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Molecular Strategies of Deoxynucleotide Triphosphate Supply Inhibition Used in the Treatment of Gynecologic Malignancies

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Abstract

Chemotherapies targeting deoxynucleotide triphosphate synthesis are of high medical interest in the treatment of gynecologic malignancies. In this article, we focus on targeted inhibitors of ribonucleotide reductase, an enzyme in charge of ribonucleotide reduction to their corresponding deoxyribonucleotide to be used as the building blocks of DNA. We also discuss human clinical trials have utilized ribonucleotide reductase subunit-specific inhibitors, particularly trials for women with cervical cancer.

Introduction

A unifying treatment stratagem for women with advanced staged gynecologic malignancies remains elusive. The American Cancer Society estimates 88,080 American women will be newly diagnosed with a gynecologic malignancy in 2011, with 29,590 (34%) fatalities attributed to gynecologic malignant disease progression [1]. Gynecologic cancers also pose a great health concern among women worldwide, with as many as 1.1 million newly diagnosed gynecologic cancers recorded by international cancer registries [2]. Of these new cases, cervical cancer will develop in an estimated 530,000 women this year worldwide [2]. In many less developed nations, cervical cancer will be responsible for a major proportion of human life years lost to cancer and cancer-related mortality [3].

In some respects, disrupted cancer cell molecular pathways can be thought of as unifying. In this review, we discuss current concepts of disrupted regulation of ribonucleotide reductase (RR) and tested treatments aimed at therapeutically modifying RR-mediated dNTP supply needed for repair of DNA damage in human gynecologic cancers. Much of this work has been the result of human translational and scientific studies conducted in cervical cancer clinical trials.

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Ribonucleotide reductase and DNA damage

RR catalyzes the substitution of the 2'-hydroxyl group of ribonucleoside diphosphate (rNDP) with hydrogen, resulting in a 2'-deoxyribonucleoside diphosphate (dNDP). Its minimal class Ia enzymatic form is an $\alpha_2\beta_2$ heterotetramer, but its higher order ($\alpha_6\beta_6$ or $\alpha_6\beta_2$) forms also exist, both as active and inactive enzymes [4]. RR functions in cells as a rate-limiting enzyme of dNTP supply. Its large subunit α (M1) contains: 1) a catalytic site where rNDP substrates bind; 2) a specificity site where adenosine triphosphate (ATP) or deoxyadenosine triphosphate (dATP) binding implies uridine diphosphate (UDP) or cytidine diphosphate (CDP) reduction, deoxythymidine triphosphate (dTTP) binding implies guanosine diphosphate (GDP) reduction, and deoxyguanosine triphosphate (dGTP) binding implies adenosine diphosphate (ADP) reduction; and 3) an activity site that controls by allosteric regulation overall RR activity by being either ATP (activation) or dATP (inhibition) occupied.

The small subunit of RR, β (M2 or p53R2 [M2b]), is functionally much simpler than the large subunit in that its job is only to stably house a free radical that shuttles to and from the catalytic site with each catalytic cycle [5]. The human genes that encode for M1 (chromosome 11), M2 (chromosome 2), and M2b (chromosome 8) are located on different chromosomes. Human tissue immunohistochemistry has confirmed that RR subunits are highly over-expressed in gynecologic cancers, especially cervical cancers [6].

The putative catalytic mechanism for RR involves proton-coupled electron transfer from the M2 or M2b diferric tyrosyl radical ($\bullet$ Y122) through an electron and proton tunnel to the catalytic site on M1 (cysteine C439). An eloquent amino acid radical intermediate chain coordinates radical transport for catalysis a lengthy 35Å away. In *Escherichia coli*, the path is believed to be one of $\bullet$ Y122/W48/Y356 in R2 followed by Y731/Y730/C439 in R1 [7]. Rigorous amino acid exchange research in *E. coli* maintains that protons at amino acids Y122 and Y356 in the small subunit (R2) move off the pathway as the radical transits. Thus overall, RR couples radical transport across long distances to short proton transfer hops at tyrosine and cysteine amino acid side chains [7]. It is believed that substrate turnover in the catalytic site of M1 is initiated by a thiyl radical (C439) that abstracts a hydrogen from the ribose ring of rNDP [7]. With this knowledge, mechanisms of some of the RR anticancer drugs can be better understood.

It is now very well-accepted that dNTP demand is met by two coordinated molecular pathways of dNTP supply: 1) new reduction of rNDPs to dNDPs via RR; and 2) salvage of deoxynucleosides (dNs) to dNMPs and ultimately dNDPs (Figure 1, [8–12]). Synthesis of dNDPs is very tightly regulated because disproportions in dNTP pools result in disruptive genotoxic stress [13]. There are at least four recognized intracellular levels of control in dNDP production, namely allosteric, transcriptional, protein-protein binding, and protein degradation regulation. Each is germane and discussed briefly here.

RR activity is regulated by an adenosine triphosphate (ATP) – deoxyadenosine triphosphate (dATP) binding to the M1 activity site [4]. ATP activates RR and dATP inactivates RR. Balanced generation of dNDPs for DNA replication or repair occurs via a feedback cascade of allosteric effectors at an M1 specificity site [14]. In the feedback cascade, thymidylate

synthase provides the sole intracellular source of *de novo* deoxythymidine monophosphates, and thus, 5-fluoracil blockade of thymidylate synthase modulates allosteric regulation of RR.

Transcriptional and degradation regulation of the M1, M2, and M2b proteins are unique to each RR subunit. Protein M1 is a long-lived protein. It can be found in low quantities in all cell cycle phases [5]. Protein M2 has a KEN-box sequence recognized by the Cdh1•anaphase-promoting complex that degrades the protein in late mitosis [15,16]. Protein M2b lacks the KEN-box and thus can be detected in low quantities in all cell cycle phases. M2b transcription is p53 dependent [15–17].

A fourth means of regulation of RR is a putative bond between p53 protein and M2b that upsets formation of an M1-M2b complex through physical interference mitosis [18]. It is presumed that the phosphorylated form of p53 changes conformation, releasing M2b to freely associate with M1 for RR activity. It is speculated that sources of dNDPs after DNA-damaging insults such as ionizing radiation include ribonucleotide reduction, first by a M1-M2b mediated process, and subsequently by a M1-M2 mechanism [9,19].

While large quantities of dNDPs are required in proliferating cells for genomic DNA duplication, meeting dNTP demand in cells after DNA-damaging therapies may be more urgent. Quantities of dNTPs demanded depend upon the type of DNA damage. For instance, cisplatin DNA adducts may demand fewer dNTPs than ionizing radiation that produces thousands of base disruptions (e.g., 8-oxoguanine) in intracellular RNA, genomic DNA, and mitochondrial DNA and ~1000 single- and ~40 double-strand DNA breaks, all of which require dNTPs (and thus, dNDPs). Ionizing radiation may necessitate large amounts of dNTPs that, to meet demand, supply must be generated not only from damage inflicted/targeted cancer cells, but also from non-targeted cells. Nature achieves this by using not only the *de novo* RR enzyme reduction of rNDPs [5,20] but also a complementary deoxynucleoside salvage system (12) that is rate limited and thus controlled by deoxynucleoside kinases [21]. Cells must coordinate the amount of dNTP flux entering through the *de novo* and salvage pathways such that their sum total equals the amount demanded. Thus, cells may draw on total body deoxynucleoside reserves of the plasma to meet the dNTP supply demands of rebuilding damaged genomic DNA and mitochondrial DNA.

Disrupted *de novo* nucleotide molecular pathways in cervical cancer

To sharpen our thinking of *de novo* RR-regulated molecular pathways in cancer, it is prudent to explore the manners in which cancer cells have disrupted their dNTP supply systems. Here, we focus on molecular events linked to human papillomavirus (HPV) pathophysiology. HPVs are non-enveloped double-stranded closed circular DNA viruses proliferating in cutaneous and mucosal epithelium of the female and male anogenital tract [22]. The circular HPV genome has eight open genome reading frames to encode six early proteins (E1, E2, E4-E7) and two late proteins (L1, L2) for viral replication in cells [22]. When perturbed to replicate, HPV disrupt host cell molecular pathways usually responsible for cell cycle termination. This allows the virus to hijack host cell enzymes needed for synthesis of DNA. To accomplish this task, HPV-E6 [23,24] binds p53, ubiquitinates it to

cause its degradation, and thus essentially removes signals that block cells in the G1/S cell cycle restriction checkpoint [25–27]. Also, the encoded protein HPV-E7 degrades hypophosphorylated RB through a proteasome-dependent pathway [28] that also promotes unchecked E2F activation [29]; this is critical because E2F transcription factors turn on S-phase proteins responsible for DNA replication. Through activation of telomerase [30], HPV extends cell life and thus increases HPV-infected cell numbers. Consequential effects of HPV viral proteins overriding cell checkpoints include genomic instability and predisposition to neoplasia and oncogenic phenotypes [22]. It is tempting to speculate that throughout its evolution HPV has targeted at least transcriptional and protein-protein regulation in cervical cancers to favor unrestricted dNDP production by RR (and perhaps its M1-M2 form specifically).

The broadest clinical effort to improve survival for women with gynecologic cancers has been directed toward radiation coordinated with co-administered chemotherapy. A radiochemotherapy approach attacks targeted primary cancer cells by ionizing radiation and simultaneously sterilizes non-primary cancer cells through systemic effects of chemotherapy. Staying with this theme, we discuss tested treatments aimed at therapeutically modifying RR activity by highlighting clinical benefits of pelvic disease control and progression-free survival for each therapeutic strategy.

Anticancer therapies directed at the tyrosyl radical of ribonucleotide reductase

Pharmacological blockade of either M2 or M2b (p53R2) renders RR inactive, with timing of the inhibitor after DNA damage being important to the extent that it can prolong DNA damage repair [9,10]. The oral anticancer agents hydroxyurea (HU) and 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) both quench the M2/ M2b diferric tyrosyl radical ($\bullet$ Y122) such that the electron and proton tunnel to the M1 active site (cysteine C439) is irrevocably disrupted. HU blocks RR activity by its one electron reductive state [31] and 3-AP disrupts RR activity by iron chelation of the differic moieties [32].

One of the first anticancer strategies targeting the tyrosyl radical of RR was pairing HU and ionizing radiation by the investigators of the Gynecologic Oncology Group (GOG-004). In this randomized trial, women with International Federation of Gynecology and Obstetrics (FIGO) stage III_B-IV_A cervical cancer received conventional daily pelvic radiation and placebo or HU (80mg/kg, oral twice weekly). Pelvic disease control at 3-years was 68% after HU-radiation, as compared to 49% after radiation alone [33]. 3-year progression-free survival was 30% after HU-radiation versus 20% after radiation alone. Profound hematopoietic toxicity due to HU was treatment-limiting on this trial.

Soon afterward, scientific and clinical investigators better understood the pharmacokinetics of cisplatin and its resultant course of DNA damage mediated through DNA adducts. In an effort to provide ‘maximum’ DNA damage for RR to supply dNDPs, cisplatin radiochemotherapy became a standard therapeutic pairing in multiple clinical trials [34–36]. Here, cells engendered damage in the form of immediate RNA, nuclear DNA, and mitochondrial DNA base damage from ionizing radiation and sustained protracted DNA damage by cisplatin.

One clinical trial randomized 526 eligible women with stage II_B-IV_A cervical cancer to one of three radiochemotherapy regimens: daily pelvic radiation plus either weekly cisplatin (40 mg/m²); or day 1 or day 29 cisplatin (50 mg/m²) plus 96-hour infusion 5-FU (4g) plus twice weekly oral hydroxyurea (2g/m²); or twice weekly oral hydroxyurea (3g/m²) [35]. Rates for control of cervical cancer disease in the pelvis were improved for cisplatin-radiation regimens (80%) as compared to HU-radiation treatment (70%). At 36 months, radiochemotherapy by cisplatin (63%) or by cisplatin, 5-FU, and hydroxyurea (62%) were superior to radiation-hydroxyurea (42%) for progression-free survival. Long-term data show that these outcomes remain durable [37].

The relatively new potent RR inhibitor 3-AP has also been paired with cisplatin-based radiochemotherapy in stage I_{B2}-IV_B cervical cancer. Here, 3-AP was timed immediately after ionizing radiation to have maximal blockade effect on dNTP supply when dNTP demand was highest [6]. A phase 1 clinical trial conducted in 10 women with stage I_{B2}-IV_B cervical cancer gave intravenous 3-AP (25mg/m², three times weekly) plus once weekly cisplatin (40mg/m²) during daily radiation [6]. The regimen was safe, and through a median follow-up of 36 months, none of the enrolled women (100%) have had disease relapse in the pelvis. 3-year progression-free survival is estimated at 90%, with no women dying from cervical cancer disease. A phase 2 study of this combination in 25 additional women with stage I_{B2}-IV_B cervical cancer has finished accrual with early indications of similar clinical benefit.

Anticancer therapies directed at allosteric sites of ribonucleotide reductase in gynecologic cancers

One clinically tested approach used daily pelvic ionizing radiation and cisplatin as DNA-damaging agents and 5FU obstructing thymidylate synthase, in due course upsetting the dNTP feedback that determines the selectivity site of RR. This clinical trial questioned whether a radiation-cisplatin-5FU combination would better eradicate primary cervical cancer, and if a cisplatin-5FU combination would better sterilize occult systemic disease, than a radiation-hydroxyurea combination. Between 1986 and 1990, a phase 3 trial was done randomizing 368 eligible women with stage II_B-IV_A to radiation with oral hydroxyurea versus radiation potentiated with cisplatin and 5-FU [34]. Control of disease in the pelvis was 70% after HU-radiation and 75% after cisplatin-5FU-radiation. Moreover, a 3-year progression-free survival benefit conferred by cisplatin-5FU-radiation (60%), as compared to HU-radiation (50%) ($P=0.03$).

Between 1990 and 1997, another clinical trial was done to randomize 388 women with stage II_B-IV_A cervical cancers to receive either pelvic plus para-aortic radiation ($n = 193$) or pelvic radiation ($n = 195$) plus two cycles of day 1 and day 22 cisplatin (75 mg/m²) plus 5-FU (4g over 96-hour infusion) [36]. Long-term outcome data have been updated [38]. 8-year progression-free survivals were 61% for radiochemotherapy and 36% for pelvic plus para-aortic radiation, reducing the hazard for disease progression by 51% ($P < 0.0001$).

Anticancer therapies directed at catalytic site of ribonucleotide reductase

Results from prospective evaluation of co-administered radiosensitizing chemotherapies during cisplatin radiochemotherapy are now coming to fruition. A randomized comparison of gemcitabine (which upon diphosphorylation covalently binds as a CDP substrate analog to the catalytic site of RR, thereby annihilating both M1 subunits of an M1₂ dimer [39]) added to cisplatin radiochemotherapy plus adjuvant cisplatin and gemcitabine versus cisplatin radiochemotherapy has been reported [40]. Eligible treatment-naïve women (stage stage II_B-IV_A cervical cancer) were assigned to once weekly cisplatin (40 mg/m²) plus gemcitabine (125 mg/m²) for 6 weeks during pelvic radiation followed by two adjuvant 21-day cycles of cisplatin (50 mg/m², day 1) plus gemcitabine (1g mg/m², day 1 and 8). Or, eligible women were assigned to once weekly cisplatin (40 mg/m²) and the same radiation. 3-year progression-free survivals were 74% after gemcitabine plus cisplatin radiochemotherapy and 65% after cisplatin radiochemotherapy. A significant reduced hazard for cervical cancer relapse or death of 32% ($P=0.023$) was observed. Controversy exists regarding a gemcitabine plus cisplatin radiochemotherapy clinical benefit, as treatment-related grade 3 or 4 gastrointestinal (diarrhea: 18% v. 5%; emesis: 8% v. 3%) and hematological (72% v. 24%) toxicities have been high [40]. In a GOG effort, this regimen was deemed too toxic [41]. In this trial, two scientific questions were posed. First, the hypothesis of adding radiosensitizing chemotherapy (gemcitabine) with intrinsic and additive anticancer effect was tested. Second, whether adjuvant post-radiation gemcitabine plus cisplatin chemotherapy was tested for consolidative anticancer effect. As such, it remains unclear whether the gemcitabine plus cisplatin treatments in the study arm simply delayed relapse or cured more patients [42]. Also, no data were given describing how many women were lost in the 48-month follow-up period. This regimen may need further testing in human clinical trials for women with gynecologic cancers.

Anticancer therapies directed at protein-protein interactions within ribonucleotide reductase

A structural basis for peptidomimetic inhibition of M1 and M2 or M2b co-association is attractive given a potential for highly selective interactions. Pharmacophores for designing highly potent nonpeptide class I RR inhibitors remains under pre-clinical development [43]. Use of nanoparticles to disrupt RR activity has recently been interrogated in pre-clinical and clinical trial forums [44].

Anticancer therapies directed at salvage pathway interplay within ribonucleotide reductase

In the salvage dNTP pathway, deoxynucleoside kinases ratelimit dNTP supply. These enzymes (TK1 and/or dCK in the cytosol, and thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) in mitochondria, Figure 1) phosphorylate deoxyribonucleosides to produce deoxyribonucleoside monophosphates (dNMPs) [45]. TK1 is S-phase specific through a mechanism similar to that of M2; the other three deoxynucleoside kinases are constitutively active across the cell cycle. Deoxynucleoside kinases are variably expressed in human normal and cancer tissues, with TK1 being elevated in cervical cancers [46]. The substrates of these salvage enzymes, deoxynucleosides, enter

cells and mitochondria passively by plasma membrane equilibrative nucleoside transporters [47]. TK1 levels become elevated after radiation and may be implicated in the facile repair of radiation-mediated DNA damage [48]. dCK is phosphorylated (and thus activated) in response to ionizing radiation [49].

To illustrate how crucial these enzymes are in the chemotherapy of nucleoside analogues, anticancer agents such as cytarabine, fludarabine, and gemcitabine require activation of dCK to be pharmacologically useful [50]. However, drugs that poison the salvage pathway remain to be developed.

Conclusion

In the era of targeted anticancer therapies, women with gynecologic cancers have benefited from a better understanding of RR enzyme structure, chemistry, and regulation, which have led to different means of RR inactivation. Whether these benefits will be found among women in both resource-rich and impoverished nations remains to be seen. Efforts to develop oral or transdermal therapies that modulate RR activity will facilitate the development of optimal strategies for inhibition of dNTP supply during treatment of gynecologic malignancies.

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Figure 1.

dNTP demand is met by two coordinated molecular pathways of dNTP supply: 1) new reduction of rNDPs to dNDPs via RR; and 2) salvage of deoxynucleosides (dNs) to dNMPs and ultimately dNDPs.