900 μl of diluted ABTS^{•+} solution ($A_{734} = 0.700 \pm 0.02$) to 100 μl of CKs or Trolox standard (final concentration 0.5-5 μM), the absorbance was taken after 15 minutes of incubation at 25 °C, using a UV/VIS spectrophotometer Cary 50 Bio (Varian, Palo Alto, CA, USA). The percentage of inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = [(A_0 - A_s)/A_0]$$

where A_0 is the absorbance of the control and A_s is the absorbance of the sample or of the standard compound. All the measurements were performed in triplicate. Results are reported as percentage of inhibition of ABTS radical versus tested compound concentrations.

7.5.2.5 DPPH Scavenging Assay

7.5.2.5.1 Reagent preparation

317.5 μM solution of DPPH was prepared daily dissolving 7.5 mg of 2,2-diphenyl-1- (2,4,6-trinitrophenyl) hydrazyl radical in 50 ml of ethanol. Then, a 1:5 dilution was effectuated to obtain a 65 μM work solution. The work solution was stable for 24h hours [82]. Fresh solutions of tested CKs and CKRs were daily dissolved in ethanol under ultrasound for 15 minutes. The concentration range of the CKs and CKRs was from 100 to 750 μM .

7.5.2.5.2 Procedure

The antioxidant activity was assessed according with Lavelli *et al.* [83] with slight modifications. Briefly, 300 μL of different concentrations of the samples in ethanol were added to 2.5 ml of 65 μM ethanolic solution of DPPH and 700 μl of ethanol. Trolox was used as reference antioxidant. The decrease in absorbance at 515 nm was determined after 30 min of incubation at room temperature (when a constant value was reached). The

percent decrease of DPPH concentration was calculated with respect to the initial value using the following formula:

$$\text{Inhibition (\%)} = [(A_0 - A_s)/A_0]$$

where A_0 is the absorbance of the control and A_s is the absorbance of the sample or of the standard compound. Triplicate solutions were analyzed for each sample.

7.6 RESULTS AND DISCUSSION

7.6.1 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of iPAdo and other cytokinins was determined at the concentration of 10 – 500 μM and the results are reported in figure 7.10.

All tested CKs react with this radical and that the scavenging activity of iPAdo is slightly higher, probably because of the allylic methylene group present in the N⁶-isopentenyl moiety that typically stabilizes the radical formation caused by the hydroxyl radical attack. It should be mentioned that the CKs did present problems of water solubility that is a necessary prerequisite for the 2-DR assay [84].

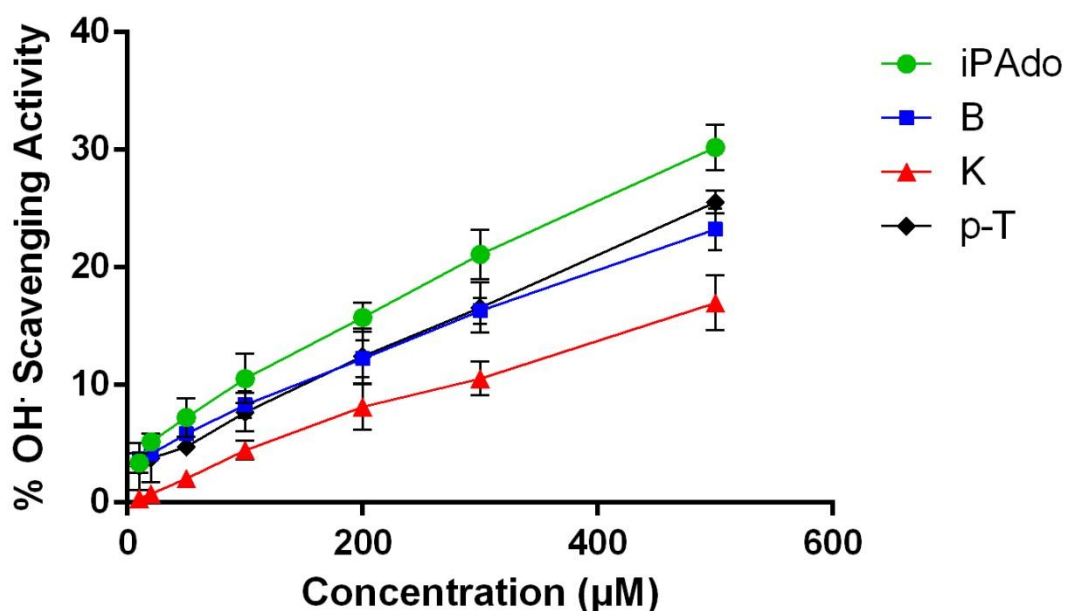


Figure 7.10 Hydroxyl radical scavenging activity of CKs.

Only iPAdo is soluble in water. The problem of water solubility of compounds to be tested by the assay is one of the major limitations of the method and this has received only a scant attention by a few researchers. In order to evaluate possible interferences of the above treatment of the bases on the result of the 2-DR assay, an aqueous solution of iPA and one with the compound dissolved with HCl/NaOH were compared and the results are reported in Figure 7.11. Figure 7.11 shows that the treatment of the N⁶-substituted adenine with NaOH/HCl does not interfere with the assay and the insoluble CKs were treated with NaOH/HCl as described above to be tested by the 2-DR assay.

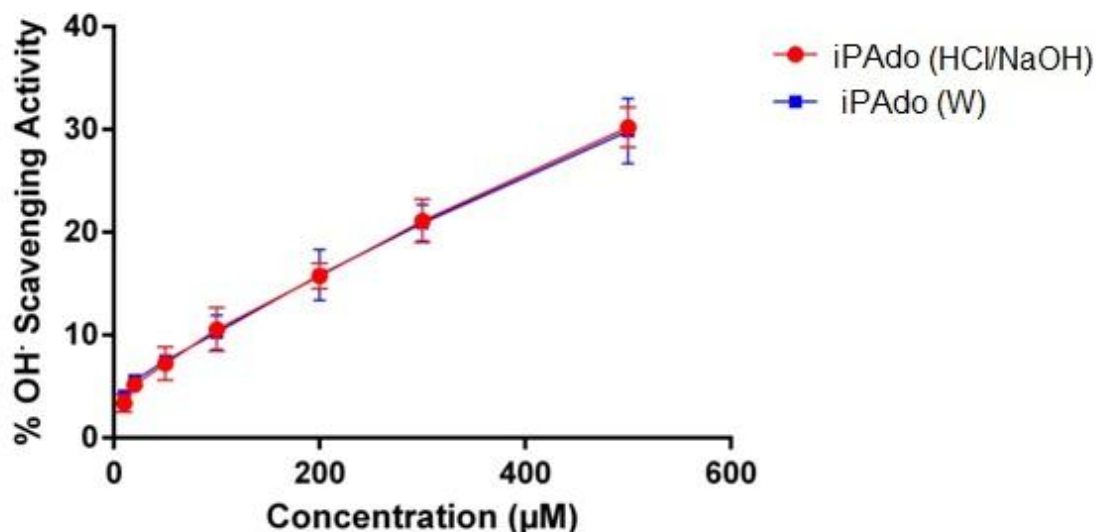


Figure 7.11 Hydroxyl radical scavenging activity of iPAdo dissolved in HCl/NaOH and in water only.

Then, we tested the corresponding CKRs against the hydroxyl radical in the same concentration range. Results are collected in Figure 7.12 and show that all compounds exhibit a similar activity with a maximum of 26-29%.

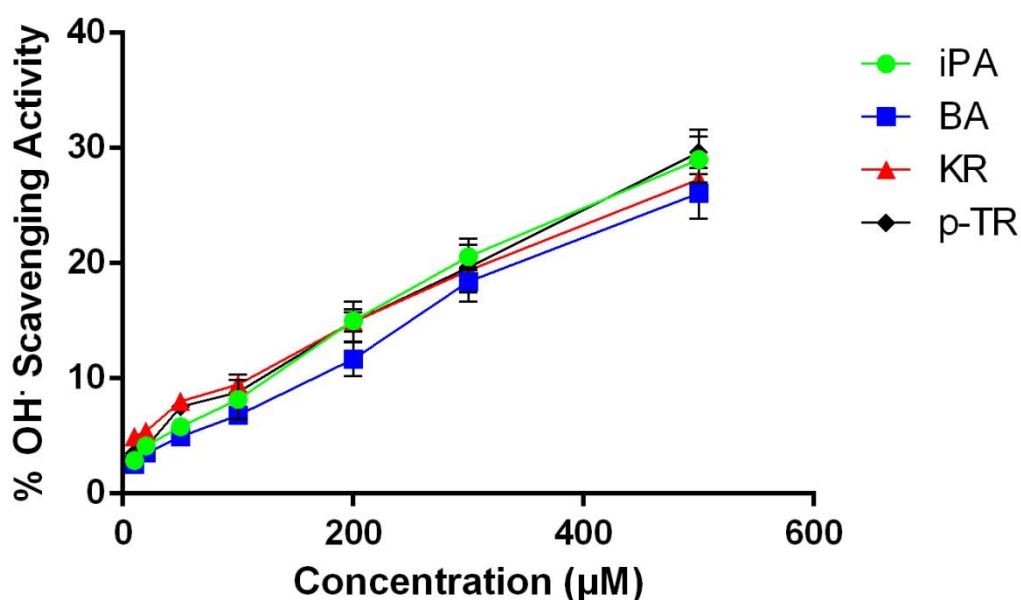


Figure 7.12 Hydroxyl radical scavenging activity of CKRs.

Unlike the CKs, the natural cytokinin ribosides iPa, BA, KR and p-TR were soluble in water. However, the synthetic N⁶-substituted adenosine derivatives, prepared as described in Chapter 4, were not soluble in water and it was not possible to dissolve 2HFEA and 2FEA with HCl/NaOH for the developing of an intense red color, due to the reaction of the

catechol with NaOH, that interfered with the 2-deoxyribose assay. Therefore, we could not perform the 2-deoxyribose degradation assay on these compounds.

Since all CKRs are N⁶-derivatives of adenosine, we have evaluated the *in vitro* scavenging activity against the hydroxyl radical of adenosine. The scavenging activity of adenosine and that of iPA taken as a reference compound, has been assayed, in order to establish the difference, if any, between the two compounds. This could furnish some information about the contribution of the N⁶-moiety to the antioxidant activity. In fact, the result reported in Figure 7.13 suggests that the isopentenyl moiety contributes to the antioxidant properties of iPA. This is confirmed by the iPAdo/adenine couple, whereas the known low scavenging power of ribose is confirmed by the results shown, all together, in Figure 7.13.

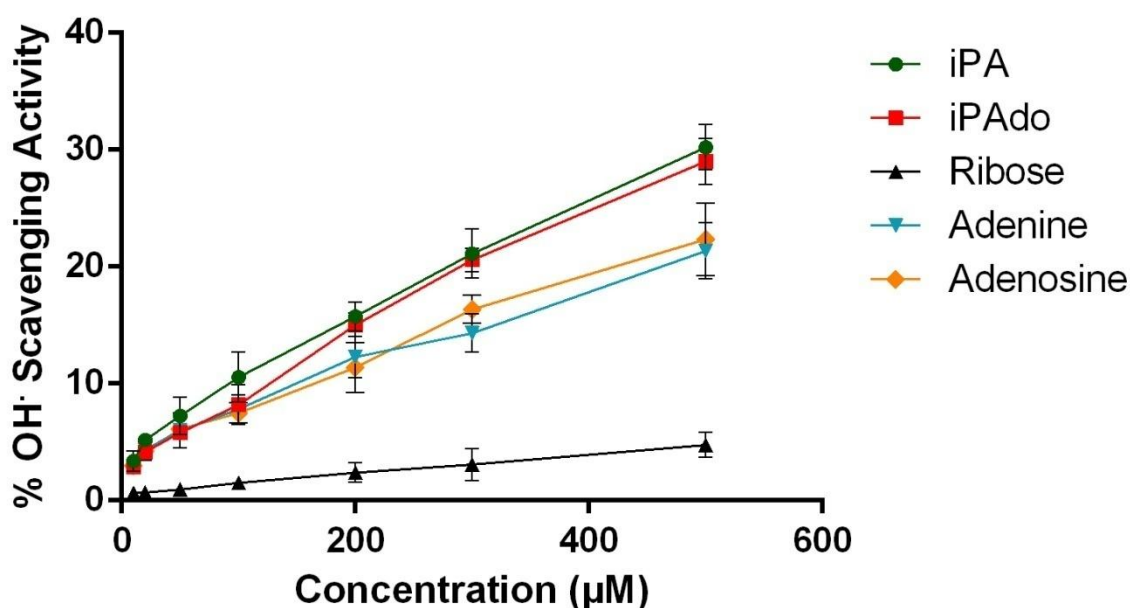


Figure 7.13 Hydroxyl radical scavenging activity of adenine, adenosine, ribose, iPA and iPAdo.

Additional contribution of the substitution at N6 in the different CKRs has been investigated as well and we can conclude that the good scavenging activity of kinetin riboside and topolin riboside can be explained by the chemical nature of N6 substitution. In fact, in kinetin contribution of the furane ring is related to the scavenging activity of this moiety, as previously reported for other furane-containing compounds [85-88]. For topolin riboside the presence of the phenolic OH in N6 can help to explain the good activity of this riboside.

7.6.2 Peroxyl radical scavenging activity

The peroxyl radical scavenging activity of CKs has been evaluated with the ORAC (O With the exception of benzyladenine (B), all the evaluated CKs are active against the peroxyl radical and the p-topolin (p-T) shows the highest activity. However, at lower concentrations, kinetin (K) is more active (Figure 7.14).

Due to the high antioxidant activity of K at low concentration, on a separate experiment, we evaluated also the antioxidant activity of kinetin (K) in a concentration range from 50 to 500 nM and the resulting data show a good peroxyl scavenging capacity (oxygen radical antioxidant capacity). The details of these results are reported in Figure 7.14 (part b) and show that the scavenging effect of kinetin is concentration dependent until 1 μM . After this value, at concentrations higher than 1 μM , the graphic goes to plateau.

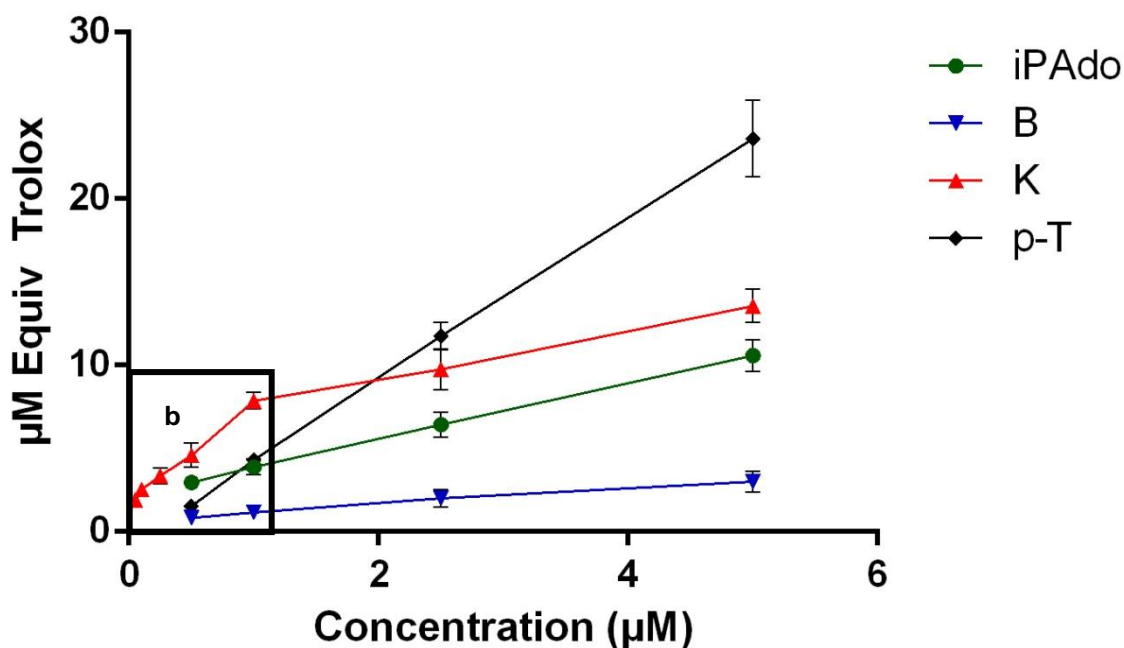


Figure 7.14 Peroxyl radical scavenging activity of CKs.

As recently observed by Amorati and Valgimigli, not always the evaluation of AUC (area under kinetic curve) values obtained by the ORAC test is sufficient to express the real antioxidant capacity of a given compound. It has been suggested that AUC values should be integrated with kinetic data in order to achieve more consistent information about the stoichiometry behind an antioxidant activity [1]. We have, therefore, recorded the time course of CKs and Trolox activities at 5.0 μM concentration (Fig. 7.15).

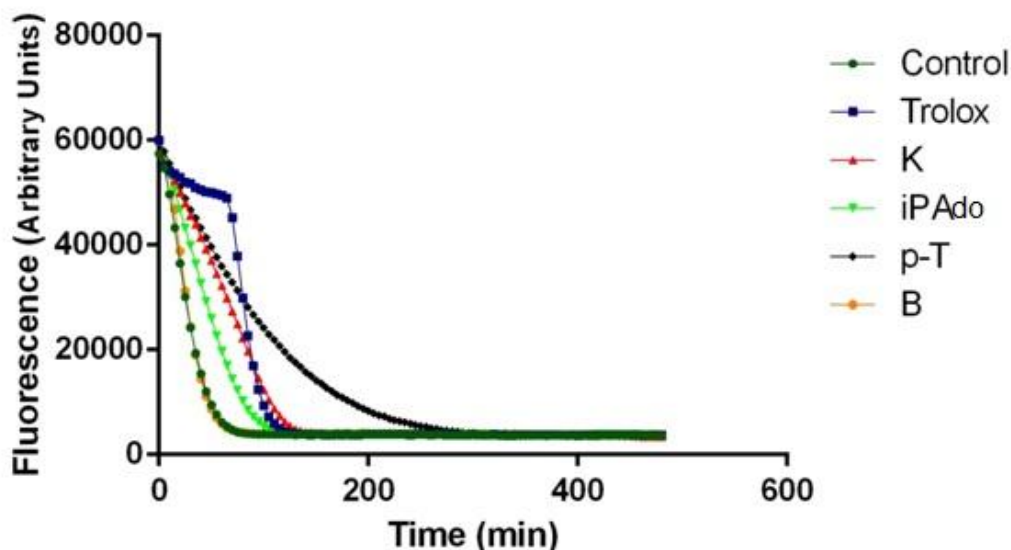


Figure 7.15 Decrease of fluorescence of CKs, control and Trolox in the ORAC assay.

As reported in Fig. 7.15, different kinetics characterize each compound and the activity of p-T is more persistent along the time. This behavior can be related to a different stoichiometry in the formation of radicals by each antioxidant at a given concentration [14]. Specifically, at higher concentrations the antioxidant activity of p-T is more efficient due to its stoichiometry higher than that of other CKs. This could be related to the presence of two sites of oxidation like the benzylic methylene and the phenolic OH groups that characterize the molecule. At lower concentrations, mainly the trapping capability of peroxy radicals can be observed, as in the case of K.

A similar trend was observed for natural CKRs as shown in figure 7.16.

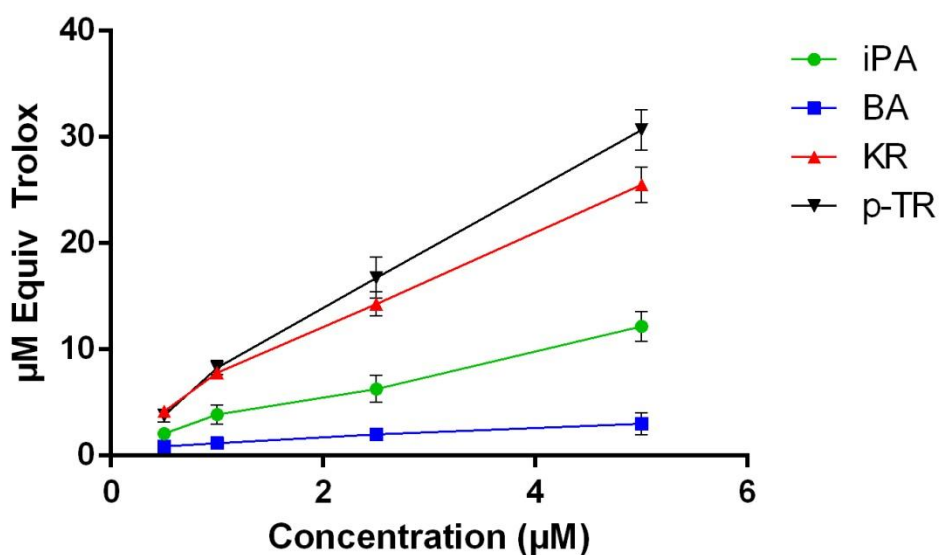


Figure 7.16 Peroxyl radical scavenging activity of natural CKRs.

If the antioxidant activity of benzyladenosine and N⁶-isopentenyl adenosine is similar to that of their corresponding bases, kinetin riboside is more active than its base, with a peroxy scavenging capacity near to the one that characterizes topolin riboside.

We, then, compared the antioxidant activity of topolin riboside with that of its synthetic derivatives, FEA, 2FEA and 2HFEA and the results are reported in figure 7.17.

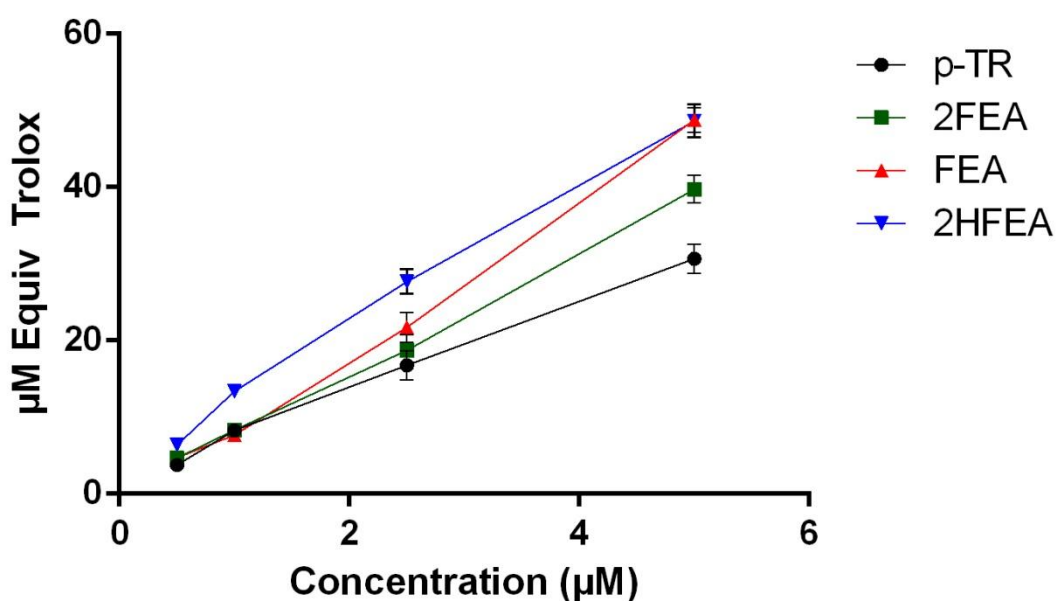


Figure 7.17 Peroxyl radical scavenging activity of synthetic N⁶-substituted adenosine derivatives.

Compared to topolin riboside, all synthetic derivatives show a higher antioxidant. The highest value is that of 2HFEA, probably due to the presence of the catechol moiety in N⁶ position. Compared to 2FEA, the distance (two methylene groups) of the catechol moiety from the purine ring than probably has a specific influence. It should be mentioned that catechols are strong peroxy radical scavengers, since a catechol group forms two hydrogen bonds with the two oxygen atoms of the lipid peroxy radical, leading to a very compact reactant complex. In this way, the catechol moiety of catechins becomes able to trap the lipid peroxy radicals in a dominant competition with the very damaging free-radical chain-lipid peroxidation reaction [89-90].

7.6.3 Superoxide anion scavenging activity

CKs have been investigated as scavengers of superoxide anion, generated by a non enzymatic system with phenazine methosulfate-NADH but they don't express any antioxidant activity toward this radical. Among the natural CKRs, only the N⁶-isopentenyl

adenosine has some activity against the superoxide radical while the synthetic N⁶ – substituted adenosine show a stronger antioxidant activity than iPA do (Figure 7.18). iPA do is active probably because of the allylic methylene group present in the N⁶-isopentenyl moiety that typically stabilizes the radical formation caused by the superoxide anion, in a way similar to that of the hydroxyl radical. 2FEA, the synthetic N⁶ – substituted adenosine with two hydroxyl groups on the aromatic ring, shows a similar antioxidant activity, due to the presence of these groups. The superoxide scavenging activity of 2HFEA is higher than 2FEA and iPA do and analogous to that of caffeic acid, used as positive control. We calculated the I₅₀ of the two compounds and the values are very

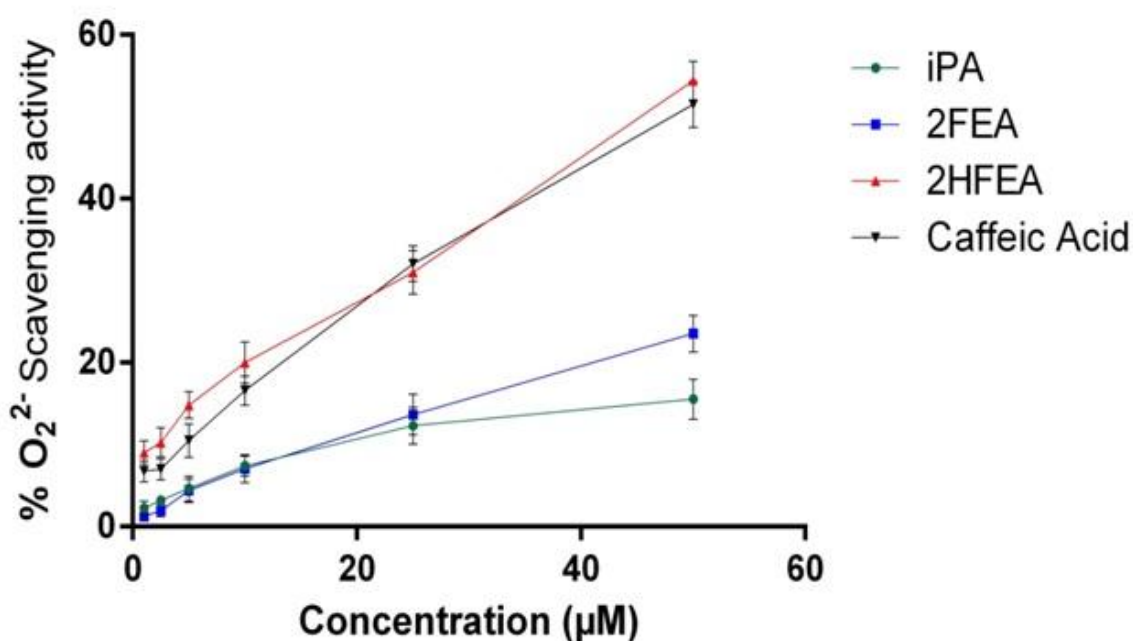


Figure 7.18 Superoxide anion scavenging activity of iPA, 2FEA, 2HFEA and Caffeic acid (positive control).

similar: 45 µM for 2HFEA and 47 µM for caffeic acid. This aspect may be explained with the analogy between the structure of the N⁶ substituted group of 2HFEA and that of caffeic acid, reported in Figure 7.19. The only important difference is the presence of a double carbon-carbon double bond. The strong antioxidant activity is probably due to the catechol system, as in the case of the peroxy radical scavenging activity.

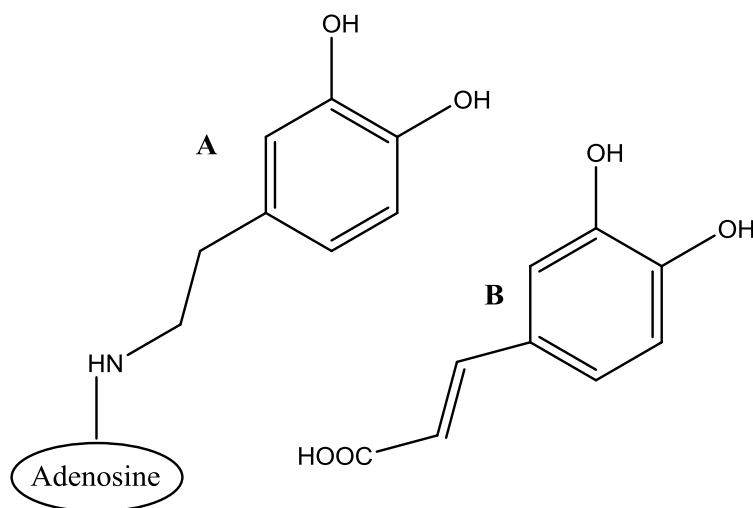


Figure 7.19 Comparison between N6 substituted group of 2HFEA and Caffeic acid.

7.6.4 TEAC (Trolox Equivalence Antioxidant Capacity) assay

The ABTS radical scavenging activity of natural CKs and CKRs has been investigated and the results, reported in Figure 7.20 with Trolox as reference compound, evince that only p-topolin and its corresponding riboside are active against this synthetic radical. This result suggests that the electron transfer from the ABTS radical is efficient only when a phenolic OH is present in the tested compound.

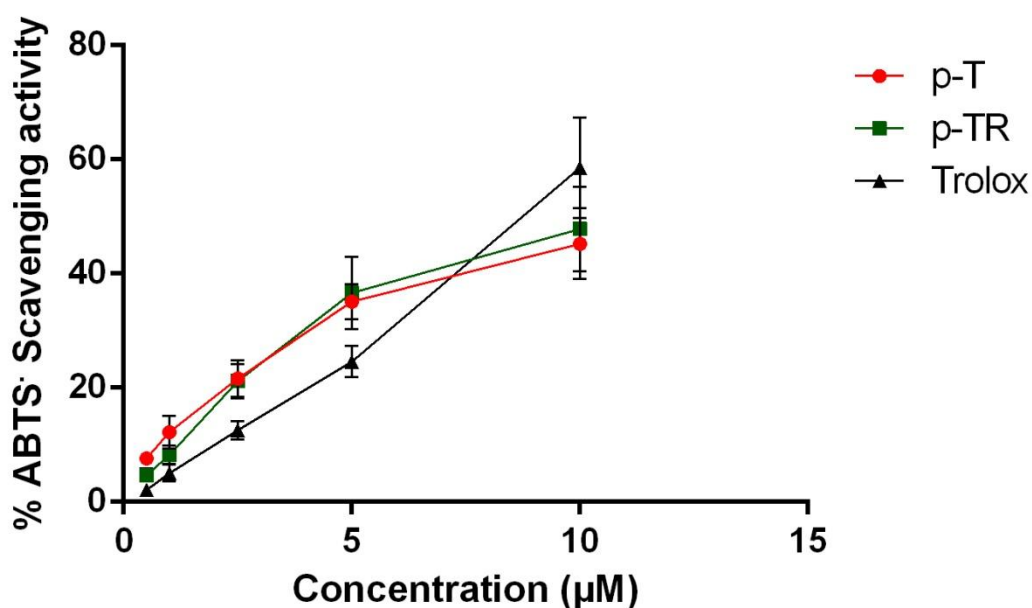


Figure 7.20 ABTS scavenging activity of p-topolin, p-topolin riboside compared with Trolox.

At a concentration range from 0.5 to 5 μM , the antioxidant capacity of p-topolin and p-topolin riboside is greater than Trolox. At higher concentrations their values go to plateau while those of Trolox grow in a concentration-dependent manner. This result may be explained by a higher reactivity of the phenol moiety of p-T with the sterically hindered reactive site of ABTS radical. We evaluated also ABTS scavenging activity of synthetic N⁶ – substituted adenosines and reported the data in Figure 7.21.

All the synthetic adenosine derivatives are active as scavenger of ABTS radical. FEA and 2FEA have an antioxidant activity lower than topoline riboside while 2HFEA is more active only at concentration higher than 5 μM . The contribution to the antioxidant capacity derives only from the N⁶ substituted group being adenosine inactive toward the ABTS radical.

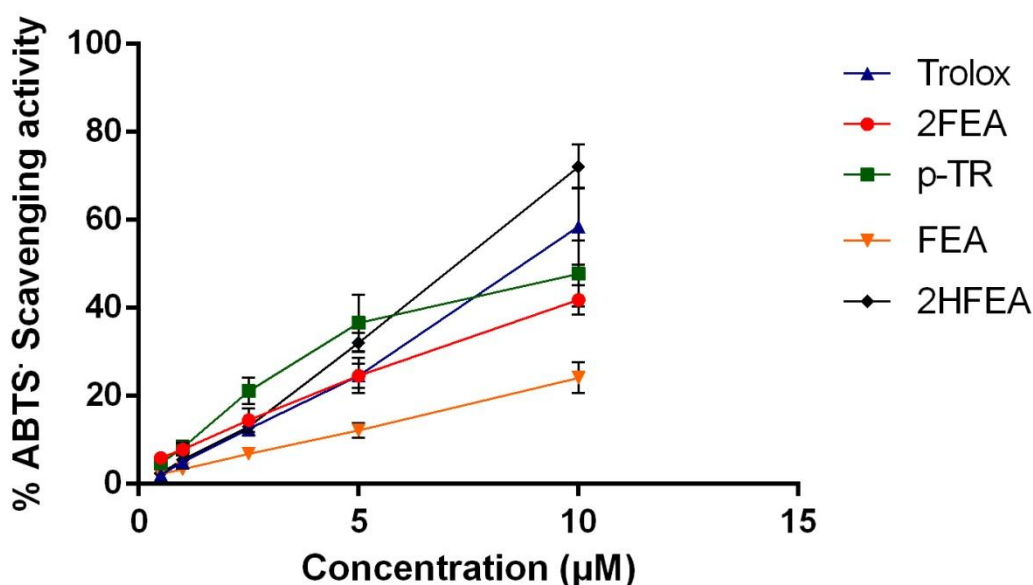


Figure 7.21 ABTS scavenging activity of synthetic N⁶ substituted adenosines compared with that of TR.

7.6.5 DPPH Scavenging Assay

Natural CKs, CKRs and the adenosine derivatives were investigated for their total antioxidant activity using the synthetic 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl radical and the results are reported in Figure 7.22. Natural CKs and CKRs don't have any scavenging antioxidant activity as the N⁶ substituted adenosine FEA. Only the other synthetic adenosine derivatives 2FEA and 2HFEA exhibit some DPPH scavenging activity. The antioxidant 2HFEA is slightly higher than 2FEA and similar to that of Trolox, using as reference compound. The antioxidant activity is probably due to the catechol system because, similar to the TEAC assay, the adenosine isn't a scavenger of the DPPH radical.

The antioxidant activity of 2FEA and 2HFEA may be explained the high reactivity of the catechol group of these compounds with the sterically hindered reactive site of DPPH radical in way similar to that of ABTS radical.

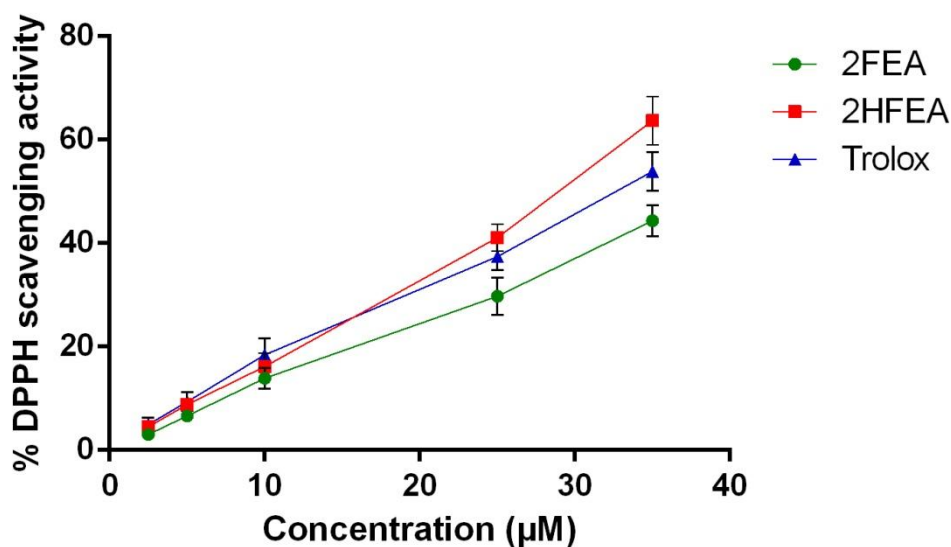


Figure 7.22 DPPH scavenging activity of 2FEA and 2HFEA compared to that of Trolox.

7.7 CONCLUSIONS

The antioxidant properties of natural purine bases CKs, their corresponding ribosides CKRs and some synthetic N⁶-substituted adenosine were investigated for their antioxidant activity, based on different chemical mechanisms. The heterogeneity of the results suggests in some instance a possible structure – activity relationship, although not all the compounds are active in every antioxidant assay at the same concentration range. This has been partially demonstrated for iPA where a part of the biological activity of CKRs can be related to an intrinsic antioxidant capacity of the purine system and in part due to the N⁶-substitution. This difference can be, in part, due to different chemical mechanisms, in turn depending on the chemical structure of the group present at the N⁶-position.

Many biological activities of CKs and CKRs in plants or in mammals can be explained by an intrinsic antioxidant activity of the compounds that could affect various biochemical parameters, in turn involved in the oxidative stress of cells. Further characterization of the antioxidant profile using other *in vitro* tests or, more significantly, specific cellular assays might contribute to explain some of the biological activities evidenced for this important class of compounds.

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8. ANTIPROLIFERATIVE ACTIVITY OF TOPOLIN RIBOSIDE AND ITS SYNTHETIC DERIVATIVES

8.1 ANTIPROLIFERATIVE ACTIVITY OF CYTOKININ RIBOSIDES

Crown gall disease is caused by the soil bacterium *Agrobacterium tumefaciens* and consists in the development of neoplastic growth on the infected plants belonging to the *Magnoliopsida* class [1]. A region of the Ti plasmid (tms locus), probably involved in *Agrobacterium tumefaciens*-transformed plant tissue, may encode IPT, the enzyme that catalyzes the first step in cytokinin biosynthesis [2]. This established a connection between CKs and induction of callus, a cluster of differentiated plant cells that are immortal and proliferate indefinitely, to re-differentiate into adventitious buds. Plant callus cells are similar to human cancer cells and CKs were expected to be able to affect the differentiation in some human cancer cells, probably, through a common signal transduction system [3]. This connection between CKs/CKRs and antiproliferative activity has been confirmed recently by the investigation on the control of differentiation and apoptosis of human myeloid leukemia HL-60 (*Human promyelocytic leukemia cells*) cells by CKs and their CKRs. Using HL-60 cell lines, it has been shown that CKs such as Kinetin, Benzyladenine and N⁶-isopentenyladenine are very effective in inducing nitroblue tetrazolium reduction and morphological changes of the cells into mature granulocytes [4]. Examining the corresponding ribosides CKRs, these compounds were more potent than the corresponding CKs for growth inhibition and apoptosis. CKRs greatly reduced the intracellular ATP content and disturbed the mitochondrial membrane potential, consequentially impairing the accumulation of reactive oxygen species. The same effect was not observed for CKs. When the cells were incubated with CKRs in the presence of ROS scavengers, antioxidant or caspase inhibitor, apoptosis was significantly reduced and differentiation was greatly enhanced. Among the CKRs, it has been shown that Benzyladenosine, Kinetin Riboside and N⁶-isopentenyladenosine are more effective than their corresponding bases [4].

8.1.1 N⁶- Isopentenyladenosine antitumor activity

In the 60's years, Gallo et al. observed N⁶- Isopentenyladenosine could exert a promoting or inhibitory effect on human cell growth, on the bases of used concentration and the cell cycle phase, reporting that iPAdo is a potent inhibitor or a stimulator of the DNA synthesis. At μM concentration, iPAdo produced inhibition while at lower values (nM concentration)

had a stimulatory effect [5]. It was demonstrated that iPAdo is cytotoxic for Sarcoma 180 cells as for the majority of the mammalian cells. It was observed that iPAdo at μM concentration inhibited the growth of Sarcoma 180 cells acting as a potent inhibitor of the uptake of purine and pyrimidine nucleosides. The authors suggested that iPAdo cytotoxicity for these cells might be due to its conversion into 5'-monophosphate that is cytotoxic at high intracellular levels affecting the enzymes involved in purine metabolism [6].

The ability of cytokinins to induce apoptosis was studied in several human cell lines and it was observed that iPAdo was the most active cytokinin, especially with respect to Caco-2 and HL-60 cancer lines [7]. Laezza *et al.* demonstrated that iPAdo in thyroid cell FRTL-5 influences the cAMP dependent organization of the microfilaments. The same authors have later demonstrated that iPAdo caused a dose-dependent arrest of G₀-G₁ cell phase transition associated with a reduction of cells in S phase. iPAdo is able to inhibit farnesyl diphosphate synthase (FPPS) and to affect protein prenylation. This may explain the arrest of tumor cells proliferation in a reversible mode, since the addition of farnesol could reverse the process. This effect was not mediated by the adenosine receptors but was due to a direct modulation of FPPS enzyme activity as a result of its uptake inside the cells [8]. This aspect remains controversial because another author [9], studying the antiproliferative activity of iPAdo in 9 human 17 epithelial cancer cell lines derived from different types of malignant tissue, showed FPPS downregulation in A549 cells was not involved in the antiproliferative activity of iPAdo [9]. Dragani *et al.* observed complete suppression of clonogenic activity in 8 of the cell lines after exposure, at μM concentration, to iPAdo where a clonogenic assay is a microbiology technique for studying the effectiveness of specific agents on the survival and proliferation of cells. Specifically, iPAdo was effective with human lung cancer cell lines NCI-H520 and NCI-H596, with breast cancer cell lines MDAMB-361 and MCF7, and nasal septum squamous cell carcinoma cell line RPMI 2650. Human lung cancer cell lines A549 and Calu-3, hepatocellular carcinoma cell line HepG2, and colorectal adenocarcinoma cell line HT-29 were also examined. Only the cell line HT-29 derived from a colorectal cancer showed a significant but incomplete inhibition upon iPAdo treatment, with about 70% colony inhibition as compared to untreated control cells [9]. Differently from the results obtained with the human myeloid leukemia cell line HL-60 only a modest increase in apoptosis after iPAdo treatment was revealed in epithelial cancer lines. Indeed, in lung cancer cells tumor growth suppression appears to be mediated by inhibition of cell proliferation due to a block of DNA synthesis rather than apoptosis [4, 9].

Laezza *et al.* studied iPAdo effects on DLD1 human colon cancer cells. iPAdo suppressed the proliferation of cells through inhibition of DNA synthesis, causing a cell cycle arrest that correlated with a decrease in the levels of cyclins A, D1 and E with a concomitant increase in the levels of cyclin-dependent kinase inhibitor p21waf and p27kip1. iPAdo induced apoptosis through an increase in the number of annexin V-positive cells, a downregulation of anti apoptotic products and caspase-3 activation. The apoptotic effects of iPAdo were accompanied by sustained phosphorylation and activation of c-jun N-terminal kinase (JNK) that induced phosphorylation of cjun. The authors concluded that JNK could play an important role in iPAdo-mediated apoptosis in DLD1 human colon cancer cells [10].

Recently, the apoptotic activity of iPAdo has been investigated on Bladder Carcinoma T24 cells where induces the alteration of cell morphology and the disorganization of the actin cytoskeleton. The inhibition of the growth is related to the arresting of the cells in G₀/G₁ phase of the cell cycle [11]. Blad *et al.* [12] evaluated the A₃ receptor antagonist activity of N⁶- isopentenyl adenosine (iPAdo). A₃ receptor might be involved in the anticancer response [13]. In a functional assay in Chinese hamster ovary cells transfected with A₃ receptor, IPA and zeatin riboside inhibited forskolin-induced cAMP formation at micromolar concentration. They demonstrated strong and highly similar antiproliferative effects of IPA and on human and rat tumor cell lines LNCaP and N1S1. The antiproliferative effect of low concentrations of IPA on LNCaP cells could be fully blocked by the selective A₃R antagonist MRS1523 while higher concentrations of iPAdo appeared to inhibit cell growth by an A₃R-independent mechanism [12].

8.1.2 Anticancer activity of Aromatic cytokinin ribosides

Only a few, recent reports are available in literature about *in vitro* antitumor activity of Kinetin riboside. It has been reported that KR along with iPAdo and BA were more potent than the corresponding N6-substituted purines for growth inhibition and apoptosis of human myeloid leukemia HL-60 cells [4].

KR shows also cytotoxic effects on M4 Beu human and B16 murine melanoma cells 1.5 and 0.2 μ M concentration. At these concentrations, cell growth is reduced by 50%, respectively, but there was no effect on the growth of mice leukemia P388 [14]. More recent results have shown that KR induces apoptosis in HeLa and mouse melanoma B16F-10 cells [15]. The apoptotic effect of KR in HeLa and mouse melanoma B16F-10 cells was explained through disruption of the mitochondrial membrane potential, induction of the release of cytochrome c, and activation of caspase-3 [15]. Mc Dermott *et al.* tested

KR against leukemia-initiating cells (L-ICs) in acute myeloid leukemia (AML). KR demonstrated comparable efficacy to standard therapies against blast cells in 63 primary leukemias. *In vitro*, KR targeted the L-IC-enriched CD34⁺ CD38⁻ AML fraction, while sparing HSPC (Hematopoietic stem/progenitor cells) enriched fractions, although these effects were mitigated on HSC assayed *in vivo*. KR reduced proliferation and induced apoptosis via caspase-3 cleavage and loss of mitochondrial membrane potential and induced cleavage of Bcl2, which may switch it from antiapoptotic to proapoptotic [16]. KR eliminated L-ICs in 2 of 4 primary AML samples when assayed *in vivo* and highlights the importance of *in vivo* L-IC and HSC assays to measure function.

In contrast, human skin fibroblast CCL-116 and bovine primary fibroblast cells show resistances to KR and no significant changes in Bad, Bcl-XL, and cleaved PARP were observed. Reported data suggest that KR selectively induces apoptosis in cancer cells through the classical mitochondria dependent apoptosis pathway [15]. Dudzic *et al.* investigated the antiproliferative and proapoptotic properties of KR on normal and cancer cell lines finding that KR inhibited growth (20–80%) of not only human cancer, but also normal cells and that this effect strongly depended on the type of cells. The anti-apoptotic Bcl-2 protein was downregulated, while proapoptotic Bax was upregulated in normal as well as in cancer cell lines, upon exposure to KR. Cytochrome c level increased in the cytosol upon treatment of cells with KR. The activity of caspases increased especially in cancer cells. The expression of procaspase 9 and its active form in the nucleus as well as in cytosol of KR-treated cells was elevated. In contrast, no effect of KR on caspase 8 expression was noted. The results indicated that non-malignant cells were less sensitive to KR than their cancer analogs and that KR most likely stimulated apoptosis mechanism of cancer cells through the intrinsic pathway [17].

In another recent study, a hypothesis about the cytotoxic effects of KR was tested. KR effects may involve interference with DNA integrity and cellular energy status leading to stress response gene expression and cell cycle arrest. Results obtained from MiaPaCa-2 pancreas carcinoma, A375 melanoma, and various other human cancer cell lines indicate that massive ATP depletion and induction of genotoxic stress occurs rapidly in response to KR exposure. This is followed by early upregulation of HMOX1, CDKN1A, and other DNA damage/stress response genes. These data suggest that early induction of genotoxicity and energy crisis are causative factors involved in KR cytotoxicity and anticancer activity [18]. Recently, Rajabi *et al* investigated the antiproliferative activity of KR on HCT-15 human cells. KR is able to inhibit the proliferation in HCT-15 human colon cancer cells in a

dose-dependent manner with a concentration of 2.5 μM , which causes 50% inhibition of cell viability. The cell cycle analysis by flow cytometry showed that KR arrested cell cycle progression in the S Phase by blocking through G2/M and G0/G1 phase in HCT-15 colon cells but the mechanisms conferring KR-induced cell death in cancer cells remain elusive [19].

Very scarce are the works about the anticancer activity of the other natural aromatic CKRs, benzyladenosine and its hydroxylated derivatives, topolin riboside.

Several authors have reported cytotoxic effects of N⁶-benzyladenosine on human cell lines derived from solid tumors [10, 15, 18]. Whether treatments resulted in cell cycle block and/or apoptosis was dependent on the cell line and the cytokinin used. Doležal et al evaluated the anticancer activity of several synthetic derivatives of N⁶-benzyladenosine against cancer lines such as HOS, K-562, MCF7, CEM, HL-60 G-361, B16 and NH 3T3 with various results. Several of them were much stronger than BA suggesting a strong relation with the chemical groups on the aromatic ring [20]. The anticancer activity of iPado, KR and BA has been demonstrated *in vivo* using several animal and xenograft models of cancer [15, 21-22]. iPado and BA have also shown promising activity against a diverse range of cancers in a limited clinical trial [23].

Furthermore, the activity pattern of ortho-topolin riboside (o-TR) against NCI60, a thoroughly characterized panel of 59 human cancer cell lines was analyzed. Finally, we report results of *in vivo* tests of the anticancer activity of o-TR against models representative of human tumors in hollow fibre assays [24].

The last part of this Ph.D project is the study of the effect of topolin riboside (TR) and its synthetic derivatives FEA, 2FEA and 2HFEA on 661W cell line and the anticancer activity on Neuro2A cell line.

8.2 EXPERIMENTAL SECTION

8.2.1 MATERIALS

p-Topolin riboside (p-TR) was synthesized by us and purchased from OIChemIm Ltd. (Olomouc, Czech Republic). N⁶- substituted adenosine derivatives FEA, 2FEA and 2HFEA were synthesized following the procedure described in Chapter 4.

Trypan blue dye was purchased from Sigma-Aldrich (S. Louis, Missouri USA). Penicillin and streptomycin (Invitrogen) were obtained from Life Technologies Italia (Monza, Italy). DMEM (Dulbecco's modified Eagle's medium) culture media and fetal bovine serum were purchased from EuroClone Life Science Division (Milano, Italy). TUNEL was performed using a specific Kit, *In situ* Cell Death Detection Kit (TMR Roche Diagnostic, Mannheim, Germany).

8.2.2 CELL CULTURES

Cone-derived cell line (661W cells) was kindly provided by Dr. Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma, Oklahoma City, USA). 661W were cloned from retinal tumor of transgenic mouse line expressing the SV-40 T antigen under control of the inter-photoreceptor retinal binding protein promoter (IRBP) [25].

661W growth doubling time is of ~24 hours in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution as preserving system. They were maintained in a sterile and controlled atmosphere incubator at 5% CO₂, 95% humidity at 37 °C. Experiments were performed in 60mm Petri dishes (3x10⁵ cells in 3 ml) starting treatments after about 24 hours.

Neuroblastoma fast-growing mouse cell line (Neuro2A cells) was obtained from LGC Standards (Teddington, U. K.). Neuro2A cells were cloned from a spontaneous tumor in an albino strain A mouse [26]. Neuro2A cells and experiments were grown in the same way of 661W cells.

8.2.3 CELL TREATMENT

p-Topolin riboside and the synthetic adenosines FEA, 2FEA and 2HFEA were dissolved in DMSO.

661W and Neuro2A were seeded and left to grow for about 24 hours in 10% FBS/1% penicillin/streptomycin DMEM. Before the treatment, the tested compounds were added to the culture media. The final concentration range of each compound was from 0 (control) to 10 μM. During the treatment, the DMEM culture media was replaced with that containing

the compounds and the dishes were incubated in a sterile and controlled atmosphere incubator at 5% CO₂, 95% humidity at 37 °C for 48 hours. At proper time point, they were collected and processed according to different assays. Each compound was tested in double.

8.2.4 METHODS

8.2.4.1 Trypan blue dye exclusion test

Trypan blue assay was used to evaluate the number of viable cells after treatments. 661W and Neuro2A cells were seeded in tissue culture Petri dishes (600mm) at 3×10^5 cells/Petri, treated and after 48 hours, counting was evaluated in experimental and control groups. For that, cells were detached using 1% trypsin for 661W cells and 2% for Neuro2A cells, for 1.5 minutes at 37°C, collected and resuspended in 1.5 ml of Phosphate buffered saline (PBS) solution, 90 µl aliquots of this cell suspension were added to 10 µl of 0,4% Trypan blue dye (1:10). Next, live and dead cells were counted using Bürker chamber under a MoticAE31 optical inverted microscope. Live and dead cells were reported as both number of cells and percentages over the control.

8.2.4.2 Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Apoptosis degree was evaluated by means of TUNEL test, trypsinized cells were washed in 1 ml cold PBS and centrifuged 5 min at 4000 rpm at room temperature. The pellet was resuspended in 500 µl of 4% cold buffered formalin for 20 min, washed in PBS two times for 5 min, and stratified on microscope glasses. After drying at room temperature, slides were maintained at -20 °C until use. Apoptosis was determined by the TdT assay in an inverted fluorescence microscope (40x magnification) Axiovert25 CFL (Zeiss, Göttingen, Germany) equipped for the detection of rhodamine (filter set 15, excitation band pass 546 nm, emission low-pass 590 nm). Nuclei were stained with the karyophilic dye Hoechst 33258 (250 ng/µl) for 3 minutes at room temperature in the dark, followed by rinsing twice in PBS and coverslipping. Slides were examined using a filter for Hoechst staining (filter set 02, excitation band pass 365 nm, emission low-pass 420 nm). Images were acquired by a digital camera (DS-2MV; Nikon, Tokyo, Japan) and the number of TdT-labeled nuclei counted (8-10 random fields in a blinded procedure). Results are expressed as number of TdT-labeled nuclei/total nuclei.

8.3 RESULTS AND DISCUSSION

The antiproliferative activity of p-TR and the synthetic CKRs FEA, 2FEA has been investigated on 661W and Neuro2A cell lines performing Trypan blue assay to evaluate the number of viable cells after treatment with each compound and the results are reported in Figure 8.1.

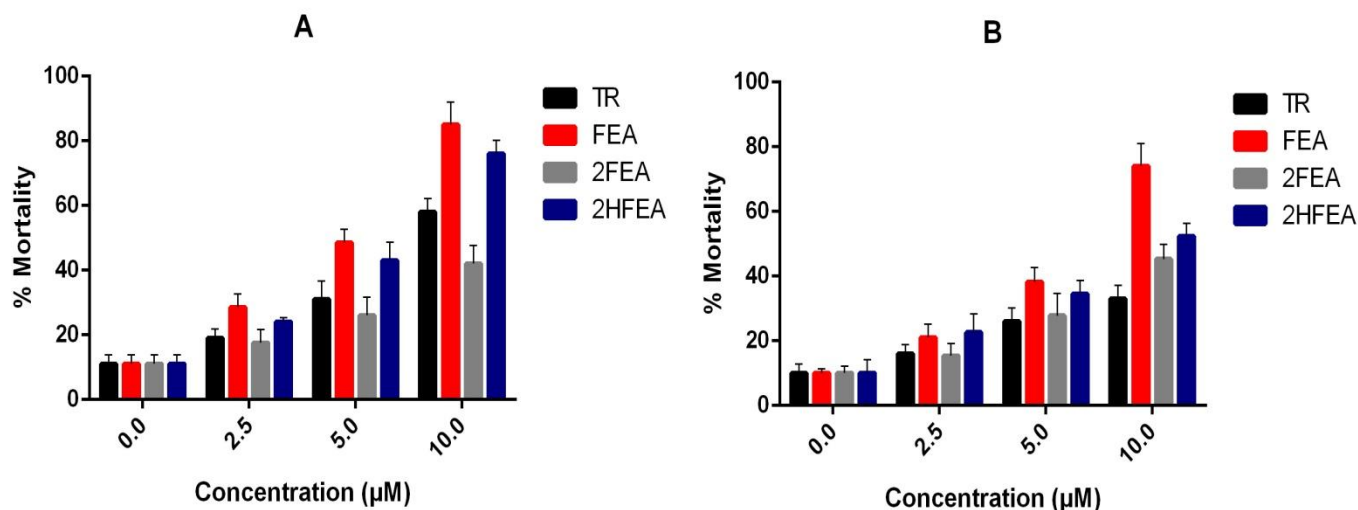


Figure 8.1 Trypan blue assay results on 661W (A) and Neuro2A (B) of p-TR and synthetic CKRs.

Trypan test results on 661W cell line show that p-TR and the synthetic adenosine derivatives FEA, 2FEA, 2HFEA have cytotoxic properties. FEA is the most cytotoxic compound followed by 2HFEA, while p-TR and 2FEA have similar cytotoxic activity. A similar trend is observed in the graph of the trypan blue assay on the Neuro2A cell line. FEA is the most effective compound respect to the other compounds that are similar among them.

The Table 8.1 reports the I_{50} values calculated for each compound. In both cell lines, FEA

Table 8.1 I_{50} values of Trypan blue assay of TR and non-natural CKRs.

Compound	661W	Neuro2A
p-TR	8.62 ± 0.96	>10
FEA	5.30 ± 0.66	6.58 ± 0.83
2FEA	>10	>10
2HFEA	6.13 ± 0.59	9.14 ± 0.99

is the most effective compound followed by 2HFEA.

The same compounds have been tested for their apoptotic induction capacity and the results are showed in Figure 8.2.

Tunel assay results and related microscope pictures on 661W cell line suggest that the examined compounds have a similar apoptotic capacity. p-TR was the best compound with a peak of 27 % at the concentration of 10 μ M. A greater difference between p-TR and

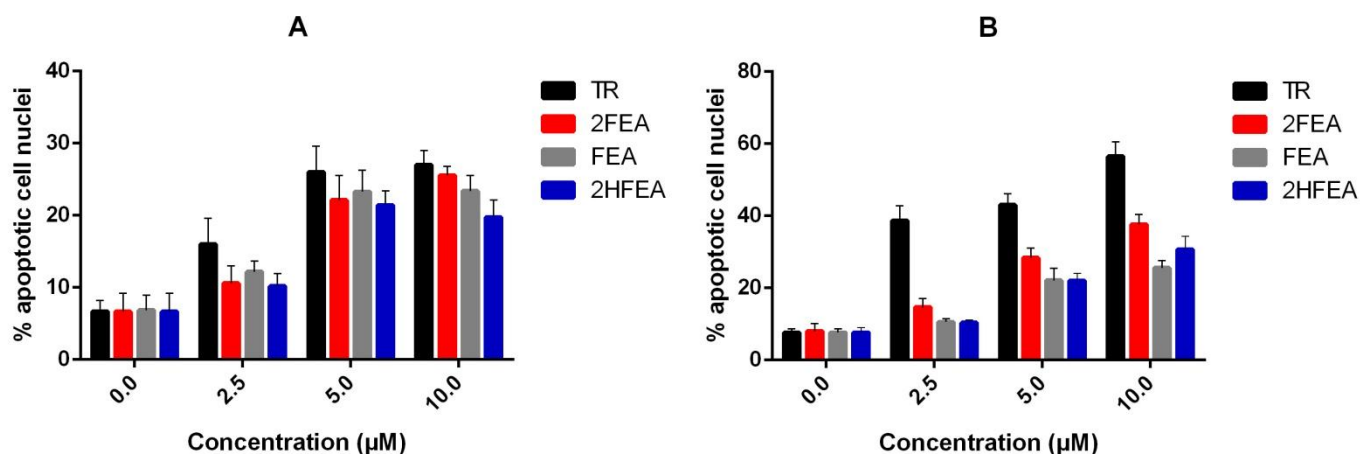


Figure 8.2 Tunel assay results on 661W (A) and Neuro2A (B) of TR and synthetic CKRs.

the synthetic adenosine derivatives was found with the test on Neuro2A cell line. The apoptotic grade of TR was 56.5% at the concentration of 10 μ M while the lowest value was that of FEA (25.5 %).

The cytotoxic and apoptotic properties of natural CKRs and synthetic adenosine derivatives might explain partially their antagonist activity toward the A_3 receptor, involved in the anticancer response. It seems to be an inverse proportion between the cytotoxic activity and the apoptotic capacity in the case of Neuro2A cell line. Another difference consists in the presence of two methylene groups of the synthetic CKRs respect to the one of p-TR. Further experiments will be necessary to understand the mechanism of action of this class of compounds.

8.4 CONCLUSION

This study was focused on structure-activity investigation with the aim of understanding the effect of a modification in the aromatic ring of the N6 group can improve the cytotoxic and apoptotic properties and for a correlation with the A_3 receptor antagonist activity. We found there is an inverse correlation between cytotoxic and apoptotic activity, especially with Neuro2A cells. p-TR showed the best apoptotic activity but weak cytotoxic activity

whereas FEA was the most cytotoxic compound but exhibited the lowest apoptotic values, following by 2HFEA. Both the compounds are antagonists of the A₃ adenosine receptor much stronger than p-TR, as reported in Chapter 5. The study of specific marker involved in the apoptotic process can contribute to explain the anticancer properties of CKRs.

8.5 REFERENCES TO CHAPTER 8

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9. CONCLUSIONS

In this Ph.D project, several biological activities of few natural occurring cytokinin ribosides (CKRs), N⁶-isopentenyladenosine (iPAdo), Kinetin riboside (KR), N⁶-benzyladenosine (BA) and ρ -Topolin riboside (TR) were investigated.

Starting from a recent observation that kinetin (K) was able to inhibit the platelet aggregation in washed human platelet (PRP), we evaluated the platelet anti aggregation activity of kinetin riboside (KR) using the simplified model of PRP. We confirmed initially the reported data on K activity (12,8 – 56,4% inhibition at 25 to 500 μ M) and found KR showed a higher activity at the same concentration range (51,3 -76,3%). As an extension of this preliminary result, the platelet anti-aggregation activity of other CKRs on PRP was evaluated. All examined CKRs revealed an anti -aggregation activity with ρ - Topolin riboside showing the highest values (54 – 83% inhibition at 25 to 250 μ M range) followed by N⁶-benzyladenosine. Through molecular modelling studies, realized in collaboration with Prof. Giulio Vistoli (associated professor of pharmaceutical chemistry, Department of Pharmaceutical Sciences, Università degli Studi of Milan), we demonstrated a good affinity for the receptor P2Y₁₂, for which a key role in platelet activation and thrombogenesis has been recently recognized. Results obtained from docking simulations showed that CKRs assumed a binding mode rather similar to that of the co-crystallized inhibitor AZJ (ethyl 6- $\{4-$ [(benzylsulfonyl) carbamoyl]piperidin-1-yl $\}-5$ -cyano-2-methylpiperidin- 3-carboxylate, AZJ) and are engaged in clear contacts within the P2Y₁₂ binding cavity. The best *in silico* interaction was observed with, ρ -TR and BA, the same CKRs that showed the highest platelet anti aggregation activity. A preliminary study on whole blood was performed using the same procedure for PRP. All the CKRs were tested at the concentration of 50 μ M and only those with an aromatic N6 substituted group showed an anti platelet aggregation activity with ρ -TR showing the best value, 26 %.

During my stay in Portugal, at the Departamento de Química e Bioquímica, Universidade do Porto (Erasmus Placement Fellowship, Tutor: Prof. Fernanda Borges), I synthesized two adenosine derivatives from 6 – chloropurineriboside as starting material using tyramine and dopamine as N⁶ substituted group. The chemical reaction was a nucleophilic substitution and the obtained products were phenylethyladenosine (FEA) and 3,4-dihydroxyphenylethyladenosine (2HFEA). Both the compounds have one methylene group more than ρ -topolin riboside and 2HFEA has an additional phenolic OH group. This synthesis was performed in structure-activity investigation with the aim of understanding

the effect of a modification in the aromatic ring of the N6 group could improve the biological properties with respect to the natural CKRs. We tested the affinity of these compounds with the human adenosine receptors A_1 , A_{2A} , A_{2B} , A_3 , a class of purinergic receptors, G protein-coupled with adenosine as endogenous ligand. The experimental part was performed during my stay in Portugal with the collaboration of Prof. Fernanda Borges and Prof. Karl N. Klotz of the University of Wurzburg, Germany. The compounds were tested on Chinese hamster ovary cell membranes in competition binding conditions using [3 H]-DPCPX (8-cyclopentyl-1,3-dipropylxanthine) as marked antagonist compound and the CKRs to be tested at different concentrations. Then K_i (inhibition constant) values were calculated from competition curves by nonlinear curve. CKRs were good antagonists of A_1 receptor with a K_i range from 21.7 to 52.4 nM. The best value was that of ρ -topolin riboside. The affinity of CKRs for A_{2A} was poor with the exception of ρ -topolin riboside that showed a K_i value of 583 nm. This result might partially explain the good anti platelet aggregation activity of TR. The same trend was observed for A_{2B} receptor. Similarly to what was observed with A_1 , CKRs were good antagonist with A_3 receptor with a K_i range from 114 to 146 nM. The best value was that of benzyl adenosine. This affinity for this receptor may explain in part the anticancer activity of cytokinin ribosides. We tested these new compounds with the adenosine receptors in the same conditions of CKRs. Results showed the synthetic compounds were antagonist of A_3 receptor much stronger than natural CKRs with a K_i range from 4.51 to 6.96 nM. 2HFEA was the best antagonist of A_{2A} with a K_i value of 458 nM while FEA was the best antagonist of A_1 with a K_i value of 10.8 nM.

During my stay in Portugal, I evaluated also the capacity of natural and non-natural CKRs to inhibit the Acetylcholinesterase (AChE) and the Monoamine oxidase B (MAO-B). We found natural and synthetic N⁶-substituted adenosine derivatives didn't show any AChE inhibition activity whereas only 2HFEA was a MAO-B inhibitor, showing a I_{50} value of 5.35 μ M, similar to that of pargyline, used as positive control. In Italy, we investigated the glycation/glycoxidation inhibition activity of kinetin and kinetin riboside on the bovine serum albumin (BSA). In contrast with reported results, in our hands K did not show the ability to decrease the glycosilation of BSA and similar negative results were obtained with the riboside KR. These controversial need further investigations. In general, further investigations on biological activity of CKRs and their synthetic analogues seem desirable. These researches should include uptake of the compounds in the cell, their mechanisms

of action and metabolic pathways. Taken altogether, the results would help to shed more light in the biological role of this class of compounds, structurally related to adenosine.

Many biological activities shown by the CKRs examined by us could be related to an effect of these compounds on the cellular oxidative stress, as recently demonstrated for iPA. Since a part of the biological activity of CKRs can be related to an intrinsic antioxidant capacity, we have determined their scavenging activity against different radicals by spectrophotometric assays. Initially, we evaluated the activity against the hydroxyl radical OH[•], using lipoic acid as positive control. At the concentration range of 10 – 500 μM, the hydroxyl radical scavenger activity of CKRs reached a maximum of 26-29%. In Portugal, I started to study the total antioxidant power of CKs and CKRs using assays that rely on the formation of the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging assay. Both tests used Trolox as standard reference compound. In the first method, the total antioxidant activity was determined by the decolourization of the ABTS[•], through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 730 nm. Only p-T and its riboside (p-TR) were able to reduce ABTS radical (p-T 7 - 35%, p-TR 5– 48%, at 0.5 – 5 μM). At this concentration range, their activity was higher than that of Trolox itself. Also the synthetic adenosine derivatives were scavengers of ABTS. 2HFEA showed the best activity slightly higher than Trolox. The DPPH assay evaluated the inhibition of the synthetic radical DPPH at 517 nm.

Whereas natural CKs and CKRs were not scavengers of DPPH, 2HFEA exhibited an activity similar to that of Trolox (2HFEA, 13 - 77%, Trolox, 13-79% at 5 – 50 μM). A similar trend was observed for 2FEA.

To complete the antioxidant profile of CKs and CKRs, ORAC (Oxygen Radical Antioxidant Capacity) and superoxide anion assays were performed. Among CKs, only B was not active whereas for the remaining CKs the activity depends on the concentration range of the assay. Specifically, in the range up to 1.0 μM, K exhibited the highest antioxidant capacity, while p-T appears as the most efficient at 2.5 μM and 5.0 μM. Among CKRs, the antioxidant activity of BA and iPA_{do} was similar to that of their corresponding CKs, KR was more active than its base, with a peroxy scavenging capacity similar to p-TR. All the synthetic N⁶-substituted adenosine derivatives showed an antioxidant activity more intense than p-TR, especially 2HFEA probably for the presence of its catechol group. In the superoxide anion scavenging assay, natural CKs did not show activity, whereas among their corresponding CKRs, only iPA_{do} was a moderate scavenger of superoxide anion.

With the exception of FEA, 2FEA and 2HFEA were active against superoxide anion. The antioxidant activity of 2FEA was similar to that of iPAdo whereas 2HFEA was a good superoxide anion scavenger, similar to caffeic acid, used as reference compound. The heterogeneity of the results suggests in some instance a possible structure – activity relationship but not all the compounds are active in every antioxidant assay. Further characterization of the antioxidant profile using *in vitro* models might contribute to explain some of the biological activities evidenced of CKs and CKRs.

During the third year of my PhD thesis, I have been involved in studies on the anti-proliferative activity of CKRs on two types of cell lines. The selected cell lines were the Murine 661W cone-like cell line and Neuroblastoma fast-growing mouse cell line (Neuro2A). Trypan blue assay was used to evaluate the number of viable cells after treatment with each compound while the terminal deoxy-nucleotidyl transferase dUTP nick end labelling (TUNEL) assay was performed to evaluate the apoptosis degree expressing the results as number of TdT-labeled nuclei/total nuclei. All the compounds were tested in a concentration range from 0 (control) to 10 μ M in both tests. Trypan test results on 661W cell line showed that p-TR and the synthetic adenosine derivatives FEA, 2-FEA, 2HFEA have cytotoxic properties. FEA was the most cytotoxic compound with a I_{50} value of 5.3 μ M. A similar trend was observed for the Trypan test on Neuro2A cell line where the best value was always that of FEA (I_{50} = 6.5 μ M). The same compounds were investigated for their apoptotic induction capacity. TUNEL results and related microscope pictures on 661W cell line suggest that the examined compounds have a similar apoptotic capacity. p-TR was the best compound with a peak of 27 % at the concentration of 10 μ M. A greater difference between p-TR and the synthetic adenosine derivatives was found with the test on Neuro2A cell line. The apoptotic grade of TR was 56.5% at the concentration of 10 μ M while the lowest value was that of FEA (25.5 %). The cytotoxic and apoptotic properties of natural CKRs and synthetic adenosine derivatives might explain partially their antagonist activity toward the A_3 receptor, involved in the anticancer response.

Acknowledgments

I'd like to thank the many people that made possible this Ph.D project.

It is difficult to overstate my gratitude to Prof. Enzo Santaniello for his guide and all his good ideas.

I'd like to thank Prof. Francesco Bonomi for helping me after the retirement of Prof. Santaniello.

I would like to thank to the people mentioned below for their personal contribute to this work:

- *Prof. Riccardo Ghidoni for his supervision after the retirement of Prof. Santaniello.*
- *Prof. Marco Cattaneo and his hematology research group for the experimental part of Chapter 3.*
- *Professor Giulio Vistoli for the modelling section of Chapter 3.*
- *Dr. Paola Bianciardi, Dr. Marta R. Reforgiato, Dr. Marco Trinchera and Dr. Anna Caretti for teaching me the cell colture.*
- *Prof. Marina Carini and Mrs. Cristina Marinello to give me the possibility to perform some of the antioxidant tests described in Chapter 6 in the laboratory of DISFARM.*
- *Prof. Karl N. Klotz for the experimental part about the adenosine receptors described in Chapter 5.*
- *Dr. Alessio Scarafoni for the experiments of inhibition of glycosilation of BSA.*

I'm grateful to my many colleagues for providing a stimulating and good environment during these three years:

- *Department of Healt Sciences c/o San Paolo Hospital: Prof. Rita C. Paroni, Prof. Michele Samaja, Dr. Paola Signorelli, Dr. Daniele Bottai, Dr. Aida Zulueta Morales, Dr. Carlotta Fabiani, Dr. Eleonora Virgili, Dr. Laura Terraneo, Dr. Jessica Rizzo, Dr. Giuseppe Campisi, Dr. Alessandra Canazza, Dr. Elena Finati, Dr. Raffaella Adami, Dr. Simona Prosperini, Dr. Eti A. Femia, Dr. Vera Caroppo, Dr. Mariangela Scavone, Dr. Ksenia Germanovich, Dr. Federico M. Rubino, Nadia Toppi.*
- *Department of Pharmaceutical Sciences: Dr. Alessandra A. Altomare, Dr. Giovanna Baron, Dr. Angela Crisciulo, Dr. Gilda Aiello, Dr. Luca Regazzoni, Dr. Mara Colzani, Dr. Fabrizio Gelmini, Dr. Simone Colombo.*

I wish to adress special thanks to:

- *Prof. Andrea Mosca for putting me aware of the Erasmus Placement Program.*
- *Prof. Luciano Saso to giving me the occasion to go to the Portugal and discovering this beautifull country.*
- *Prof. Fernanda Borges for the kind hospitality in her laboratories and the supervision during my stay in Portugal.*
- *Dr. Alexandra N. Gaspar and Dr. Fernando Cagide for theaching me a lot about organic synthesis and for the precious help to the synthesis of some adenosine derivatives. Alexandra helped me also in some antioxidant tests.*
- *Dr. Joana Reis for the kind support in the enzymatic tests.*
- *Dr. Mariana Andrade for the NMR spectra of some of my compounds.*
- *Dr. Enrico Caneva and Mr. Marco Pappini for the NMR and mass spectra of some adenosine derivatives.*

I want to do a special dedication to my colleague and friend Dr. Jelena S. Lazarevic for her kind company and precious suggestions during the first year of my doctorate.

Lastly, and most importantly, I wish to thank my family: my parents Marco and Rosella, my grandmother Lidia, my sister Laura and my brother in law Marco. To them I dedicate this thesis.