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A ribonucleotide reductase inhibitor with deoxyribonucleoside-reversible cytotoxicity

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ABSTRACT

Ribonucleotide Reductase (RNR) is the sole enzyme that catalyzes the reduction of ribonucleotides into deoxyribonucleotides. Even though RNR is a recognized target for antiproliferative molecules, and the main target of the approved drug hydroxyurea, few new leads targeted to this enzyme have been developed. We have evaluated a recently identified set of RNR inhibitors with respect to inhibition of the human enzyme and cellular toxicity. One compound, NSC73735, is particularly interesting; it is specific for leukemia cells and is the first identified compound that hinders oligomerization of the mammalian large RNR subunit. Similar to hydroxyurea, it caused a disruption of the cell cycle distribution of cultured HL-60 cells. In contrast to hydroxyurea, the disruption was reversible, indicating higher specificity. NSC73735 thus defines a potential lead candidate for RNR-targeted anticancer drugs, as well as a chemical probe with better selectivity for RNR inhibition than hydroxyurea.
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1. Introduction

Ribonucleotide Reductase (RNR) is an essential enzyme present in all free living organisms as well as in some double stranded DNA viruses. RNR catalyzes the reduction of ribonucleoside diphosphates into deoxyribonucleoside diphosphates and is rate-limiting for dNTP synthesis. The mammalian enzyme consists of two subunits, one large substrate binding subunit denoted R1 (α, gene id RRM1), and one small radical generating subunit denoted R2 (β, gene id RRM2). There is also an alternative form of the small subunit, the p53R2 isoform (gene id RRM2B). RNR activity is mainly present in actively dividing cells that need to synthesize DNA, whereas terminally differentiated cells that have stopped dividing has much lower RNR activity. The activity of RNR is regulated with respect to the cell cycle with a peak at S-phase, which is achieved by a distinct increase in the expression level of the R2 subunit (Chabes and Thelander, 2000; Eriksson et al., 1984). In contrast, the level of the R1 subunit is more constant throughout the cell cycle (Engström et al., 1985). In addition to regulation at the expression level, the RNR activity is controlled by a sophisticated allosteric mechanism to keep the dNTP pool in balance (Hofer et al., 2012), as unbalanced dNTP pools are mutagenic (Mathews,

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2006). The relative ratios between the dNTPs are controlled by the allosteric specificity site (the s-site), where binding of ATP/dATP induces CDP/UDP reduction and dTTP and dGTP induce GDP and ADP, respectively (Hofer et al., 2012). The human RNR has an additional allosteric activity site (the a-site), an N-terminal ATP cone domain (Aravind et al., 2000), which controls overall activity and hence the absolute dNTP concentrations; when ATP is bound to the a-site the enzyme is active, and when dATP is bound the enzyme activity is shut off (Hofer et al., 2012). In all studied eukaryotic RNRs, binding of ATP or dATP to the a-site results in hexamerization of the R1 subunit into α_6 complexes (Ando et al., 2016; Crona et al., 2013; Fairman et al., 2011; Kashlan et al., 2002; Rofougaran et al., 2006). The dATP-inhibited α_6 complex forms a ring structure that binds the R2 dimer (β_2) in such a way that the crucial electron transport chain between the R1 and R2 subunits is disrupted (Fairman et al., 2011). Less is in known about the ATP-induced α_6 complex, but mutagenesis studies of the *Saccharomyces cerevisiae* RNR indicated that it is structurally different from the dATP-induced complex (Fairman et al., 2011). Furthermore, a recent study of the human RNR indicates that the ATP-induced α_6 complex is less stable than the dATP complex as the ATP-induced complex changes conformation upon β_2 binding, and higher order filamentous structures were also seen at high ATP concentrations (Ando et al., 2016).

The alternative small subunit, p53R2, is induced by p53 and is therefore associated with DNA repair (Guittet et al., 2001; Tanaka et al., 2000) and is crucial for mitochondrial DNA synthesis (Bourdon et al., 2007; Guittet et al., 2001; Pontarin et al., 2012; Tanaka et al., 2000).

All three RNR subunits, but particularly R2, have been found to be overexpressed in many cancer tissues and in some instances the expression levels of RNR subunits can serve as prognostic markers (Aye et al., 2015; Hsu et al., 2011; Jordheim et al., 2011; Kidd et al., 2005; Liu et al., 2011, 2013, 2006; Xi et al., 2008; Zheng et al., 2007). RNR is also pharmaceutically relevant as the main target of the anticancer drugs hydroxyurea (HU) and gemcitabine. In addition, RNR has been the focus of many new drug discovery efforts, both in the field of cancer and antibiotics, *e.g.* in clinical trials involving RNR gene silencing (Davis et al., 2010; Jin et al., 2010; Sridhar et al., 2011), in clinical studies of triapine as an anticancer drug targeted to RNR (Nutting et al., 2009; Traynor et al., 2010), and as a target for novel antibiotics (Tholander and Sjöberg, 2012). In a recent study, Faiz Ahmad et al. identified a set of bioactive RNR inhibitors via *in silico* screening (Ahmad et al., 2015). In several cases, drugs and drug candidates affects the oligomer state of RNR, for example clofarabine, cladribine, fludarabine and phthalimide could increase the amount of α_6 complex (Ahmad et al., 2015; Aye and Stubbe, 2011; Wisitpitthaya et al., 2016) and gemcitabine was found to induce $\alpha_6\beta_6$ complex formation (Wang et al., 2009). Undoubtedly, RNR defines an interesting drug target with proven clinical relevance.

Tholander & Sjöberg previously identified a set of compounds active as inhibitors of the *Pseudomonas aeruginosa* class I RNR (Tholander and Sjöberg, 2012), of which only a few also possessed activity against *P. aeruginosa* cells. In contrast, according to available data for bioactivity in the NCI-60 cell line panel, most of the compounds are active in various human tumor cell lines. Here we show that these compounds are potent inhibitors of human RNR. One of the compounds, NSC73735, has particularly interesting properties suggesting a potential for lead development or usefulness as a chemical probe. Data mining shows that NSC73735 exhibit a bioactivity profile that correlates with that of hydroxyurea, and also with the expression of RNR genes. A combination of thermal shift experiments and quaternary structure determinations indicate that NSC73735 binds to the R1 subunit and interferes with oligomerization. In addition flow cytometry experiments show that the cytotoxicity of the compound causes a decrease in dNTP levels and a disruption of the cell cycle that is reversible by addition of deoxyribonucleosides, which together suggest that RNR is a cellular target.

2. Materials and methods

2.1. Materials

HU, deoxyribonucleosides, Triton X-100, propidium iodide (PI), and all standard chemicals were from Sigma–Aldrich, Sweden. Glutamine and RPMI medium were from Lonza, RNase A from Macherey-Nagel, and fetal calf serum (FCS) from Life Technologies. NSC73735 (redoxal, 2-[(4-{4-[(2-carboxyphenyl)amino]-3-methoxyphenyl}-2-methoxyphenyl)amino]benzoic acid) were from the Drug synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, USA, and previously found to inhibit a bacterial Ribonucleotide Reductase by a PCR-based HTS method (Tholander and Sjöberg, 2012). Custom gene synthesis and subcloning into expression vectors were performed by Epoch Lifescience Inc., USA.

2.2. Cloning, protein expression and protein purification

The coding sequences of human R1 and R2 were codon optimized, custom synthesized and cloned into pET24 vectors. A 6×His-tag was added to the N-termini of all protein constructs. The proteins were expressed in *Escherichia coli* BL21 (DE3) in LB media (for R2) or in TB media (for R1). Overnight pre-cultures were diluted 20 times into 1.5 L of fresh media and cultivated at 37 °C until the OD_{600} reached approximately 0.5, at which point IPTG was added (250 μM for R2 and 50 μM for R1) to induce protein expression. Cultivation was continued at 15 °C overnight and the cultures then harvested by centrifugation at 4000 × g for 15 min. The collected cells were then resuspended in a buffer solution (50 mM Tris, 500 mM NaCl, 10 mM Imidazole, pH 7.5, and with the addition of complete protease inhibitor tablets from Roche according to the manufacturer's instruction) and lysed by sonication. Cell debris was removed by centrifugation at 10.000 × g for 15 min. All extraction steps were performed with the samples kept chilled.

Extracts of R2 were filtered (0.45 nm) and loaded onto a Ni-chelate affinity resin (Qiagen) using an automated liquid chromatography system. Weakly bound protein was washed away (buffer: 20 mM Tris–HCl, 500 mM NaCl, and 10 mM Imidazole, pH 7.5) and the protein eluted with a buffer containing 20 mM Tris–HCl, 500 mM NaCl and 500 mM Imidazole, pH 7.5).

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