# Characterization of Hammerhead Ribozymes Potentially Suitable for the Treatment of Hyper-Proliferative Vascular Diseases

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**Abstract** Nucleic acid based drugs have emerged as attractive and novel alternatives to commonly used drugs for the treatment of different human diseases. Hammerhead ribozymes were the first type of nucleic acid based drugs to be extensively studied and predicted to be of potential practical utility. These are catalytic RNA molecules capable of inducing the site-specific cleavage of a phosphodiester bond within an RNA molecule. Thus, they can be used to reduce the intracellular level of a specific mRNA coding for a protein which affects cellular metabolism or environment, causing disease. Here we present a description of hammerhead ribozyme cleavage kinetic properties, some of the problematic related to their delivery from viral vectors and the description of their potential application in the cardiovascular field with a particular accent on the pathological condition known as artery-restenosis.

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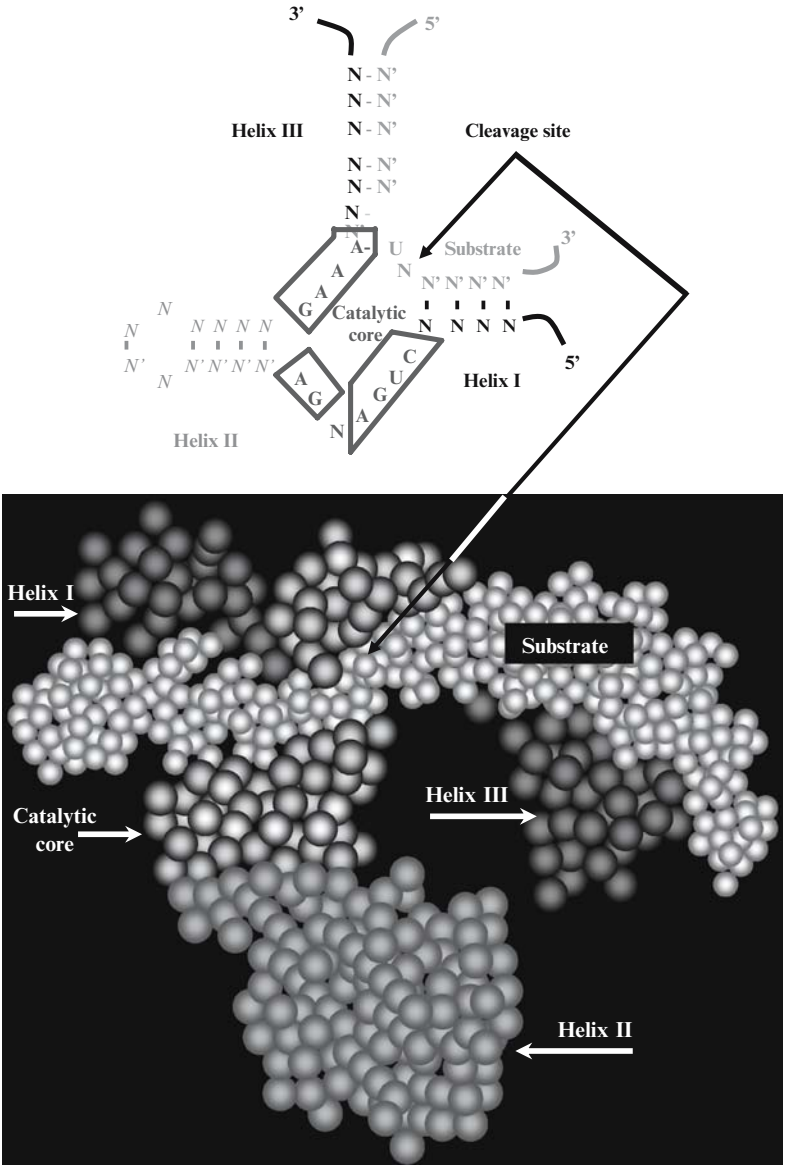
## 1 Introduction

In the last 20 years, considerable progress has been made in the identification of RNA molecules (Scherr et al. 2003) with potential therapeutic values. Among these, ribozymes were the first to be discovered (Kruger et al. 1982). The name ribozymes derives from the fact that these molecules are made of ribonucleic acid stretches (ribo-) and behave like true enzymes (-zymes) which are able to specifically catalyze the cleavage of an RNA phosphodiester back-bone. This feature implies that ribozymes can be used to cut and destroy deleterious RNAs (Puerta-Fernandez et al. 2003). Their potential therapeutic value has been demonstrated in a number of pathological conditions including virally-induced diseases, cancer, cardiovascular, neurological and genetic diseases (Grassi et al. 2004; Grassi and Marini 1996; Puerta-Fernandez et al. 2003), against which conventional therapies have little success. Among the huge scientific production in this field, we will focus here on the most commonly used form of ribozyme, namely hammerhead ribozyme (HRz), describing its therapeutic potential in the treatment of a particular human pathological condition, i.e., artery restenosis, the prototype of hyper-proliferative vascular diseases. A complete description of the potential use of HRz in the treatment other human hyper-proliferative diseases, such as glomerulonephritis, rheumatoid arthritis, ocular pathologies and tumors, has been reported elsewhere (Grassi et al. 2004). To provide a comprehensive picture relative to the potential use of HRz as therapeutics, an overview about their selection, kinetic characterization and delivery precedes the description of their potential use in the treatment of artery restenosis.

## 2 Hammerhead Ribozyme

HRzs represent a class of ribozyme (Grassi and Marini 1996) which were originally isolated from viroid RNA (Uhlenbeck 1987). They are capable of inducing the site-specific cleavage of a phosphodiester bond within an RNA molecule (Birikh et al. 1997b). Despite the fact that naturally occurring HRzs are *cis*-acting molecules, meaning that the ribozyme and the substrate are on the same molecule, it is possible to design *trans*-acting HRzs able to cut a separate RNA molecule (Symons 1992). The *trans*-acting HRz has three essential features (Fig. 1): (1) three double helices numbered I, II, and III with helices I and III also called binding arms; (2) a triplet cleavage site within the target RNA which is composed of the tri-nucleotide triplet NUH where N represents any nucleotide and H stands for A, C, or U (but HRzs which can cleave triplets ending with G have been also described; Vaish et al. 1998); and (3) two highly conserved sequences representing the catalytically active core (Haseloff and Gerlach 1988). HRzs specifically bind to their RNA targets by the binding arms which recognize complementary nucleotide regions on the target. As the binding arms are not conserved (Symons 1992), it is in principle possible to generate HRzs with flanking regions complementary to a substrate of any sequence.




**Adapted from P HeinzW.; Nature (1994), 372: 68-74**

**Fig. 1** Structure of hammerhead ribozymes. Two (*top*) and three (*bottom*) dimensional structure of a hammerhead ribozyme in the complex with its target. Hammerhead ribozyme structure consists of three double helices marked *I, II*, and *III*, and a triplet cleavage site within the target RNA composed of the tri-nucleotide NUH where N represents any nucleotide and H represents A, C, or U. Two stretches of highly conserved sequences (*boxed* in the two-dimensional structure) represent the catalytically active core

Following binding, a $Mg^{+2}$-dependent *trans*-esterification reaction takes place breaking a covalent bond 3′ of the triplet cleavage site (Dahm and Uhlenbeck 1991). The HRz kinetic pathway terminates with the release of the products (cut target RNA) from the HRzs, free to bind another target molecule (Stage-Zimmermann and Uhlenbeck 1998) as detailed in Sect. 2.3 (see also Fig. 2).

## 2.1 Selection and Characterization of Active HRzs

The design of HRzs for the selective destruction of a target RNA is not an easy task as not all HRzs can efficiently reach and cleave the target RNA. Due to the RNA folding (secondary and tertiary structure), not all of the triplets are available for HRz cleavage. In this regard, it is assumed that triplets surrounded by an RNA stretch forming an open region (loop), can be efficiently reached and cleaved by the HRz. In contrast, triplets present in RNA regions involved in the binding with other RNA stretches of the same molecules are considered to be far less accessible for HRz cleavage. Computer algorithms (Zuker and Jacobson 1998; Mercatanti et al. 2002) were firstly developed to predict the folding of the target RNA. The limits of this approach suggested that experimental strategies should have been also developed. We and others (Grassi et al. 2001; Birikh et al. 1997a; Cairns et al. 1999) have used a completely randomized DNA oligonucleotide library together with RNaseH to map HRz accessible cleavage sites. Others have followed even more articulated approaches (Warashina et al. 2001).

Following the identification of the accessible cleavage sites, an initial in vitro determination of HRz kinetic constants $k_{cat}$, representing the chemical step to form products, and $K_m$, which reflects the affinity of the HRz for the substrate, are required (see Sect. 2.3 for details). For in vivo applications, it is usually suggested to select HRzs with low $K_m$ values as they can function efficiently at comparatively

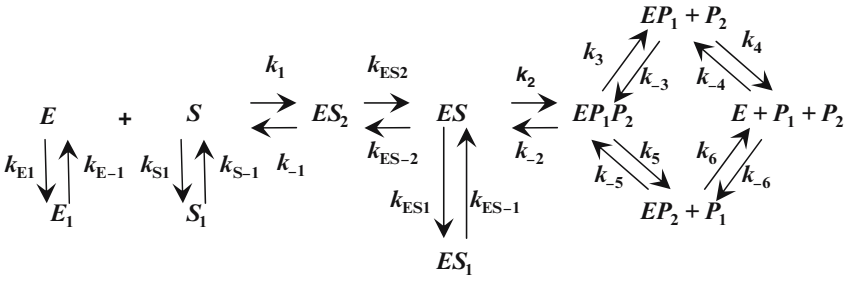



**Fig. 2** Ribozyme cleavage kinetic pathway. In the hammerhead ribozyme cleavage kinetic pathway different ribozymes (*E, E1*) substrates (*S, S1*) and activated complex (*ES, ES1, ES2*) conformations can occur; moreover, different mechanisms can rule the conversion of the complex EP1P2 (hammerhead ribozyme and the cleavage products) to E and P1–P2. All these variables eventually determine hammerhead ribozyme cleavage kinetics

low concentrations (Birikh et al. 1997b). To correctly calculate kinetic constants the appropriate experimental conditions and mathematical models are required. Whereas experimental conditions were defined (Birikh et al. 1997b), less effort has been put into the development of an optimal mathematical model. Although currently used mathematical systems (Birikh et al. 1997b) show an acceptable accuracy in kinetic constant calculation, in the presence of extra ribozyme sequences (Platz et al. 2007) (promoter-derived sequences appended for delivery requirements; see Sect. 2.2 for details) and/or when HRz are targeted against long and structured targets, they may not be reliable. For these specific cases, the calculation procedure, detailed below, and involving equations (10) and (12), (12′), is strongly recommended (Grassi et al. 2002).

## *2.2 Delivery Systems: Exogenous and Endogenous Approaches*

If administered as naked ribonucleic molecules, only a minor fraction of HRzs would reach the target resulting in negligible biological effects. The barrier which HRzs have to pass is firstly represented by the extra-cellular matrix compartment and its fluids where there are substances able to rapidly induce HRz degradation. Subsequently, HRzs have to cross the cellular membrane which, due to the negatively charged phosphate groups present in the HRz structure, cannot be crossed by passive diffusion but by endocytosis. Finally, once in the cellular environment, HRzs are susceptible to further degradation by cellular nucleases. To try to overcome these obstacles, two major strategies have been followed so far: the delivery of pre-synthesized HRz conjugated with a carrier (exogenous method), and the delivery of HRzs embedded in viral vectors (endogenous method).

The exogenous approach implies the use of chemically synthesized HRzs to be applied to the target cell/tissue complexed with different molecules generally termed “tranfection agents.” Among these, liposomes have been the most extensively used. These are minute hollow spheres composed of a lipid membrane surrounding an aqueous sphere (Balicki and Beutler 2002) which confer a certain protection against HRz degradation and allow a relatively efficiently crossing of the cellular membrane. Other substances used to trasnfer HRz to the target cells include polyethylenimine, chitosan and even polymeric nano-particles (Grassi et al. 2004). We are in the process of studying the delivery of liposome-complexed HRz from a polymeric matrix for endovascular application (Grassi et al. 2006a).

A limitation of the exogenous delivery systems is the relatively reduced release time. Viral systems (endogenous systems) can in part overcome this problem providing an extended expression of the HRz. Among the several viral vectors tested so far (Balicki and Beutler 2002), baculoviral vectors can be considered a novel and attractive tool for cardiovascular delivery. Baculovirval vectors are derived from the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV), an insect virus characterized by a large double-stranded circular DNA genome packaged in a rod-shaped capsid enveloped by a membrane (Ghosh et al.

2002). These vectors bear great potential in the field of gene therapy as they do not replicate in mammalian cells, have large insert capacity, and give rise to only minor cytopathic effects observable microscopically (Kost and Condreay 2002). Interestingly, baculoviral vectors have the ability to infect a broad range of mammalian cells (Sarkis et al. 2000) and our data (Grassi et al. 2006b) indicate that this vector is also particularly suited to transfer HRzs into human cardiomyocytes and VSMCs.

In addition to the viral vector choice, the successful administration of HRzs also depends on the promoter system employed to express the HRz. The promoter can influence HRz activity in the cell environment in variable ways which includes promoter transcription rate and intracellular localization of the transcript (Rossi 1995; Bertrand et al. 1997). Moreover, any HRz transcribed from promoters will invariably carry various promoter-derived sequences which can unpredictably affect HRz-cleavage. This last fact implies that, for the proper characterization and choice of HRz to be expressed from promoters, an evaluation of the effects of promoter derived sequences on HRz cleavage efficacy is necessary through an appropriate mathematical approach (see Sects. 2.3 and 2.4).

## *2.3 Enzymatic Mechanisms of HRz Action*

The creation of mathematical models able to describe HRz cleavage reaction is of utmost importance to calculate kinetic constants thus allowing the selection of the most appropriate molecules. In the minimal kinetic pathway of HRz cleavage reaction four main species are present: the HRz ($E$, molar concentration), the substrate ($S$, molar concentration), the HRz-substrate complex ($ES$, molar concentration) and the HRz-product complex ($EP_1P_2$; $P_1$ and $P_2$ represent the two parts in which the HRz splits the substrate; molar concentration). Accordingly, $E$ reacts with $S$ (direct reaction kinetic constant $k_1$, inverse reaction kinetic constant $k_{-1}$) to form $ES$ that, in turn, converts into $EP_1P_2$ (direct reaction kinetic constant $k_2$, inverse reaction kinetic constant $k_{-2}$) to finally give $E$ plus $P_1$ and $P_2$ passing through two different $EP_1P_2$ decomposition paths (direct reactions kinetic constants $k_3$, $k_4$, $k_5$ and $k_6$; inverse reactions kinetic constants $k_{-3}$, $k_{-4}$, $k_{-5}$ and $k_{-6}$) (Fig. 2), (Grassi et al. 2002). Unfortunately, this minimal kinetic pathway does not always reflects the real situation as alternate structures of $E$, $S$ and $ES$ (named, respectively, $E_1$, $S_1$, $ES_1$ and $ES_2$) (Fig. 2) can occur in solution (Stage-Zimmermann and Uhlenbeck 1998). In order to take into account the presence of these alternate structures, the simple reaction scheme needs to comprehend other reversible reactions accounting for $E$, $S$ and $ES$ conversion into possible alternate forms ($E_1$, $S_1$, $ES_1$ and $ES_2$, respectively) and characterized by proper kinetics constants ($k_{E1}$, $k_{E-1}$, $k_{S1}$, $k_{S-1}$, $k_{ES1}$, $k_{ES-1}$, $k_{ES2}$ and $k_{ES-2}$). In this regard, we have developed a general model (Grassi et al. 2002) to describe HRz kinetic reaction. The time variation of $E$, $S$, $S_1$, $ES$, $ES_1$ and $ES_2$ can be theoretically determined by means of the following set of differential equations:

$$\frac{dE}{dt} = -k_{E_1} E + k_{E-1} E_1 - k_1 ES + k_{-1} ES_2 + k_3 EP_1P_2 - k_{-3} EP, \tag{1}$$

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -k_{s_1} S + k_{s-1} S_1 - k_1 ES + k_{-1} ES_2, \tag{2}$$

$$\frac{\mathrm{d}E_1}{\mathrm{d}t} = k_{E_1} E - k_{E-1} E_1, \tag{3}$$

$$\frac{\mathrm{d}S_1}{\mathrm{d}t} = k_{s_1} S - k_{s-1} S_1, \tag{4}$$

$$\frac{dES}{dt} = k_{ES_2} ES_2 - (k_{ES_{-2}} + k_{ES_1} + k_2) ES + k_{ES-1} ES_1 + k_{-2} EP_1P_2, \tag{5}$$

$$\frac{dES_1}{dt} = k_{ES_1} ES - k_{ES-1} ES_1, \tag{6}$$

$$\frac{dES_2}{dt} = k_1 ES - (k_{-1} + k_{ES_2}) ES_2 + k_{ES_{-2}} ES, \tag{7}$$

$$P = S_0 - (S + S_1) - (E_0 - (E + E_1)), (P = P_1 + P_2), \tag{8}$$

$$EP_1P_2 = E_0 - E - E_1 - ES - ES_1 - ES_2, \tag{9}$$

where $t$ is time while $E_0$ and $S_0$ are, respectively, the initial HRz and substrate molar concentrations. While (1)–(7) represent kinetics equations, (8), (9) are expressions of species mass balances, made up on the whole reaction volume and regard $P$ (sum of $P_1$ and $P_2$) and $EP_1P_2$. This mathematical model assumes, for the sake of simplicity, that the $EP_1P_2$ conversion into $E$, $P_1$ and $P_2$ depends only kinetic constants $k_3$ and $k_{-3}$ (this means that $k_5 = k_6 = k_{-5} = k_{-6} = k_{-4} = 0$ and $k_3$, $k_{-3}$ are $\ll k_4$). Due to the non-linear character of this system of differential equations, a fifth order adaptive step size Runge Kutta method is used to get the numerical solution (Press et al. 1992). In the absence of alternate conformations, assuming $E_0 = 10\,\mathrm{nM}$, $S_0 = 1\,\mathrm{nM}$ and typical values for the kinetics constants (Grassi et al. 2002) [$k_1 = 0.05$ (nM min)$^{-1}$, $k_{-1} = 0.06\,\mathrm{min}^{-1}$, $k_2 = 0.01\,\mathrm{min}^{-1}$, $k_{-2} = 0.0001\,\mathrm{min}^{-1}$, $k_3 = 55\,\mathrm{min}^{-1}$, $k_{-3} = 0.05$ (nM min)$^{-1}$] product molar concentration ($P = P_1 + P_2$) follows, approximately, an exponential increase (see Fig. 3a, solid thick line). When, on the contrary, alternate conformations occur, $P$ trend can be highly modified. For example, in the case of an irreversible conversion into alternate conformations occurring "*off*" the kinetic pathway ($E_1$, $S_1$ and $ES_1$), a complete $S$ conversion can not take place as shown in Fig. 3a,c (dashed line) in the case of $ES$ conversion into $ES_1$ ($k_{\mathrm{ES1}} = 0.02\,\mathrm{min}^{-1}$ and $k_{\mathrm{ES-1}} = 0$; $E_1$ and $S_1$ absent) and $S$ conversion into $S_1$ ($k_{\mathrm{S1}} = 0.2\,\mathrm{min}^{-1}$ and $k_{\mathrm{S-1}} = 0$; $ES_1$ and $E_1$ absent). Interestingly, if an $ES$ fast conversion into $ES_1$ ($k_{\mathrm{ES1}} = 0.2\,\mathrm{min}^{-1}$ ($k_{\mathrm{ES1}} > S_0 k_1$), $k_{\mathrm{ES-1}} = 0.05\,\mathrm{min}^{-1}$) slows down $P$ increase (see Fig. 3a, solid thick line), an $ES$ slow conversion into $ES_1$ [$k_{\mathrm{ES1}} = 0.02\,\mathrm{min}^{-1}$ ($k_{\mathrm{ES1}} < S_0 k_1$), $k_{\mathrm{ES-1}} = 0.005\,\mathrm{min}^{-1}$]

leads to a biphasic behavior characterized by an initial fast $P$ increase followed by a slower $P$ increase (see Fig. 3a, dotted line). The presence of $ES_2$ confers to $P$ increase a sigmoidal character that is more pronounced in the case of slow conversion (see Fig. 3b: slow conversion, dotted line, $k_{ES2} = 0.02\,\text{min}^{-1}$, $k_{ES-2} = 0.005\,\text{min}^{-1}$; fast conversion, dotted line, $k_{ES2} = 0.2\,\text{min}^{-1}$, $k_{ES-2} = 0.05\,\text{min}^{-1}$). Our general model (1)–(9) predicts that the smaller the $ES_2$ conversion to $ES$, the lower the product concentration increases. Finally, the presence of $S_1$ reflects into a reduction of $P$ formation kinetics as witnessed by Fig. 3c. Interestingly, in this case, the difference between fast (thin solid line, Fig. 2c; $k_{S1} = 2\,\text{min}^{-1}$, $k_{S-1} = 0.5\,\text{min}^{-1}$) and slow (dotted line, Fig. 3c; $k_{S1} = 0.2\,\text{min}^{-1}$, $k_{S-1} = 0.05\,\text{min}^{-1}$) conversion is neatly less evident than in previous cases ($ES_1$, $ES_2$).

These considerations lead to the conclusion that if a biphasic behavior is usually due to the presence of $ES_1$, a simple $P$ lowering may be due to the presence of $S_1$, $E_1$ and $ES_2$. Obviously, the situation becomes more difficult if more than one alternate conformations exist contemporarily.

Despite the complexity depicted above, in the absence of alternate conformations, HRz kinetics path can be considerably simplified assuming that the kinetic constant $k_{-2}$ is negligible in comparison with $k_2$ (Birikh et al. 1997a,b). This implies an $ES$ conversion to $EP_1P_2$ ruled only by $k_2$ and that $EP_1P_2$ conversion to $E$, $P_1$ and $P_2$ develops instantaneously (*multiple turnover*). Accordingly, kinetics path reduces to the well known Michaelis and Menten mechanism (Michaelis and Menten 1913):

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftarrows}} ES \xrightarrow{k_2\,(=k_{cat})} P + E \quad \text{multiple turnover},$$

where $k_2$ is usually called $k_{cat}$ and $P$ represents the sum of $P_1$ and $P_2$. This reaction scheme is acceptable only for HRz carrying the minimal structural motif and targeted against short RNA substrates (in this case $E_0$ can be small in comparison to $S_0$) (Birikh et al. 1997b). On the contrary, when long and structured RNA substrates and/or HRz carrying additional RNA stretches are present, the *multiple turnover* scheme no longer holds. Indeed, in this case, substrate and/or HRz dimensions can yield to a very slow HRz-product ($EP_1P_2$) conversion to free HRz ($E$) and product ($P = P_1 + P_2$) in comparison to the $ES$ conversion to $EP_1P_2$. Accordingly, the reaction product is represented by the complex $EP_1P_2$ and, consequently, HRz regeneration does not occur. This is the reason why this condition is termed *single turnover*:

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftarrows}} ES \xrightarrow{k_2\,(=k_{cat})} P(=EP_1P_2), \text{ single turnover},$$

In this case, the reaction pathway needs an excess of HRz over the substrate (Birikh et al. 1997b). We (Grassi et al. 2002) demonstrated that when $E_0 > 10S_0$, *single turnover* condition (that is, ultimately, a simplification of the whole reaction scheme shown in Fig. 2 and mathematically represented by equations (1)–(9)) leads to the following product time dependence:

$$P = \frac{S_0}{\alpha_1 - \alpha_2}[\alpha_1(1 - e^{\alpha_2 t}) - \alpha_2(1 - e^{\alpha_1 t})], \tag{10}$$

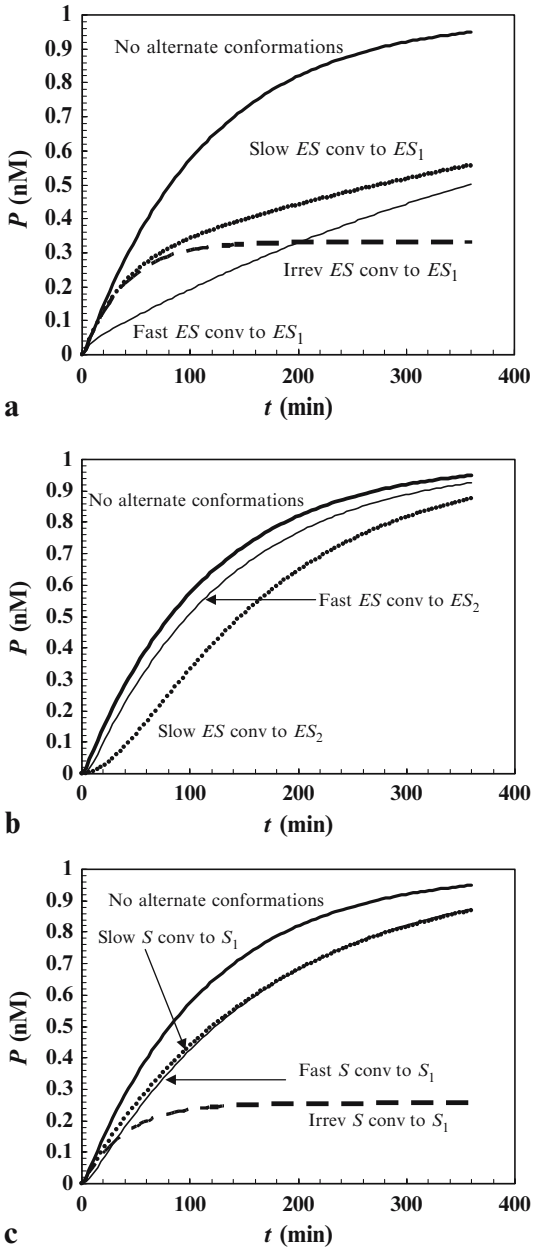



**Fig. 3** Product (*P*) formation vs time (*t*) in presence of alternate conformations. (**a**) $ES_1$, (**b**) $ES_2$, (**c**) $S_1$, according to the proposed model (1)–(9). *Solid lines* indicate the absence of alternate conformation, *dotted lines* indicate slow conversion into the alternate conformation, *solid thin lines* indicate fast conversion into the alternate conformation and *dashed lines* indicate irreversible conversion into the alternate conformation

where $\alpha_1$ and $\alpha_2$ are constants depending on $k_1$, $k_{-1}$, $k_{cat}$, $E_0$ and $S_0$. If, additionally, $S$ conversion to $ES$ is very fast (in presence of long HRz/substrate this in not always the case), equation (10) becomes the usual equation considered for kinetic constants calculation (Birikh et al. 1997a; Heidenreich et al. 1994; Heidenreich and Eckstein 1992)

$$P = S_0\left(1-\exp\left(-\frac{k_{cat}\,E_0}{K_m+E_0}t\right)\right),\ K_m=\frac{k_{cat}+k_{-1}}{k_1} \tag{11}$$

Due to the particular mathematical nature of equation (10), the determination of the kinetics constants ($k_1$, $k_{-1}$, $k_{cat}$) resorting to a data fitting performed on the time course curve (the experimental $P$ vs $t$ data) is not possible. However, what is biologically relevant is just the determination of $k_{cat}$ (representing the chemical step to form products) and $K_m$ (defined in (11) and representing the affinity of the HRz for the substrate). This can be accomplished according to a double fitting procedure. Equation (10) has to be firstly fitted on each time course curve ($P$ vs $t$) corresponding to different $E_0$ concentrations ($S_0$ constant and $\leq 10E_0$) in order to get $\alpha_1$ and $\alpha_2$ dependence on $E_0$. Consequently, it is possible to build the pseudo-experimental curve $R$ vs $E_0$, where $R$ is defined by:

$$R=-\frac{\alpha_1\alpha_2}{\alpha_1+\alpha_2}. \tag{12}$$

As it can be demonstrated that the following relation holds (Grassi et al. 2002):

$$R=\frac{k_{cat}\,(E_0-S_0)}{K_m+(E_0-S_0)}, \tag{12′}$$

$k_{cat}$ and $K_m$ determination comes from equation (12′) fitting on pseudo-experimental data $R$ vs $E_0$ calculated according to equation (12).

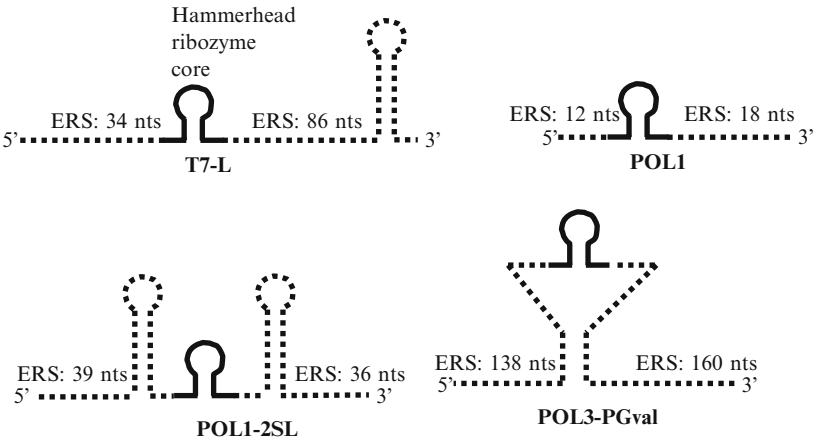
## 2.4 Calculation of Cleavage Kinetic Constants in the Presence of Promoter-Derived Sequences: An Example

In this paragraph, we show the application of our developed mathematical model (10), (12), (12′) to calculate HRz kinetic constants under single turnover conditions. In particular, we focus on the kinetic constant determination for a HRz directed against cyclin E1. This is a pivotal cell cycle gene whose non-regulated expression is present in many human pathologies including artery restenosis (Grassi et al. 2005). To achieve prolonged biological effects, the cyclin E1-HRz, as all HRzs, needs to be introduced into target cells by specific constructs (such as viral vectors) able to drive their expression from a defined expression cassette (promoter). This strategy implies the presence of promoter-derived sequences bound to the HRz-structure which can variably and unpredictably affect HRz-cleavage kinetic

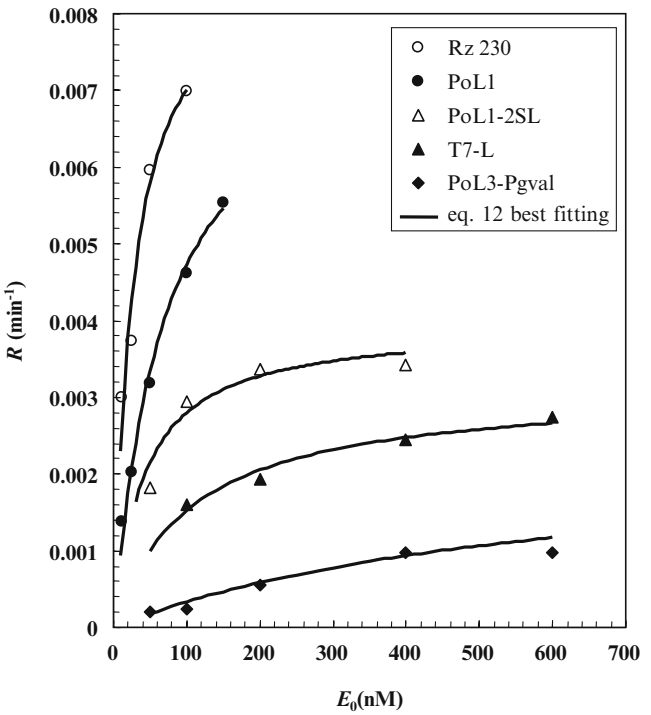
constants. To select the most appropriate expression cassette, sequences derived from different promoters were added to the minimal HRz. The constructs, described in details elsewhere (Platz et al. 2007), are derived from the RNA bacteriophage T7 promoter (T7-L), RNA polymerase I promoter (PoL1 and PoL1-2SL) and RNA polymerase III (PoL3-pGval) (Fig. 4a). T7-L contains 34 and 86 nucleotides before and after the minimal HRz core, respectively. In the 86-nucleotide stretch, a stem loop region, required for polymerase termination, is contained. In the PoL1 construct, the minimal HRz core is preceded and followed by 12 and 18 nucleotides, respectively. PoL1-2SL is preceded and flanked by longer stretches of 39 and 36 nucleotides, respectively. In both stretches, a stem lop region, known to increase stability in the cellular environment, was added. Finally, in the pGval construct, the HRz is placed in a stem-loop region preceded and followed by 138 and 160 nucleotides, respectively. The stem-loop region is thought to confer higher HRz stability in the cellular environment.

HRz cleavage assays were performed under *single turn-over* conditions using a target-RNA concentration ($S_0$) of 1nM in the presence of increasing molar ratios of HRz-constructs/target-RNA ($E_0$) as described in (Grassi et al. 2002). For each different molar ratio ($E_0/S_0$), the percentage of cleaved target-RNA ($P/S_0$) was evaluated at different time intervals, after resolving the undigested substrate and products on a 40-cm denaturing gel (3.5% polyacrilamide 7M urea). Substrate- and product-radioactivity was quantified by a Fuji PhosphorImager (Tokyo, Japan). As discussed in paragraph 2.3, $K_m$ and $k_{cat}$ were evaluated on the basis of a double fitting procedure. Figure 4b, showing equation (12′) best fitting (solid line) on pseudo-experimental data (symbols) $R$ vs $E_0$ for the minimal HRz structure and for the other four variants considered, represents the second step of this procedure. It is evident that HRz cleavage efficiency reduces when different promoter sequences are added to the minimal core. Indeed, the $R$ vs $E_0$ curve competing to the four variants is always lower than that competing to the minimal core (open circles). This effect becomes more and more evident passing from PoL1 (filled circles) to PoL1-2SL (open triangles), T7-L (filled triangles) and PoL3-pGvall (filled diamonds). This behavior translates in the following $k_{cat}$ and $K_m$ values: minimal core ($k_{cat}$ = (8.8 ± 0.8)$10^{-3}$ $min^{-1}$, $K_m$ = 25 ± 7 nM), PoL1 ($k_{cat}$ = (7.9 ± 0.6)$10^{-3}$ $min^{-1}$, $K_m$ = 67 ± 11 nM) PoL1-2SL ($k_{cat}$ = (3.9 ± 0.4) $10^{-3}$ $min^{-1}$, $K_m$ = 41 ± 16 nM), T7-L ($k_{cat}$ = (3.1 ± 0.2)$10^{-3}$ $min^{-1}$, $K_m$ = 104 ± 22 nM) and PoL3-pGvall ($k_{cat}$ = (2.3 ± 0.8) $10^{-3}$ $min^{-1}$, $K_m$ = 595 ± 100 nM).

These data show the general negative effects exerted by promoter derived sequences on HRz cleavage reaction, stressing the importance of the proper promoter choice. Moreover, these data indicate the general tendency of an inverse relation between the length of the promoter derived sequences and the cleavage efficiency of HRz-cyclinE1. The phenomenon may be explained with the formation of alternate conformations of the different reaction molecules which eventually affect cleavage kinetic constants. The complex cleavage reaction pathway clearly requires an appropriate mathematical tool to calculate kinetic consents. Our model allows the proper calculation graphically displaying, through the relation between $R$ and $E_0$, the efficacy of HRzs.




a




b

**Fig. 4** Structure and cleavage efficiencies of a hammerhead ribozyme bearing different promoter-derived sequences. (**a**) A minimal hammerhead ribozyme core targeted against the mRNA of cyclin E1 has been embedded into sequences derived from the RNA bacteriophage T7 promoter (*T7-L*), the RNA polymerase I promoter (*PolI* and *PolI-2SL*) and RNA polymerase III (*pGval*) promoter; (**b**) the effects on the cleavage efficiency of the different promoter derived sequences are compared to that of the minimal hammerhead ribozyme core in terms of *R* (see equation (12)) vs $E_0$ (initial ribozyme concentration)

## 3 Potential Use of HRzs as Therapeutics for Artery Restenosis

The most common cause of small and large artery occlusions (stenosis) is the progressive development of atherosclerosis. When this pathologic process involves coronary arteries, clinical symptoms ranging from angina pectoris to heart attack can occur. In order to revascularize stenotic coronary arteries, since 1979 the so-called percutaneous transluminal coronary angioplasty (PTCA) has been introduced (Gruntzig et al. 1979). This non-surgical method, shown to be safe and effective (Pocock et al. 1995), involves: (1) advancing a balloon catheter to an area of coronary narrowing, (2) inflating the balloon, and (3) retrieving the catheter following balloon deflation (Fig. 5a). More than 500,000 percutaneous coronary intervention procedures are performed yearly in the USA, and about 1 million procedures worldwide (American Heart Association 2001). However, PTCA has been shown to induce (Fig. 5b) the development of symptomatic re-occlusion (restenosis) caused by early elastic recoil, intimal thickening, late constricting remodeling of the vessel (Ruygrok et al. 2003) and formation of mural thrombus in about 30–50% of treated patients (Califf 1995).

To try to overcome the PTCA related problems, the expansion of the balloon during angioplasty has been associated with the deployment of a stent. This is an expandable metal tubular mesh (Fig. 5c) (Sigwart et al. 1987) which has been shown to significantly reduce restenosis rate down to 20–30% (Serruys et al. 1994; Fischman et al. 1994). The partial success of the stents is due to the induction of the intimal thickening (in-stent restenosis, ISR), a phenomenon particularly evident in small caliber vessels (Ruygrok et al. 2003; Moreno et al. 2004). This pathological event is characterized by an exuberant proliferation of vascular smooth muscle cells (VSMCs) which migrate from the tunica media of the artery, where they normally reside in a quiescent state, to the intima artery layer where they synthesize extracellular matrix and give origin to the intimal thickening, also called neointima (Edelman and Rogers 1998; Ferns and Avades 2000). To try to overcome this problem, devices able to locally deliver anti-proliferative drugs such as Sirolimus and Paclitaxel (drug-eluting stent, DES) have been developed. DES have significantly reduced ISR compared to bare metal stents (Moses et al. 2003; Stone et al. 2004), in patients with discrete, de novo lesions in native vessels. However, their revascularization benefit is attenuated in high-risk patients (diabetes; the acute coronary syndromes, including ST-segment elevation myocardial infarction; smaller-diameter lesions and longer lesions; several stents or overlapping stents), compared to low risk patients displaying, for example, 26% ISR rate in coronary bifurcation lesions (Colombo et al. 2004). Moreover, some other concerns such as stent thrombosis (Tung et al. 2006; Serruys and Daemen 2007) are now emerging with regard to the use of DES. These limitations suggest that alternative approaches and/or the selection of novel antiproliferative drugs, such as HRz, may be beneficial for ISR treatment.

Platelet-derived growth factor A (PDGF-A), a known stimulator of VSMC proliferation (Hart et al. 1988), has been directly implicated in the pathogenesis of arterial proliferative diseases (Nilsson et al. 1985) which includes atherosclerosis,

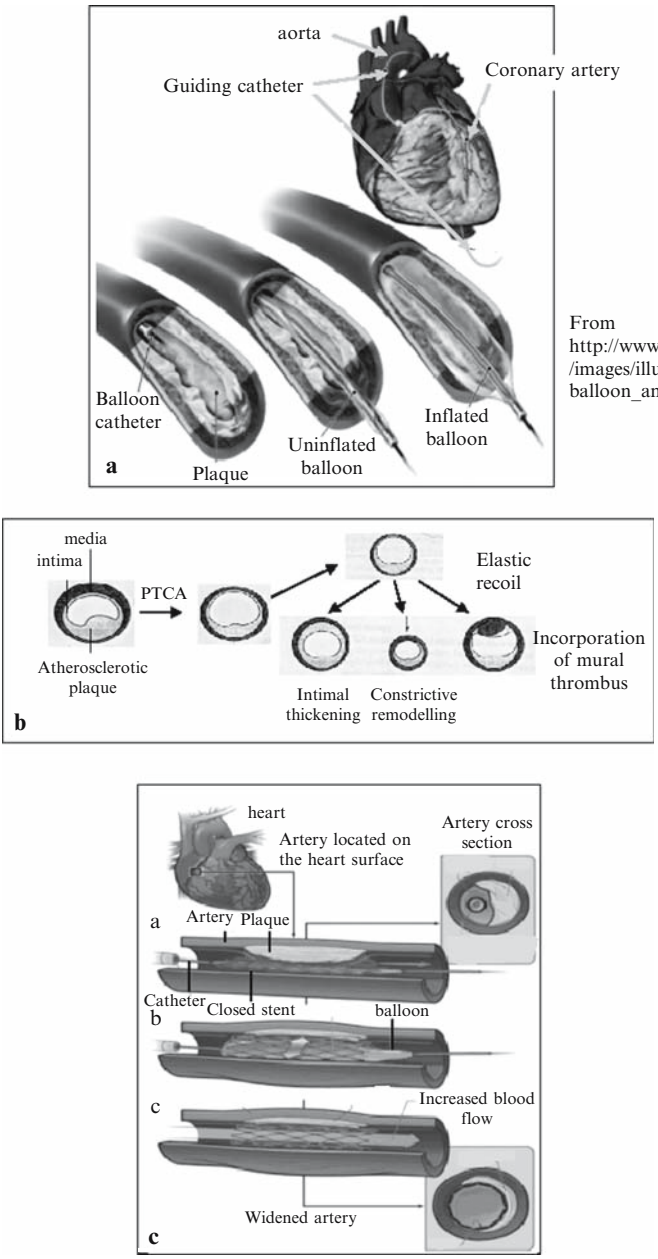



**Fig. 5** Coronary revascularization procedure and its complications. (**a**) Percutaneous transluminal coronary angioplasty (*PTCA*) dilates the area of vascular narrowing by means of a balloon catheter inflated in the artery lumen; (**b**) *PTCA* can be followed by early artery elastic recoil, intimal thickening, incorporation of mural thrombus and late constrictive remodeling, all phenomenon which determines the re-occlusion of the treated vessel; (**c**) the implantation of endovascular implants (*stent*) following PTCA efficiently prevent the early artery elastic recoil and late constrictive remodeling. However, stents trigger a complex series of patho-biological events, among which the exuberant proliferation of vascular smooth muscle cells, which lead to the formation of a neo-intima with consequent reduction of the artery lumen

hypertension and artery restenosis. Thus, HRz-based approaches against PDGF-A have been explored. A PDGF-A targeted HRz has been shown to be successful in inhibiting human VSMC proliferation in vitro (Hu et al. 2001b). Notably, the authors have used for their experiments VSMC isolated from a spontaneously hypertensive rat strain (SHR) which are characterized by an higher growth rate compared to cells isolated from normotensive rats. This indicates that the HRz approach followed might also be effective in the presence of increased VSMC proliferation rate, an un-favorable condition typically found in hypertensive and diabetic patients. Finally, the authors could show a good correlation between the in vitro (cell-free environment) HRz cleavage efficacy and that observed in cultured rat VSMCs (ex vivo). This observation shows the good predictive power of HRz cell free analysis for ex vivo/in vivo applications.

A chimeric DNA-RNA HRz targeted against PDGF-A mRNA induced, in a rat model, about 45% reduction in the ratio between neointima/media (Kotani et al. 2003), a measurement commonly chosen to evaluate the efficacy of anti restenotic treatment. Interestingly, in this work, the authors also investigated, by microarray analysis, the gene expression pattern in vessel treated by the anti-PDGF-A HRz. As a result of this analysis, they could show that only the expression of PDGF-A was completely inhibited, thus indicating a high specific action of the selected HRz. The authors also observed a substantial reduction in the expression of some cell cycle related genes such as Cdc-2-related protein kinase, cyclin D3, cyclin D1 and cyclin B. This reduction was interpreted as a consequence of the inhibition of PDGF-A and might have contributed to the reduced intima hyperplasia. However, some other cell cycle genes such as cyclin D2 and cdk4 kinase were upregulated, possibly suggesting a compensative reaction of VSMCs to the anti-proliferative action of the HRz. This last observation may contribute to explain why the inhibition of intimal growth did not reach 100%.

Significant but partial success in inhibiting the neo-intimal formation was also achieved by expressing an anti-PDGF-A HRz from an adenoviral vector (Lin et al. 2004). In this case, the intima/media ratio decreased down to 30% of control in a rat model of restenosis. Comparable degree of reduction was achieved in a rat model (Yamamoto et al. 2000) by an HRz targeted against the mRNA of transforming growth factor β 1 (TGFβ-1). TGFβ-1 has been chosen as target due to its involvement in the pathogenesis of artery restenosis (Nikol et al. 1992). The expression of this protein, typically increased in human vascular restenosis lesions, has been shown to have mitogenic effects. Moreover, it plays a pivotal role in the synthesis of extracellular matrix protein such as collagen, laminin and fibronectin by a variety of cells, which all contribute to the thickening of the neointima. The anti-restenotic effect of targeting TGFβ-1 was also confirmed in another study where a chimeric DNA-RNA HRz significantly decreased VSMC proliferation in vitro and reduced the intima/media ratio down to 30% of control in the rat (Ando et al. 2004).

Despite the significant efficacies of the tested HRzs, complete prevention of neointima formation was not achieved. Among the several variables which can explain this observation, one may be represented by the choice of the target gene.

It is possible that the blockade of a single pathway, i.e., PDGF-A or TGFβ-1 cascade, is not sufficient to prevent VSMC proliferation because of the existence of alternative/redundant signaling pathways. In this regard, a PDGF-A targeted HRz successfully reduced the proliferation of VSMC stimulated by $TGF\beta_1$, but only modestly, after angiotensin II stimulation (Hu et al. 2001a).

The inhibition of genes shared by multiple pathways and directly involved in the control of cell cycle progression was also explored. A chimeric DNA-RNA HRz against the mRNA of the proliferating cell nuclear antigen (PCNA) achieved a reduction of about 30% in neo-intimal thickening compared to controls, in a pig model of artery restenosis (Frimerman et al. 1999). PCNA was chosen as target as it is a cofactor for DNA polymerase and it is required for DNA synthesis and S-phase progression (Fairman 1990). Moreover, it forms complexes with different cyclins and cyclin-dependent kinases, pivotal regulators of cell cycle progression. Notably, the authors evaluated the anti-restenotic effect of the PCNA-HRz in the presence of a stent. This experimental design is particular relevant as it perfectly matches the condition of the common clinical practice where PTCA is followed by stent implantation. The reported results are also particularly interesting considering that the delivery system used (cationic liposomes) are not ideal for in vivo delivery. It is reasonable to assume that optimized delivery systems (i.e., polymeric mediated delivery) may further improve the efficacy.

Another cell cycle related gene targeted by HRz is the proto-oncogene *c-myb*. High expression level of this protein has been correlated to increased cell proliferation rates. Conversely, reduced *c-myb* expression has been associated with cell differentiation. Moreover, in VSMCs, the expression level of *c-myb* has been directly correlated to cell proliferation rate (Brown et al. 1992). In rat cultured VSMC, a HRz targeted against the mRNA of *c-myb* (Jarvis et al. 1996a,b), decreased cell proliferation down to 14% of controls in vitro and reduced the neo-intimal/media ratio down to 50% of controls in a rat model (Macejak et al. 1999). In this last case, the authors delivered the HRz by an adenoviral vector, a commonly used strategy to transfer genes for gene therapy purposes. The reason for the partial success of this approach can, at least in part, depend on the fact that the adenoviral vector may not represent the optimal delivery system. In vitro it has been show that adenoviral vector can efficiently transduce both VSMC and endothelial cells (Grassi et al. 2006b). This implies that the anti-proliferative HRz can be up-taken also by the endothelium with consequent retardation in endothelium growth, i.e., in the so-called re-endothelization of the injured artery, a fact known to favor restenosis (Serruys and Daemen 2007).

We proposed a novel anti-proliferative approach based on the specific knock-down of two pivotal cell cycle promoting genes which regulate G1/S transition of the cell cycle, i.e., cyclin E and the transcription factor E2F1 (Dyson 1998; Ohtsubo et al. 1995). The inhibition of the expression of these genes is particularly attractive because of the existence of a feed-forward loop between them which amplifies the G1 to S phase promoting signals (Geng et al. 1996). Therefore, the downmodulation of the interaction between these two genes might augment the proliferation inhibition effect. Additionally, the fact that they are

synthesized de novo at each new G1 phase and then rapidly degraded at the protein and mRNA levels with the progression of the cell cycle, implies that no active protein and mRNA is left for the next cycle. Thus, their knockdown during G1 phase may efficiently reduce the intracellular level preventing their biological functions. Finally, the observation (O'Sullivan et al. 2003) that, compared to normal VSMCs, cyclin E is over-expressed in in-stent restenotic VSMCs, points towards the central role of cyclin E in sustaining VSMC proliferation, and makes it an ideal target to prevent in-stent stenosis. In cultured human VSMC (Grassi et al. 2001, 2005), we observed a dose- and time-dependent decrease in the amount of S phase cells which, 2 days after transfection and at HRz concentration of 420 nM, corresponded to one-fifth/one-tenth of controls. Moreover, the concomitant targeting of cyclin E and E2F1 resulted in a more pronounced inhibition of VSMC proliferation compared to the independent targeting of each of the two genes, at the same HRz concentration.

Alternative approaches to downregulate VSMC include the promotion of cell death rate, instead of the reduction of cell proliferation. Over-expression of Bcl-2 protein, first identified as an oncogene, inhibits apoptosis induced by a variety of circumstances including growth factor withdrawal, DNA damage and conflicting sub-cellular signal events. Thus, the downregulation of its expression may in principle be useful to favor VSMC death diminishing their accumulation within the neointima. In this regard, a HRz targeted against the mRNA of the Bcl-2 greatly increased apoptotic cell rate compared to controls in cultured rat cells (Perlman et al. 2000). In vivo, the neo-intimal/media ratio was reduced to about of 50% of controls with a concomitant reduction of the cell number within arterial media layer. Despite the effectiveness of this approach, it cannot be excluded that it might induce a weakening of the arterial wall possibly resulting in the formation of secondary artery lesions. A tight regulation of the expression of the pro-apoptotic HRz should be therefore be considered in order to keep an appropriate apoptotic rate.

The concomitant prevention of different patho-biological events which concur to the development of artery re-occlusion, such as VSMC proliferation, migration and deposition of extracellular matrix components, has also been explored. An attractive target is represented by the leukocyte-type 12-lipoxygenase (12-LOX) mRNA whose protein mediates the stimulatory growth effect of Ang II and the chemotactic effect of platelet derived growth factor (PDGF)-BB on VSMCs. A chimeric DNA-RNA HRz successfully downmodulated leukocyte 12-LOX expression in cultured porcine VSMC (Gu et al. 1995). Moreover, an analogous HRz, significantly reduced, in cultured rat VSMC, the PDGF-induced cell migration and deposition of fibronectin, a key matrix protein (Gu et al. 2001). The same HRz decreased the neo-intimal/media ratio to 40% of controls in a rat carotid artery model of restenosis. It should be noted that 12-LOX is also expressed, in addition to VSMC, in monocytes/macrophages, and that 12-LOX products mediate monocytes adhesion to endothelial cells. This implies that the anti-12-LOX HRz may have not only downregulated VSMCs proliferation/migration but also the adhesion of inflammatory cells (monocytes) to endothelial cells. In turn, this may have reduced, in the

injured area, the inflammation, a relevant factor which promotes and sustain artery restenosis.

Whereas all the different HRzs described here efficiently reduced VSMC proliferation ex vivo, in vivo (animal models) their effectiveness was reduced. A relevant contribution to this discrepancy may depend on the sub-optimal delivery systems available so far. The major challenge for the future will be the identification of efficient and controllable delivery systems for local HRz release. Moreover, an optimized and standardized HRz release system will allow the comparison of the efficacies of different HRzs and will contribute to select the best candidate genes to knock out.

## 4 HRzs and Small Interfering RNAs in the Cardiovascular Field

The potential applications of HRz in the cardiovascular field are not limited to artery-restenosis since other vascular and non-vascular pathologies might benefit from a HRz-based approach. A HRz has been successfully directed against the mRNA of the endothelial 12/15 lipoxygenase (12/15LO), a protein which favors monocytes binding to endothelial cells (Hatley et al. 2003). This is considered an early inflammatory event which may contribute to vessel damage, especially in diabetics. It follows that the downregulation of 12/15LO expression by HRzs may be of therapeutic benefit, as this strategy would reduce monocyte adhesion to vascular endothelial cells thus attenuating inflammation-dependent damage of the vessel. These findings confirm a previous report (see above; Gu et al. 2001) of anti-restenotic and anti-inflammatory effects achieved by downregulating the expression of 12LO. Another HRz has been directed against the tissue factor (TF), a glycoprotein bound to the membrane of vascular cells that initiates the clotting cascade (Cavusoglu et al. 2002) causing vessel occlusion with dangerous ischemic consequences. The reduction of the TF mRNA levels in cultured vascular smooth muscle cells by HRzs indicated that it is potentially feasible to think of an anti-thrombotic strategy based on the reduction of the expression levels of the pro-thrombotic protein TF. Another potentially interesting application of HRzs deals with their ability to downmodulate the expression of the mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGF2R) in cardiomyocytes (Chen et al. 2004). M6P/IGF2R is a multi-functional protein implicated in the regulation of cardiomyocytes growth and apoptosis. The regulation of its expression levels may thus represent a novel approach to favor cardiomyocytes survival, essential for the treatment of myocardial pathological conditions such as myocardial infarction and heart failure. Finally, HRzs have also been used to knockdown gene expression as a strategy to understand the contribution of a given gene product in cardiac-related biochemical pathways (Montagnani et al. 2002; Wang et al. 2002; Katsuyama et al. 2002).

Up to the end of the last century, HRzs represented one of the most commonly used nucleic acid molecules potentially able to down-regulate gene expression in the cardiovascular system as well as in other human organs. From the year 2000 on, a novel class of nucleic acid molecules named small interfering RNAs (siRNAs) become more and more popular (see Agostini et al. 2006 for a review) to knock-down gene expression. siRNA are short double-stranded RNA molecules able to drive, in a sequence-specific manner, the degradation of virtually any RNA when bound to a cellular protein complex named RNA-induced silencing complex (RISC). The specificity of action is given by the antisense strand, present in the RNA duplex, which directs RISC to the complementary sequence on the target RNA thus triggering its catalysis. Despite the relatively recent identification of siRNAs, several potential applications as therapeutics for different human pathological conditions have been shown (Scherer and Rossi 2004). With regard to the cardiovascular field (Agostini et al. 2006), three main research topics can be identified: (1) studies devoted to ameliorate and to understand the mechanisms regulating cardiomyocytes functionality; (2) studies aimed at the prevention of viral myocarditis; and (3) studies dealing with vascular pathologies. In the first case, studies were focused on the development of novel approaches to treat heart failure and to ameliorate cardiac damages following hypoxia-ischemia. In the second case, attention was put on the attenuation of cardiac infection sustained by coxsackieviruses of group B, the most relevant etiological cause of viral myocarditis. Finally, investigations related to the vascular system focused on the identification of novel approaches to treat atherosclerosis, both by interfering with the atherosclerotic process itself and by improving the outcome of the technique used to re-vascularize vessels occluded by atherosclerotic plaques.

Despite the different mechanism of action, both HRzs and siRNA action results in the destruction of the target RNA in a sequence-specific fashion. This implies that, in principle, the use of one or the other nucleic acid molecule for gene expression knockdown purposes is equivalent. So far, however, it is not completely clear which, among the two kind of molecule, is the most convenient. Few studies have been conducted to compare the knockdown efficiency of HRzs and siRNAs (Miyagishi et al. 2003) targeted against the same substrate RNA. We have compared, in VSMCs, the anti-proliferative effects of two HRzs (Grassi et al. 2005) and two siRNAs (Dapas et al. 2007) all directed against two pivotal cell cycle promoting genes, i.e., cyclin E1 and the transcription factor E2F1. Under optimized conditions, we did not detect significant differences between the anti-proliferative effects of HRz (evaluated 2 days after treatment at a concentration of 220 nM) and siRNA (evaluated 3 days after treatment at a concentration of 220 nM). In progress is the evaluation of the reduction of the intracellular levels of the target mRNAs induced by HRzs and siRNAs. The reported examples are obviously not enough to draw final conclusions about the efficacy of HRs and siRNAs. Despite this fact, it is becoming evident that the selection of efficient siRNAs represents an easier task compared to the selection of efficient HRzs. Computer-based approaches to select active siRNA (Mercatanti et al. 2002) are now highly efficient (more than 75%

success). In contrast, computer-based approaches to select active HRzs are far less reliable and prolonged experimental work is required to select active molecules.

## 5 Conclusion

HRzs base the biological effect on the sequence-specific recognition of the target RNA leading to its destruction. Thus, they can be used to reduce the intracellular level of a specific RNA coding for a protein which affects cellular metabolism or environment causing disease. The data presented indicate that they have the potential to become of therapeutic value for human pathologies such as artery restenosis. However, despite the encouraging results achieved, HRz technology can and should be optimized to bring it closer to clinical practice.

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