Ribonucleotide Reductase as a Target to Control Apicomplexan Diseases

James B. Munro and Joana C. Silva*

Department of Microbiology and Immunology and Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, MD 21201, USA

Abstract
Malaria is caused by species in the apicomplexan genus Plasmodium, which infect hundreds of millions of people each year and kill close to one million. While malaria is the most notorious of the apicomplexan-caused diseases, other members of the eukaryotic phylum Apicomplexa are responsible for additional, albeit less well-known, diseases in humans, economically important livestock, and a variety of other vertebrates. Diseases such as babesiosis (hemolytic anemia), theileriosis and East Coast Fever, cryptosporidiosis, and toxoplasmosis are caused by the apicomplexans Babesia, Theileria, Cryptosporidium and Toxoplasma, respectively. In addition to the loss of human life, these diseases are responsible for losses of billions of dollars annually. Hence, the research into new drug targets remains a high priority. Ribonucleotide reductase (RNR) is an essential enzyme found in all domains of life. It is the only means by which de novo synthesis of deoxyribonucleotides occurs, without which DNA replication and repair cannot proceed. RNR has long been the target of antiviral, antibacterial and anti-cancer therapeutics. Herein, we review the chemotherapeutic methods used to inhibit RNR, with particular emphasis on the role of RNR inhibition in Apicomplexa, and in light of the novel RNR R2_e2 subunit recently identified in apicomplexan parasites.

Introduction
The Apicomplexa are a group of single-celled, eukaryotic organisms that, together with the ciliates and dinoflagellates, form the major lineages in the Alveolates. All Apicomplexa, save the predatory flagellates Colpodellida, are pathogenetic, obligate, intracellular parasites (Adl et al., 2005; Morrison, 2009). They are characterized by the presence of an apical complex, a structure involved in host-cell invasion, which is located at the anterior end of the cell. Most apicomplexans also possess a specialized organelle called the apicoplast, a secondary endosymbiotic plastid believed to be of red algal origin (Blanchard and Hicks, 1999; Fast et al., 2001; Janoušková et al., 2010). Within the apicoplast occur processes essential for the parasite's survival, such as heme and lipid biosynthesis. Another defining characteristic of apicomplexans is their inability to synthesize purine rings de novo and hence their need to salvage exogenous purines via a variety of different pathways (Booden and Hull, 1973; Chaudhary et al., 2004; de Koning et al., 2005; Cassera et al., 2008; Madrid et al., 2008). These organisms have complex (indirect) life cycles, and they often exploit multiple hosts/vectors and transition between life cycle stages is dependent upon a diverse array of environmental cues.

The biological characteristics that differentiate apicomplexans from their vertebrate hosts have often been considered optimal targets of new therapeutics to control these eukaryotic pathogens. Alternatively, essential and strongly conserved proteins can be targeted, provided that they differ from their vertebrate homologs in such a way that minimizes potential cross-reaction and toxicity.

The enzyme ribonucleotide reductase (RNR) is one such example. RNR utilizes free radical chemistry to catalyze the reduction of ribonucleotides to deoxyribonucleotides (Thelander and Reichard, 1979; Reichard, 1988). It provides the only de novo means of generating the essential building blocks for DNA replication and repair across all domains of life and, as such, it is the rate-limiting step in DNA synthesis (Jordan and Reichard, 1998; Lundin et al., 2009). Additionally, RNR is critical for maintaining a balanced pool of DNA precursors during chromosome replication (Herrick and Sclavi, 2007). An unbalanced deoxyribonucleotide triphosphate pool may lead to an increase in mutation and disease (Lin and Elford, 1980; Reichard, 1988; Chabes et al., 2003; Wheeler et al., 2005; Gon et al., 2006; Mathews, 2006; Kumar et al., 2010).

Here we review the chemotherapeutic methods used to inhibit the essential enzyme RNR, with particular emphasis on the novel RNR R2_e2 subunit recently identified in apicomplexan parasites (Munro et al., submitted) and on the malaria-causing genus Plasmodium. The R2_e2 subunit is unique to the Apicomplexa and as such, it can potentially be used to specifically target apicomplexan pathogens.

Apicomplexan parasites are responsible for devastating infectious diseases
The phylum Apicomplexa consists of more than 4,000 described species (Levine, 1988), many of which are of medical, agricultural, and economic importance and whose adverse impact on human society cannot be overstated. Among the most notorious are Plasmodium, Babesia, Theileria, Cryptosporidium, and Toxoplasma the causative agents of malaria, babesiosis, theileriosis and East Coast fever, cryptosporidiosis, and toxoplasmosis, respectively. They are responsible for causing millions of human deaths and billions of dollars in productivity and material losses each year (Sachs and Malaney, 2002; Corso et al., 2003; Rowe et al., 2006; Spielman, 2009). Currently, five species of Plasmodium are known to cause malaria in humans, P. falciparum, P. knowlesi, P. malariae, P. ovale, and P. vivax (Rougemont et al., 2004; Singh et al., 2004; Cox-Singh et al., 2008), of which P. falciparum is the most deadly and P. vivax the most geographically widespread. The life cycle of Plasmodium alternates between a vertebrate host and mosquito vector and involves four major developmental stages in the vertebrate host: sporozoites, merozoites, trophozoites, and gametocytes (Bledsoe, 2005; Brown and Catteruccia, 2006).

*Corresponding author: jcsilva@som.umaryland.edu
Prioritization of apicomplexan drug targets

There is currently no fully efficacious vaccine on the market against any apicomplexan species and drug treatment is the method of choice in the management of apicomplexan diseases. Modern approaches to drug design, made possible with the advent of genome sequencing, emphasize defining and targeting metabolic and molecular differences between host and parasite to avoid host side effects (Croft, 1997). This may be achieved with the selective targeting of parasite-specific enzymes or by targeting those which are highly divergent or have distinct binding sites and are thus sufficiently different to be selectively targeted (Coombs, 1999; Cerqueira et al., 2017). Also important is that the protein target be essential to the growth, reproduction, or survival of the parasite. Finally, knowledge of the gene expression pattern of potential target proteins is necessary to link drug administration with the critical and relevant stages of the pathogen’s life cycle. This is particularly pertinent to organisms characterized by differentially expressed, stage-specific, and often stage-unique gene expression, such as apicomplexans (Coulson et al., 2004). It has been suggested that targeting the intraerythrocytic life stages of Plasmodium (the ring forms, trophozoites, schizonts, and merozoites), when clinical symptoms are manifested, is of particular interest (Yeh and Altman, 2006). However, targeting multiple life stages, for example controlling both the liver and blood stages of Plasmodium, may be more conducive to the ultimate goal of disease eradication (Alonso et al., 2011).

Current control strategies and challenges

Drug targets for control of apicomplexans have focused on parasite metabolic processes. These include processes within the cytosol, mitochondrion, digestive vacuole, synthesis of macromolecular and metabolic enzymes, and processes involved in membrane synthesis and signaling (Olliaro and Yuthavong, 1999; Padmanaban, 2003; Fidock et al., 2004; El Bissati et al., 2006). Enzymes in the purine salvage pathways, essential to these pathogens, are also potential drug targets (Tracy and Sherman, 1972; Krug et al., 1989; Parker et al., 2000; Gardner et al., 2002; Raman and Balaram, 2004; Striepen et al., 2004; Ting et al., 2005; Downie et al., 2008), as are all proteins and processes related to the apicomplexan-specific organelles, the apicoplast (Foth et al., 2003; Sato and Wilson, 2005; Wiesner et al., 2008; Lizundia et al., 2009). Antibiotics like doxycycline, which specifically target plastid pathways and impair the expression of apicoplast genes, can be used to target apicomplexans (Ralph et al., 2001; Leander and Keeling, 2003; Dahl et al., 2006). Apicoplast drug targets have included the apicoplast’s metabolic pathways (e.g. DNA replication, transcription, protein translation, fatty acid biosynthesis, and isoprenoid biosynthesis), or targeting proteins encoded by the host’s nuclear genes that are destined for the apicoplast (McFadden and Roos, 1999; Roos et al., 1999; Roos et al., 2002; Ralph et al., 2004; White, 2004; Waller and McFadden, 2005; Dahl and Rosenthal, 2008; Prusty et al., 2010).

The availability of genome sequences from several species and isolates of Plasmodium and other Apicomplexa facilitates the identification of novel, potential drugs for the control of apicomplexan parasites (Doolan et al., 2003; Yeh et al., 2004; Carvalho and Ménard, 2005; Winzeler, 2008; Mu et al., 2010). For example, evolutionary patterning has been proposed as a means to combat the emergence of drug resistance (Durand et al., 2008). Genes with high rates of nonsynonymous changes have been associated with drug resistance in P. vivax (Dharia et al., 2010), and may be responsible for vaccine evasion in P. falciparum (Takala and Plowe, 2009). In contrast, evolutionary patterning focuses on finding and targeting protein residues that are under strong purifying selection, which will in principle reduce the instances of drug resistance mutations.

The tremendous health burden imposed by malaria has made Plasmodium the primary target for many of these approaches. Despite a diversified arsenal of potential tools to combat malarial infection, multiple drug resistance to existing anti-malarial compounds is becoming increasingly common in Plasmodium (Greenwood and Mutabingwa, 2002; Anderson, 2009; Bustamante et al., 2009; Takala and Plowe, 2009). A case in point is the antifolate drugs used to treat malaria. Antifolate drugs bind enzymes necessary for folate biosynthesis, thus targeting essential precursors for purine and pyrimidine synthesis. Antifolates have been used against Plasmodium with success, but resistance to these drugs has become widespread (Gregson and Plowe, 2005; Mkulama et al., 2008; Sridaran et al., 2010). Currently, the primary treatment for malaria is based on artemisinin, which is administered in combination with other drugs in order to prevent, or delay, the onset of resistance. However, there are clear indications that artemisinin resistance is emerging (Chrubasik and Jacobson, 2010; Dondorp et al., 2010; Enserink, 2010; Fidock, 2010). Therefore, the development of new chemotherapeutic and prophylactic antimalarial drugs and vaccines remains a priority (Greenwood and Mutabingwa, 2002; Anderson, 2009; Bustamante et al., 2009; Takala and Plowe, 2009).

RNR classification and distribution

RNRs are classified into one of three classes, I-III (Jordan et al., 1994; Reichard, 1997; Fontecave, 1998; Nordlund and Reichard, 2006). All three classes use a thyl radical to remove the ribose OH-group. The distinction between these classes relies on differences in radical generation chemistry and the cofactor needed to produce the organic radical. Class I RNRs are oxygen-dependent, typically require a tyrosyl radical and a diiron center, and are characteristic of eukaryotes and common among bacteria. Class II RNRs are indifferent to oxygen, form a thyl radical via adenosylcobalamin, and are characteristic of Archaea and bacteria. Class III RNRs are anaerobic, form a glycycl radical using an iron-sulfur center in the presence of S-adenosylmethionine and reduced flavodoxin, and are also characteristic of Archaea and bacteria.

Class I RNRs have been further categorized as class Ia, Ib, or Ic. Class Ia is typical in eukaryotes and bacteria, while bacteria and Archaea primarily encode classes Ib and Ic RNRs (Harder, 1993; Sjöberg, 2010). Class Ic RNRs are further categorized as R2c proteins and while the R2-homolog R2lox proteins are described as “R2c-like”, they are not believed to form active RNR holoenzymes (Högborn et al., 2004; Andersson and Högborn, 2009). This classification, based on structural and chemical properties, has shortcomings since classes Ia and Ic are not monophyletic clades, i.e., discrete, mutually exclusive groups, which contain a most recent common ancestor and all of its descendants. Instead, class Ia is polyphyletic and Ic
is paraphyletic. This problem was addressed by Munro et al. (submitted) (Figure 1) who recognized four distinct class I RNR clades, namely the eukaryotic-specific clades $R2_{e1}$ and $R2_{e2}$, and the clades containing primarily archaean and bacterial RNRs, namely $R2_{ab}$ and $R2_c$. The study also revealed that the newly discovered clade $R2_{e2}$ is unique to the Apicomplexa (Munro et al., submitted). In fact, the most significant mammalian-infecting Apicomplexa genera, such as *Plasmodium*, *Cryptosporidium*, and *Babesia*, all encode one $R2_{e2}$ subunit.

**Class Ia holoenzyme regulation and formation**

Apicomplexans encode Class Ia RNRs, which are thus the focus of this review. Class Ia RNR enzymes are composed of two distinct subunits, R1 and R2. Subunit R1, the larger of the subunits, contains a catalytic site (substrate binding) and two allosteric effector-binding sites. dATP and ATP bind to the first allosteric site, the A-site, and serve as inhibitor and stimulator, respectively, while binding of dATP, ATP, dGTP and dTTP to the S-site determines substrate-binding preference (Brown and Reichard, 1969; Reichard et al., 2000). Allosteric regulation is accomplished by changes in the conformation of loop 2 which spans both the A- and S-sites, and which is determined by the molecule bound to the A-site (Reichard, 2010). Reviews and additional details can be found in (Eriksson et al., 1979; Reichard et al., 2000; Reichard, 2002; Crona et al., 2010; Logan, 2011).

Subunit R2, the smaller subunit, contains an amino acid residue that harbors the organic radical (Nordlund et al., 1990; Nordlund and Eklund, 1993). A long-range electron-coupled pathway connects the R2 radical to a cysteine in R1 (the thiyl radical) via hydrogen-bonded amino acid residue side chains (Nordlund et al., 1990; Stubbe et al., 2003; Kolberg et al., 2004). Binding of the R2 subunit to the R1 subunit involves the C-terminus residues of the R2 subunit interacting with a hydrophobic cleft in the R1 subunit and it has been suggested that oligomerization of R1 is a prerequisite (Climent et al., 1992; Rova et al., 1999; Uppsten et al., 2006).

The most current model for RNR in eukaryotes suggests a holoenzyme with eight subunits and is of the form $\alpha_6\beta_2$, where alpha stands for the R1 subunit and beta for R2 (Rofougaran et al., 2006). It has been proposed that in the absence of the effectors dATP, ATP, dGTP and dTTP, the R1 subunit is an inactive monomer; however, once dTTP or dGTP are bound to the S-site, an $\alpha_6\beta_2$ heterodimer is formed (Ingemarson and Thelander, 1996). With increasing dATP concentration (which induces enzyme inhibition), R1 monomers form dimmers and eventually inactive hexamers (formation of intermediate tetramers remains in question), while in the presence of the enzyme activator ATP, the holoenzyme adopts an $\alpha_6\beta_2$ conformation (Fairman et al., 2011).

**Paralogous copies of the R2 subunit**

Many eukaryote genomes encode two or more distinct copies of the small R2 subunit (Lundin et al., 2009). For example, humans have the R2 and p53R2 paralogs and *S. cerevisiae* the Y2 and Y4 paralogs. It is clear that these copies have different functional roles, be it de novo creation of deoxyribonucleotides, maintenance of the deoxyribonucleotide pool, mitochondrial DNA replication, or DNA damage repair (Elledge and Davis, 1990; Huang and Elledge, 1997; Tanaka et al., 2000; Lin et al., 2004; Bourdon et al., 2007). In such instances, a $\beta^6$ configuration is believed to contribute to the active holoenzyme, although $\beta^8$ and $\beta^6$ configurations have been reported (Wang et al., 1997; Nguyen et al., 1999; Chabes et al., 2000; Ge et al., 2001; Guittet et al., 2001; Voegtli et al., 2001; Perlstein et al., 2005; Ortigoso et al., 2006).

While most eukaryotes encode two R2 genes belonging to the typical eukaryotic clade $R2_{e1}$, apicomplexans encode one $R2_{e1}$ subunit and one $R2_{e2}$ subunit. *Toxoplasma* appears to be an exception, as so far two $R2_{e1}$-encoding genes have been identified but no $R2_{e2}$ has been found in its genome.

The conservation of functionally important R1 active site cysteines, and electron transfer cysteine and tyrosine residues, as well as the conservation of R2 residues involved

---

**Figure 1.** Unrooted phylogenetic relationships between the RNR class Ia and Ic subunits and the R2lox R2 homolog proteins (Munro et al., submitted). Class Ic includes the $R2_c$ proteins; however, this classification proved to be paraphyletic as it failed to include the clade of proteins now designated as $R2_{ab}$. Former class Ia proved to be polyphyletic, including the novel $R2_{ab}$ clade, which does not share a recent common ancestor with the monophyletic $R2_{e1}$ and $R2_{e2}$ clade. Reference to the clades $R2_c$, $R2_{ab}$, $R2_{e1}$, and $R2_{e2}$ now allows for unambiguous reference to these RNR subunits.
in iron binding, electron transport, free radical transfer, and the formation of the hydrophobic pocket around the radical, implies that both eukaryotic pathogens and their hosts utilize the same free radical chemistry to synthesize deoxyribonucleotides (Hofer et al., 1997; Roshick et al., 2000; Akiyoshi et al., 2002; Shao et al., 2006). As such, it would appear that targeting apicomplexan RNR by chemotherapeutic means might have an adverse effect on the human host. This is not necessarily so.

While prokaryotic and eukaryotic R1 and R2 subunits are highly conserved at, and around, the functionally important residues (Chakrabarti et al., 1993; Sjöberg, 1997; Roshick et al., 2000; Voegtl et al., 2001; Högbom et al., 2004; Högbom, 2010), there is considerable variation in the sequences at both the N- and C-termini (Ingram and Kinnaird, 1999). In particular, the eukaryotic orthodox R2 subunit, R2_e1, presents distinct differences between apicomplexans and mammals, including differences in key functional regions of the R2 protein; perhaps most notable are those differences between C-terminal sequences (Bracchi-Ricard et al., 2005). Furthermore, and more pertinent to this review, is the fact that the apicomplexan-specific R2 subunit, R2_e2, offers additional unique regions for drug-targeted inhibition (Munro et al., submitted). It is these differences between apicomplexan and mammalian host sequences that may best be exploited when designing chemotherapeutic drugs to specifically target the Apicomplexa, making RNR an appealing option as a drug target.

RNR has a long history as chemotherapeutical target

Much research has focused on the relationship between the class Ia R1 and R2 subunits in the context of human cancer. It has been hypothesized that normal or over-expression of R1 results in suppression of malignant cells (Yen, 2003) and Fan et al. (Fan et al., 1997) demonstrated that the R1 subunit had tumor-suppressing activity. Cao et al. (Cao et al., 2003) utilized a recombinant adenovirus that encoded and over-expressed the human R1 gene, which reduced proliferation of human colon adenocarcinoma cells, yet had no effect on normal cells. On the other hand, inhibition of the R2 subunit may have an antineoplastic effect, serving to inhibit and combat the development of cancer cells. Expression of R2 in conjunction with activated oncogenes impacts a cell's malignant potential (Fan et al., 1998; Desai et al., 2005). Overexpression of R2 is linked to increased drug resistance and increased invasive potential in cancer cells (Yen, 2003).

RNR inhibition has been applied to the control of viruses (Gaudreau et al., 1987; Moss et al., 1993; Bianchi et al., 1994; Szekeres et al., 1997; Robins, 1999), bacteria (Yang et al., 1997; Mdluli and Spigelman, 2006; Ericsson et al., 2010; Lou and Zhang, 2010; Torrents and Sjöberg, 2010), and certain cancers (Cory, 1988; Nocentini, 1996; Gwilt and Tracewell, 1998). Because inhibition of RNR ceases, or severely reduces, DNA replication, it has long been considered an ideal target for the control of pathogens. As such, inhibition of RNR to control eukaryotic pathogens has also been suggested (Dormeyer et al., 1997; Ekanem, 2001), in particular those belonging to Apicomplexa (Chakrabarti et al., 1993; Barker et al., 1996; Akiyoshi et al., 2002). In fact, RNR was included in a set of 57 “gold standard” essential enzymes with experimentally documented antimalarial effects (Huthmacher et al., 2010). These, and other studies, have resulted in a considerable array of approaches to inhibit RNR, which we briefly describe next.

Methods of RNR inhibition

RNR may be targeted at the translational or protein levels. RNR inhibitors are loosely categorized as those that prohibit the formation of an active holoenzyme or those that inhibit the translation of mRNA. The different types of RNR inhibitors are shown in the flowchart in Figure 2.

**Translation Inhibitors**
- RNAi
- Antisense oligonucleotides

**Dimerization Inhibitors**
- R2 C-terminus peptidomimetic sequences

**Catalytic and Allosteric Inhibitors**
- Nucleoside analogues (R1)
- Allosteric inhibitors (R1)
- Radical scavengers (R2)
- Iron chelators (R2)

---

**Figure 2.** Means of RNR inhibition. A flowchart showing the progression from mRNA to the formation of the holoenzyme (ovals) and how translation, dimerization and catalytic and allosteric inhibitors act along this process.
function of an already fully-formed holoenzyme (Cerqueira et al., 2007). At the translation level, synthesis of the enzyme subunits is blocked, while at the protein level, inhibitors may be employed to prevent the formation of the holoenzyme or inhibitors may be used to inactivate either the R1 or R2 subunit, or both (Cerqueira et al., 2005) (Figure 2). The use of ribozymes, single strand antisense oligonucleotides, and small interfering RNA (siRNA) can all be defined as anti-mRNA strategies, or subunit synthesis inhibitors (see (Aboul-Fadl, 2005) for a review of the implementation, optimization, and practical application of these methodologies), while dimerization, catalytic, and allosteric inhibitors focus on the inhibition of formed proteins.

Subunit synthesis inhibitors
Ribozymes
Ribozymes are catalytic RNA molecules with distinct three-dimensional configurations, which principally exhibit trans-cleavage properties. Ribozymes can be specifically designed to cleave a targeted RNA sequence, thereby inactivating gene transcripts (Haseloff and Gerlach, 1988; Norris et al., 2000; Citti and Rainaldi, 2005). Ribozymes and their applications have been extensively reviewed (Puerta-Fernández et al., 2003; Nayak and Kohli, 2005; Khan, 2006; Tedeschi et al., 2009). Ribozymes offer a productive avenue for gene therapy and have been designed for use against inborn metabolic disorders, viral infections, and cancer (Lewin and Hauswirth, 2001).

Both in vitro and in vivo studies demonstrated promising use of ribozymes to target the survivin gene, which when expressed, leads to cell proliferation, typical in most human carcinomas (Choi et al., 2003). Ribozymes designed to target mouse telomerase RNA were successfully administered and systemically expressed in vivo, and served to reduce the metastatic progression of B16_F10 murine melanoma metastases (Nosrati et al., 2004). In vitro targeting of Rhoc by ribozymes showed reduction of invasiveness in human breast cancer cells and thus demonstrated the utility of ribozymes in gene therapy (Lane et al., 2010). Similarly, ribozymes designed for targeting specific sites for cleavage in human telomerase RNA were demonstrated to be effective in arresting cell growth and induction of spontaneous cell apoptosis in colon cancer cells (Lu et al., 2011).

In the context of apicomplexan control, ribozymes were successfully used to reduce malarial viability up to 55% when targeting P. falci-parum-specific inserts in the carbamoyl-phosphate II synthetase gene (Flores et al., 1997). Accordingly, C-terminus insertions present in the R1 subunit enzyme of Plasmodium and Theileria offer sites uniquely different from those of their hosts, which may be specifically targeted by ribozymes (Ingram and Kinnaird, 1999).

RNA interference
RNA interference (RNAi) utilizes segments of double-stranded RNA to interfere with gene expression and it usually relies on the enzyme Dicer and the RNA-induced silencing complex (Scherr et al., 2004). Theoretically, the chemotherapeutic applicability of RNAi, and that of variants on the RNAi theme, is extensive; however, caution is advised as there are safety and specificity concerns (Grimm and Kay, 2007). There have been recent advances in the reduction of non-target effects and improved specificity in the silencing of target genes with chemically synthesized small interfering RNA (siRNA) (Lee and Sinko, 2006; Vaish et al., 2010), which typically utilizes much shorter lengths of double stranded RNA (20 to 25 bp). siRNA has proven useful in the suppression of pS3R2 expression, leading to the inhibition of tumor growth and an increase in sensitivity to anticancer drugs (Yanamoto et al., 2005).

Antisense RNA has been documented in Plasmodium (Militello et al., 2005) and there are numerous examples where RNAi has reportedly been successfully used to silence genes in Plasmodium (Kumar et al., 2002; Malhotra et al., 2002; McRoberts and McConkey, 2002; Mohmmad et al., 2003; Dasaradhi et al., 2005; Gissot et al., 2005; Crooke et al., 2006; Sunil et al., 2008; Tuteja et al., 2008; Srivilaijaroen et al., 2009). However, in stark contrast to these findings, are those where support for RNAi in Plasmodium is lacking. In fact, using both an experimental and bioinformatics approach, Baum et al. (Baum et al., 2009) suggested that Plasmodium lacks RNAi functionality and the conserved enzymes necessary for RNAi activity such as Dicer and Argonaute-like proteins, or their analogs. It has further been suggested that documented RNAi activity in Plasmodium may be the result of general toxicity to the introduced RNA, or an alternative (non-RNAi) antisense mechanism, and not the result of specific gene targeting by RNAi (Ullu et al., 2004). Additional authors have also questioned RNAi activity or the presence of a classical RNAi pathway in Apicomplexa, particularly in Plasmodium (Aravind et al., 2003; Blackman, 2003; Cerutti and Casas-Mollano, 2006; Xue et al., 2008). Further calling into question the utility of RNAi is the fact that these studies show down-regulation, but not the elimination, of gene function, and the degree to which protein expression is suppressed depends on a variety of factors (Brown and Catteruccia, 2006).

RNA antisense oligonucleotide inhibitors
Antisense oligonucleotides (AOs) are short (10 to 30 nucleotides), single strands of RNA or DNA that serve to inhibit gene expression. The sequence of an AO is complementary to a chosen target mRNA sequence, to which it will bind via canonical Watson-Crick base pairing. A variety of modifications to antisense oligonucleotides may be employed to prevent degradation, increase affinity and potency, and to reduce non-target toxicity (Chan et al., 2006; Sahu et al., 2007; Li et al., 2010). Further improvements come in terms of selective delivery systems for oligonucleotides (Ming et al., 2010). AOs may knockdown a target mRNA molecule by means of three distinct processes: (1) steric inhibition, where protein translation is inhibited once AOs are bound to the target mRNA, (2) the non-specific endonuclease, ribonuclease H (RNase H), may be activated and catalyze the cleavage of a DNA/mRNA duplex; alternatively, (3) pre-mRNA targeting, which includes inhibition of splicing, inhibition of the 5’ cap formation, or de-stabilization of the pre-mRNA, would inhibit mRNA maturation (Ho et al., 1996; Baker and Monia, 1999; Achenbach et al., 2003; Sahu et al., 2007).

Genes encoding both the large R1 RNR subunit and small R2 subunit have been targeted with antisense inhibition. Inhibition of expression of the herpes simplex virus was achieved using AOs to target the R1 translation initiation site (Au-relian and Smith, 2000). The R2-directed AO, GT1-2040, has shown promising selectivity and specificity in its...
antitumor activity against a variety of human cancers (Lee et al., 2003; Desai et al., 2005). AOs were designed to target both the R1 and R2 subunits in oropharyngeal KB cancer cells; however, only the targeted inhibition of R2 expression reduced enzyme activity and inhibited cell growth (Chen et al., 2000).

AOs have also proven to be effective against a variety of gene products in Plasmodium (Rapaport et al., 1992; Barker et al., 1998; Gardiner et al., 2000; Patankar et al., 2001; Kyes et al., 2002; Noonapakdee et al., 2003; Gunasekera et al., 2004). RNR activity was inhibited by targeting the region surrounding the translation initiation codon with AO phosphorothioates for the P. falciparum R2_e1 subunit, (Chakrabarti et al., 1993).

Protein inhibitors

Dimerization (polymerization) inhibitors

Dimerization inhibitors bind to one or more partners in a protein-protein interaction and hence prevent formation of the holoenzyme. In the case of RNR, they are small peptidomimetic sequences that mimic the R2 subunit C-terminal residues. As such, they competitively bind to the hydrophobic cleft in the R1 subunit, to the exclusion of R2, and prevent formation of the holoenzyme (Paradis et al., 1992; Gaudreau et al., 1990; Yang et al., 1990; Cosentino et al., 1991). It is the differences between host and parasite R2 C-terminal sequences that have so far lent themselves to specific targeting of a parasite's RNR.

RNR enzyme activity was inhibited in the Herpes simplex virus with the introduction of a peptide that corresponded to the C-terminus amino acid residues of the viral R2 subunit (Gaudreau et al., 1992; Liuzzi et al., 1994). In addition to the Herpes simplex virus, RNR dimerization inhibitors have been extensively studied in E. coli, hamster, mouse, yeast, and human cells (Cohen et al., 1986; Dutia et al., 1986; Climent et al., 1991; Cosentino et al., 1991; Fisher et al., 1993; Davis et al., 1994).

Likewise, in the case of apicomplexans, it is the difference in the C-terminal sequences of the R2 subunits between parasites and their hosts that may be best exploited by chemotherapeutic means (Chakrabarti et al., 1993; Fisher et al., 1993; Cerqueira et al., 2005). Peptidomimetic inhibitors based on the C-terminus of the small subunit have been proposed as a means of disrupting the formation of the RNR heterodimer complex in P. falciparum (Bracchi-Ricard et al., 2005). In fact, targeting malarial RNR activity of P. falciparum-infected erythrocytes was accomplished by use of synthetic peptidomimetic peptides, which prevented binding of the R1 and R2 subunits (Rubin et al., 1993).

Catalytic and allosteric inhibitors

Catalytic protein inhibitors target the active RNR holoenzyme and may act on either the R1 or R2 subunits, or both. Catalytic inhibitors may function by a variety of means, be it: (1) the destruction of the R2 subunit radical by radical scavengers or iron chelators, (2) inactivation of the R1 subunit active site, or (3) via substrate/nucleoside analogs, thus primarily acting on the R1 subunit. Allosteric inhibitors, which are also nucleoside analogs, target the R1 effector binding sites.

Radical scavengers such as hydroxyurea, irreversibly destroy the tyrosol radical of the R2 protein (Krakoff et al., 1968; Lepoiivre et al., 1991; Szekeres et al., 1997; Fontecave et al., 1998; Guittet et al., 1999). Hydroxyurea was shown to stop DNA synthesis in P. falciparum (Inselburg and Banyal, 1984). Improved control of malaria utilizing a combination therapy of erythropoietin and iron sulfate in conjunction with hydroxyurea has been hypothesized (Saei and Ahmadian, 2009). In contrast to the scavenging of radicals, iron chelators, which may destroy or prevent the formation of the radical (Nyholm et al., 1993; Richardson, 2002; Hodges et al., 2004; Whitnall et al., 2006), do not necessarily cause permanent inhibition. Iron chelators target cellular iron, leading to iron deprivation, which has been suggested to result in RNR inhibition (Pradines et al., 1996). Iron chelators have been demonstrated to be effective against the P. falciparum trophozoite and ring stages, which, unlike host cells, demonstrated a limited to irreversible loss of capacity for recovery after the chelator was removed (Lytton et al., 1994).

Substrate analogs are also referred to as suicide inhibitors and were recently reviewed (Perez et al., 2010). They are recognized as “normal” ribonucleotide substrates; however, their interaction with the holoenzyme's active site leads to inactivation of the enzyme. A case in point is inactivation of the R1 active site by use of nucleoside-diphosphate analogs, which bind and result in alkylation of the protein (Pereira et al., 2004; Pereira et al., 2006). Note that some substrate analogs such as gemcitabine and tezacitabine have additional inhibitory effects on the R2 subunit (Salowe et al., 1993; Shao et al., 2006).

As noted earlier, nucleoside-triphosphates bind to the allosteric effector sites and serve to either activate or inhibit the reduction of nucleoside diphosphates (Theland and Reichard, 1979). Allosteric effector analogs, which are typically nucleoside-triphosphate analogs, thus interact with the two R1 allosteric effector-binding sites, i.e. the A- and S-sites. dATP normally acts as an inhibitor; however, some deoxyadenosine analogues have an even more powerful affect due to their higher affinity for the R1 effector site (Cory and Mansell, 1975; Harrington and Spector, 1991; Jeha et al., 2004; Shao et al., 2006). Interference of the allosteric binding sites will have an influence on the activity and substrate binding affinity of the R1 subunit. While the structure of the allosteric sites in the R1 subunit may be similar between the RNR of an eukaryotic parasite and that of its host, there are usually unique substitutions between the two, which in turn may lead to differences in allosteric regulation. That is the case of the RNR of humans and of Trypanosoma brucei, the causative agent of sleeping sickness (Hofer et al., 1997). Chakrabarti et al. (Chakrabarti et al., 1993) suggested that differences in the N-terminus sequence of the R1 subunit of P. falciparum might indicate that it too utilizes a different allosteric regulation mechanism relative to humans. The authors suggested that conservation across other protozoans, in terms of the residues whose function it is to bind dTTP, indicates that they too may employ an allosteric regulation mechanism that differs from mammalian hosts. Such a difference has the potential to be exploited via suicide substrate inhibitors or nucleoside analogues (Ingram and Kinnaid, 1999).

Challenges to using RNR to control apicomplexan parasites

First and foremost, the function of the apicomplexan-specific R2_e2 subunit remains unknown and, in particular, whether
this subunit is essential to the formation of a functional RNR holoenzyme has yet to be determined. Munro et al. (submitted) have identified considerable variability among apicomplexans in the amino acid residue that purportedly harbors the free radical in the R2_e2 subunit, as well as in additional residues of functional importance. For example, the lack of conservation of one of the two phenylalanine residues in the C-terminus (Figure 3A) raises the possibility of a difference in the interaction between the R2_e2 and R1 subunits relative to that observed with R2_e1. Following the residue notation of Fisher et al. (Fisher et al., 1993), the C-terminal residues F^7 and F^7 appear to be particularly influential in dictating the interaction/bindng-specificity to subsites of the R1 subunit (Pellegrini et al., 2000; Pender et al., 2001). While the R2_e2 C-terminal residue equivalent to F^1 is maintained as phenylalanine and conserved across R2_e2, the residue equivalent to F^7 is instead substituted for isoleucine in all sequences sampled, save the cryptosporidians. This may, however, not be a concern; it has been established that F^7 need not be stringently conserved because the R1 subsite interacting with this R2 subunit position can accommodate a variety of hydrophobic residues (Pender et al., 2001; Gao et al., 2002; Cooperman, 2003). Also, Tyr370 in mouse R2 was determined to be essential in the radical transport pathway (Rova et al., 1999) and yet an equivalent to this residue is lacking in the apicomplexan-specific R2_e2 subunit.

There are further challenges to RNR chemotherapy. Two widely used anti-cancer RNR inhibitors, hydroxyurea and gemcitabine, are toxic to humans (Banach and Williams, 2003). Also, Tyr370 in mouse R2 was determined to be essential in the radical transport pathway (Rova et al., 1999) and yet an equivalent to this residue is lacking in the apicomplexan-specific R2_e2 subunit.

Bolstering support for use of RNR inhibition to control apicomplexan parasites

Since its discovery in 1961 (Reichard et al., 1961), ribonucleotide reductase has been featured in almost 5,500 publications in a variety of fields, such as biochemistry, molecular biology, oncology, cell biology, and chemistry. As such, there is a wealth of information regarding this protein. The literature is replete with studies of RNR inhibitor use in the control of cancer and of human pathogens. As detailed earlier, RNR has also received attention for its potential use as a target to control the apicomplexan parasites *Plasmodium*. RNR inhibitors have already been shown to have some antimalarial activity. Examples include RNA antisense oligonucleotide inhibitors (Chakrabarti et al., 1993) and radical scavengers such as the substituted/modified benzohydroxamic acids, specifically the vicinal dihydroxybenzohydroxamates (Holland et al., 1998).

Knowledge of protein structure and localization can greatly aid in the design of chemotherapeutic drugs. For example, it is essential to determine if the region being targeted is exposed on the protein’s surface, whether it is functional, and whether the residues surrounding the target region in its native conformation are similarly conserved (Durand et al., 2008). While the structure of the

**Figure 3.** RNR subunit R2 C-terminus. A) Sequence logo representation of human and apicomplexan R2_e1 and apicomplexan R2_e2 terminal residues. F^1 and F^7 are crucial phenylalanine residues that interact with the R1 subunit. The numbering of these residues follows (Fisher et al., 1993), while subsequent authors appear to have reversed the order (Pellegrini et al., 2000; Pender et al., 2001; Gao et al., 2002; Cooperman, 2003). Created with WebLogo 3 (Schneider and Stephens, 1990; Crooks et al., 2004). B) Terminal residues for human and apicomplexan R2_e1 and apicomplexan R2_e2 from which the logos were derived. Data derived from Munro et al. (submitted). *Plasmodium* and human sequences are underlined for comparative purposes (see text). Amino acid substitutions: . = semiconserved, : = conserved, * = identical.
apicomplexan-specific R2 subunit is not known, structure of the orthodox R2_e1 subunit from *Plasmodium vivax* (201Z) and *P. yoelii* (2P1I) are deposited on the RCSB Protein Data Bank (www.pdb.org; Berman et al., 2000).

Advances have been made in the production of apicomplexan recombinant proteins, a process that has been historically hampered by the A+T-biased nature of the plasmodial genome and uncommon codon usage (Weber, 1987; Anonymous, 2006; Brombacher 2006). However, see Vedadi et al. (Vedadi et al., 2007) who found *E. coli* to effectively produce apicomplexan proteins on a large-scale basis. Codon optimization (Hedfalk et al., 2008) and codon harmonization (Hillier et al., 2005; Angov et al., 2008; Chowdhury et al., 2009) have been used to improve expression of *Plasmodium* proteins in *E. coli*. Further advances have been made in the area of the use of a phylogenetically similar, or “pseudoparasite”, expression system (Fernández-Robledo and Vasta, 2010). Furthermore, in vitro protocols for *P. falciparum* are established, although in vivo animal models are based on the murine *Plasmodium* species *P. berghei, P. chabaudi, P. vinkei*, and *P. yoelii*, and not those that parasitize humans (Fidock et al., 2004).

To a large degree, the utility of RNR as an antimalarial chemotherapeutic target is dependent upon the timing of the protein’s expression. In mammals and yeast, the large subunit R1 has a half-life of 24 hours and is maintained at a constant level throughout a cell’s life cycle, while the small subunit R2 has a half-life of around 3 hours and its expression is restricted from the S-phase through to late mitosis, at which time it is rapidly degraded (Eriksson et al., 1984; Engström et al., 1985; Björklund et al., 1990; Elledge et al., 1992). The non-canonical human p53R2 protein is expressed during periods of DNA repair (Hákansson et al., 2006). In mammalian cells, it has been demonstrated that it is the presence or absence of the R2 protein that regulates RNR activity (Chabes and Thelander, 2000). In contrast to this, in *S. cerevisiae* it is the R1 subunit whose transcription is increased during S-phase, thus controlling RNR activity (Ortigosa et al., 2006). The utility of RNR inhibition in the control of certain cancers has relied in part on the fact that RNR is most needed when cells are rapidly proliferating, rendering cancerous cells particularly vulnerable to RNR inhibition (Smith and Karp, 2003). In *P. falciparum*, ribonucleotide biosynthesis begins as early as the ring and early trophozoite stage; however, deoxyribonucleotide metabolism occurs later, with both R1 and R2_e1 subunit transcription detected in the red blood cells (RBCs) at 10 hours post RBC infection and peaking 31 to 33 hours post-infection, which coincides with the late trophozoite/early schizont stage (Chakrabarti et al., 1993; Bozdech et al., 2003; Bozdech and Ginsburg, 2004). Comprehensive expression studies in *P. falciparum* show that the expression profile of R2_e2, albeit less intense, matches that of the other to subunits (Bozdech et al., 2003; Linás et al., 2006). The timing of RNR expression is not surprising, since it is during the intraerythrocytic stages that the malarial parasite undergoes logarithmic growth and requires RNR for DNA synthesis (Yeh and Altman, 2006). Additionally, expression of PFR4 (R2_e2) has been detected in the sporozoite and gametocyte stages of the parasite life cycle (Bracchi-Ricard et al., 2005), and in the case of *P. yoelii*, both R1 and R2_e1 transcripts were detected in the liver stage of infection (Nivez et al., 2000). These expression profiles are well-suited for chemotherapeutical intervention, since it is the intraerythrocytic stages of *Plasmodium* that cause clinical symptoms (Yeh and Altman, 2006). Additionally, human RBCs are not nucleated, thus precluding an alternative means for the parasite to exploit host RNR (Rubin et al., 1993).

The utility of RNR as an antimalarial/antipathogen target depends upon the ability to specifically target the pathogen’s RNR and not that of the host, as RNR is essential to both species. R1 and R2 subunits are highly conserved between prokaryotes and eukaryotes in the regions containing the functionally important residues (Chakrabarti et al., 1993; Roshick et al., 2000; Voegtl et al., 2001; Högbom et al., 2004; Högbom, 2010); however, they differ in the N- and C-terminal sequences (Ingram and Kinnaird, 1999). Novel RNRs are additional potential targets for new drugs, especially if they provide distinct differences between host and parasite sequences. The necessity for target specificity to avoid side, or non-target, effects in humans cannot be overstated. The use of antisense oligonucleotides in the control of some cancers has shown that the drugs in question have favorable toxicity profiles, in part because of their ability to specifically target segments of RNA (Davies et al., 2003). The unorthodox R2_e2 apicomplexan subunit provides a distinct and additional opportunity for specific drug targeting. One example is the C-terminus of the R2 subunit, which differs considerably between the human R2_e1 subunits and both the R2_e1 and R2_e2 apicomplexan subunits (Figures 3A and 3B). This difference in the C-terminal sequences of the R2 subunits between apicomplexans and their hosts can be ideally targeted by chemotherapeutic means (Rubin et al., 1993; Fisher et al., 1995; Ingram and Kinnaird, 1999).

Finally, it is worth noting that *Plasmodium*-infected erythrocytes demonstrate an increase in cell membrane permeability (Baumeister et al., 2011). In vitro uptake of small pieces of RNA becomes less of an issue as RBCs that are infected with malaria exhibit an enhanced and selective uptake of such molecules in comparison to non-infected RBCs (Rapaport et al., 1992), a difference attributed to the presence of a parasitophorous ducts in infected RBCs (Pouvelle et al., 1991).

**Summary**

Extensive research has already established that RNR has potential as an antimalarial drug. What is particularly appealing about RNR inhibition as a means of controlling Apicomplexa is the potential control of not one, but two, copies of the R2 subunit, both of which are distinct from that of the host. Additionally, in the case of the malarial parasite *Plasmodium*, RNR expression occurs from the sporozoite through the gametocyte life cycle stage, offering multiple opportunities for chemotherapeutic targeting. This may well be the case for other Apicomplexa parasites that undergo rapid clonal expansion in the host.

Of the eight established methods of RNR inhibition discussed, RNAs seems the least promising in terms of controlling apicomplexan parasites as the necessary enzymes appear to be lacking. Ribozyme approaches have been successfully implemented in *Plasmodium*; however their use against RNR has not yet been demonstrated. In contrast, substrate analogs and allosteric effector analogs have been effectively used to inhibit RNR, but their use...
in *Plasmodium* has yet to be demonstrated. Antisense oligonucleotide inhibitors, dimerization inhibitors, radical scavengers, and iron chelators have all been successfully used to target RNR in *Plasmodium*. Ribozymes, antisense oligonucleotide inhibitors, and dimerization inhibitors show the most promise in terms of future anti-apicomplexan drug development. On the other hand, resistance to some radical scavengers and iron chelators has been established, they can not be used to selectively target a peptide, and the fact that the effects of iron chelators are generally reversible, makes them less appealing prospects.

The difference in sequence similarity between the parasite and human R2_e1 subunits is considerable, and the difference is even more accentuated when the parasite's R2_e2 subunit is considered (Munro et al., submitted). Assuming that this protein is essential for RNR function, ribozymes, antisense oligonucleotide inhibitors, and dimerization inhibitors can all be optimized for target specificity and thus used to take advantage of the unique R2_e2 protein. The fact that the R2_e2 gene is present in several apicomplexan genera, each of which contains species of significant health and socio-economic impact, holds promise that the results of any research would be translatable to several very important diseases.

**References**


Huang, M., and Elledge, S.J. (1997). Identification of
Holland, K.P., Elford, H.L., Bracchi-Ricard, V., Annis, C.G.,
Högbom, M., Stenmark, P., Voevodskaya, N., McClarty, G.,
Högbom, M. (2010). The manganese/iron-carboxylate
Hofer, A., Schmidt, P.P., Gräslund, A., and Thelander, L.
Ho, S.P., Britton, D.H., Stone, B.A., Behrens, D.L., Leffet,
Hedfalk, K., Pettersson, N., Oberg, F., Hohmann, S., and


Further Reading

- **MALDI-TOF Mass Spectrometry in Microbiology**
  Edited by: M Kostrzewa, S Schubert (2016)
  www.caister.com/malditof

- **Aspergillus and Penicillium in the Post-genomic Era**
  Edited by: RP Vries, IB Gelber, MR Andersen (2016)
  www.caister.com/aspergillus2

- **The Bacteriocins: Current Knowledge and Future Prospects**
  Edited by: RL Dorfl, SM Roy, MA Rilety (2016)
  www.caister.com/bacteriocins

- **Omics in Plant Disease Resistance**
  Edited by: V Bhadauria (2016)
  www.caister.com/opdr

- **Acidophiles: Life in Extremely Acidic Environments**
  Edited by: R Quatrini, DB Johnson (2016)
  www.caister.com/acidophiles

- **Climate Change and Microbial Ecology: Current Research and Future Trends**
  Edited by: J Marxsen (2016)
  www.caister.com/climate

- **Biofilms in Bioremediation: Current Research and Emerging Technologies**
  Edited by: G Lear (2016)
  www.caister.com/bioremem

- **Microalgae: Current Research and Applications**
  Edited by: MN Tsaiologlou (2016)
  www.caister.com/microalgae

- **Gas Plasma Sterilization in Microbiology: Theory, Applications, Pitfalls and New Perspectives**
  Edited by: H Shintani, A Sakudo (2016)
  www.caister.com/gasplasma

- **Virus Evolution: Current Research and Future Directions**
  Edited by: SC Weaver, M Denison, M Roossinck, et al. (2016)
  www.caister.com/virusvol

- **Arboviruses: Molecular Biology, Evolution and Control**
  Edited by: N Vasiliakos, DJ Gubler (2016)
  www.caister.com/arbo

- **Shigella: Molecular and Cellular Biology**
  Edited by: WD Picking, WL Picking (2016)
  www.caister.com/shigella

- **Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment**
  Edited by: AM Romani, H Guasch, MD Balaguer (2016)
  www.caister.com/aquaticbiofilms

- **Alphaviruses: Current Biology**
  Edited by: S Mahalingam, L Herrero, B Herring (2016)
  www.caister.com/alpha

- **Thermophilic Microorganisms**
  Edited by: F Li (2015)
  www.caister.com/thermophile

- **Flow Cytometry in Microbiology: Technology and Applications**
  Edited by: MG Wilkinson (2015)
  www.caister.com/flow

- **Probiotics and Prebiotics: Current Research and Future Trends**
  Edited by: K Venema, AP Carmon (2015)
  www.caister.com/probiotics

- **Epigenetics: Current Research and Emerging Trends**
  Edited by: BP Chadwick (2015)
  www.caister.com/epigenetics2015

- **Corynebacterium glutamicum: From Systems Biology to Biotechnological Applications**
  Edited by: A Burbkovski (2015)
  www.caister.com/cory2

- **Advanced Vaccine Research Methods for the Decade of Vaccines**
  Edited by: F Bagnoli, R Rappuoli (2015)
  www.caister.com/vaccines

- **Antifungals: From Genomics to Resistance and the Development of Novel Agents**
  Edited by: AT Coste, P Vandeputte (2015)
  www.caister.com/antifungals

- **Bacteria-Plant Interactions: Advanced Research and Future Trends**
  www.caister.com/bacteria-plant

- **Aeromonas**
  Edited by: J Graf (2015)
  www.caister.com/aeromonas

- **Antibiotics: Current Innovations and Future Trends**
  Edited by: S Sánchez, AL Demain (2015)
  www.caister.com/antibiotics

- **Leishmania: Current Biology and Control**
  Edited by: S Adak, R Datta (2015)
  www.caister.com/leish2

- **Acanthamoeba: Biology and Pathogenesis (2nd edition)**
  Author: NA Khan (2015)
  www.caister.com/acan2

- **Microarrays: Current Technology, Innovations and Applications**
  Edited by: Z He (2014)
  www.caister.com/microarrays2

- **Metagenomics of the Microbial Nitrogen Cycle: Theory, Methods and Applications**
  Edited by: D Marco (2014)
  www.caister.com/n2

Order from caister.com/order