Hypoxia-targeted bioreductive tyrosine kinase inhibitors with glutathione-depleting function

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Tyrosine kinase inhibitors may serve as ligands for kinases that are involved in normal cell differentiation or repair, thereby leading to toxicity. It may be possible to target such inhibitors to tumor cells by coupling them to hypoxia-activated bioreductive molecules. Such coupling can utilize or incorporate bonds that have a propensity to be preferentially oxidized by thiols such as intracellular glutathione (GSH). The resulting depletion of GSH may increase redox-mediated apoptosis. The resultant molecule is hence projected to act via multiple cell killing mechanisms: (i) inhibition of tumor kinases, (ii) tumor DNA disruption and (iii) causing increased redox-mediated apoptosis. *Anti-Cancer Drugs* 17:21–24 © 2006 Lippincott Williams & Wilkins.

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The epidermal growth factor receptor (EGFR) is a molecular target of cancer therapies because it is over-expressed in various epithelial tumors [1]. An EGFR inhibitor used as a monotherapy in non-small cell lung cancer (NSCLC) however, has been associated with interstitial lung fibrosis. The inappropriate regeneration of sequentially injured epithelium represents an important process leading to pulmonary fibrosis [2]. Previous studies have demonstrated that epithelial expression of EGFR is upregulated in fibrotic lung tissue, suggesting that EGFR-mediated signaling is involved in epithelial regeneration in fibrotic lung disease. Thus, in NSCLC patients with underlying or a predisposition to lung injury, inhibition of the EGFR may also trigger interstitial pulmonary fibrosis. In addition, EFGR inhibition may also augment fibrosis by stimulating apoptosis in alveolar epithelial cells [3,4], by inhibiting epithelial differentiation [5] or by inhibiting pulmonary angiogenesis [6].

Members of the vascular endothelial growth factor (VEGF) play a critical role in angiogenesis [7]. Therefore, the disruption of the VEGF pathway represents an attractive target for anti-cancer therapy. Angiogenesis inhibitors that target VEGF have been associated with increased incidences of thromboembolic events [8]. VEGF has been shown to be involved in hemostasis by altering the hemostatic properties of endothelial cells [9]. A low basal level of VEGF activity has been postulated to keep non-tumoral endothelial cells in a non-activated state, partly by inducing nitric oxide synthetase expression and nitric oxide production [10,11]. Blockage of endothelial VEGF results in significant elevations of s-E-selectin and s-tissue factor reflecting a pro-thrombotic state and a decrease in the level of the coagulation cascade. The pro-thrombotic state is aggravated by concomitant administration of cytotoxic agents that induce thrombocytopenia because platelets are carriers of VEGF [12].

It is now becoming evident that therapies that target multiple targets or signaling pathways in malignant diseases are likely to be more efficacious than receptor- or molecule-specific drugs [13–15]. In particular, the ability of kinases to mutate in response to the selective pressure created by treatment with tyrosine kinase inhibitors (TKIs) alone provides a strong rationale of hitting more than one essential target at the same time in the tumor cells [16]. This can be achieved, for example, by linking TKIs to hypoxia-activated pro-drugs or by engineering a single TKI to inhibit multiple target receptors. For example, SU6668 is a potent inhibitor of VEGF receptor-2, platelet-derived growth factor receptor (PDGFR) and fibroblast growth factor receptor [17] – three transmembrane tyrosine kinases involved in different phases of tumor angiogenesis. Imatinib mesylate (Gleevec) targets the Abl tyrosine kinase, c-kit (stem cell factor receptor) as well as PDGFR tyrosine kinases [18]. The better clinical outcomes and relative non-toxic profile of these drugs may be attributed in part to their molecular 'promiscuity' against multiple targets and blocking multiple steps in tumor growth. As more ATP competitive inhibitors are developed, however, it is possible that some of these may also serve as ligands for other kinases whose inactivation may result in undue host toxicity.

How can such drugs be made to 'differentiate' between cells that are proliferating due to impaired apoptotic

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control mechanisms (tumors) versus those that are dividing (i) either normally or (ii) as a response to cellular or organ injury or microbial attack? For example, such a TKI must be able to differentiate between overexpressed EGFR in tumors versus overexpressed EGFR that serves to ameliorate pulmonary fibrosis and low basal levels of EGFR signaling by normally functioning quiescent endothelial cells. Such 'differentiation' between tumor cells and normally dividing cells may be achieved by mechanisms that serve to target the TKI to tumor cells alone. One approach is to link the TKI to a hypoxia-activated pro-drug.

In 1924, Otto Warberg hypothesized that 'the prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar' [19]. Aerobic glycolysis has since been shown to be elevated in over 90% of all metastatic cancers [20]. The products of such glycolysis, lactate and pyruvate, have been linked to a lower extracellular tumor pH and to a stimulation of the accumulation of hypoxia-inducible factor (HIF)-1α [21]. The resultant hypoxia induces proteomic and genomic changes within the tumor cells that result in malignant progression and treatment resistance [22,23].

Low oxygen tensions distinguish hypoxic tumor cells from normal cells [24]. Therefore, it may reasonably be assumed that the redox environment of tumors is different from that of normal cells. Indeed, it has been demonstrated that an electron-rich reducing environment exists in tumors [25] along with an elevated concentration of the primary intracellular buffer – glutathione (GSH) [26]. GSH has been implicated in protection against the induction of apoptotic and necrotic cell death in a variety of cell types [27–29], in part by redox modulation of cytochrome *c* and caspase release [30,31]. Depletion of cellular reduced GSH has also been shown to abolish the multidrug resistance-associated protein MRP1-mediated resistance against vinca alkaloids and anthracyclines, thereby making GSH-depleted cells more vulnerable to chemotherapy [32,33]. It has been speculated that reducing the elevated levels of GSH in tumors could inhibit aerobic glycolysis, which in turn may result in normalization of oxygen perfusion and pH [34]. Consequently, the depletion of GSH in hypoxic tumor tissue may have beneficial effects on the treatment of tumors [35,36]. GSH-influenced intracellular redox state is coupled to the oxidation state of cysteine residues in proteins [37] by complex thiol/disulfide exchange mechanisms that in turn influence the activity of a variety of enzymes [38].

Hypoxia-selective bioreductive pro-drugs, rather than undergoing selective reduction, undergo reduction events in all cells. These reductive events are reversible in the presence of molecular oxygen (via superoxide generation) [37], but irreversible in its absence. Excellent reviews of hypoxia-activated drug design can be found [39–44]. In hypoxic tumors, such pro-drugs are reduced by ubiquitous cellular reductases (cytochrome P450 reductase, xanthine oxidase) to yield electrophilic traps for DNA amino acid nucleophiles [44]. Since the efficiency of redox cycling depends upon the rate of catalytic turnover, optimum activity results when the one-electron redox potential of the pro-drug is in between that of cellular reductants, around −0.4 V versus normal hydrogen electrode (NHE) and that of the $O_2/O_2^{-\bullet}$, around −0.15 V versus NHE in an aqueous buffered medium, at pH 7.0 [45]. Molecules with too low a rate of reduction (low electron affinities) are expected to be inadequately activated by bioreductive enzymes even under hypoxic conditions, while molecules with too high a rate of reduction (high electron affinity) when compared with the rate of radical reactivity with oxygen will be cytotoxic toward normo-oxic tissue. It would hence appear that molecules with electron affinities less than −0.35 and greater than −0.15 V will show little activity against hypoxic cells *in vivo*.

The concept that GSH (as opposed to cellular reductase enzymes) can be used to reduce hypoxia-selective bioreductive pro-drugs has not received much attention.

The redox potential of the GSSG/2GSH couple is −0.25 V. Therefore, a bioreductive pro-drug may be preferentially reduced by cellular reductases rather than by GSH due to the larger redox potential difference between the pro-drug and the reductase than between the pro-drug and GSH. Furthermore, a bioreductive pro-drug whose redox potential falls between −0.25 (GSSG/2GSH) and −0.15 V ($O_2/O_2^{-\bullet}$) could potentially be reduced (and consequently be cytotoxic) in normo-oxic tissue. Even if a pro-drug could be designed so that its redox potential is lower at physiological pH that exists in normo-oxic tissue, but increases at the lower pH that exists in hypoxic tissue, it would still be preferentially reduced by cellular reductases instead of by GSH. Therefore, modulation of redox potential for design of a hypoxia-specific GSH reducible cytotoxin does not seem particularly attractive.

Another approach would be to design pro-drugs that are reducible preferentially by thiols. For example, KW-2149 (an analog of mitomycin C) is activated by non-protein thiols rather than by bioreductive enzymes, presumable due to the presence of a disulfide bond [46]. A novel class of Pt(IV) dicarboxylate compounds has been developed which are preferentially reduced and activated by thiols [47] occurring via a reductive elimination process through an attack by sulfur at one of the mutually *trans* chloride ligands [48]. A heteroaromatic *N*-oxide, tirapazamine, is the first hypoxia-selective pro-drug registered for clinical use [49]. Its low aqueous solubility however, and slow extracellular diffusion have prompted a search for analogs

with more favorable physicochemical characteristics [50]. Linking this molecule to a suitable hydrophilic TKI by a disulfide bond as well as imparting a more acidic character may increase opportunities for favorable modulation of the physicochemical properties as well as inducing thiol-specific reductivity. Some possible constructs are shown in Fig. 1. Figure 1(I) shows such a possible tirapazamine–TKI construct, linked by a disulfide bond. Figure 1(II) shows the possibility of modulating the solubility and diffusion kinetics of Fig. 1(I) by attachment of a platinum pro-drug (JM335) [48] to the *N*-oxide ring of tirapazamine. The platinum chelate was chosen because it can also (in addition to the disulfide bond) be reduced by thiols, thereby increasing the molecule's GSH-depleting ability.

It should be emphasized that the above constructs represent 'thought experiments'. The authors have no data to show that such molecules will actually be effective as envisaged *in vivo*. Nevertheless, these structures may serve as good first approximations for the design and testing of the hypoxia-mediated TKI paradigm. It is also evident that cleavage of the disulfide bond by GSH will admittedly leave a residual thiol group on the released TKI molecule. However, if positioned away from the catalytic ATP-binding site, such an added -SH group may not be detrimental to the TKI-binding affinity.

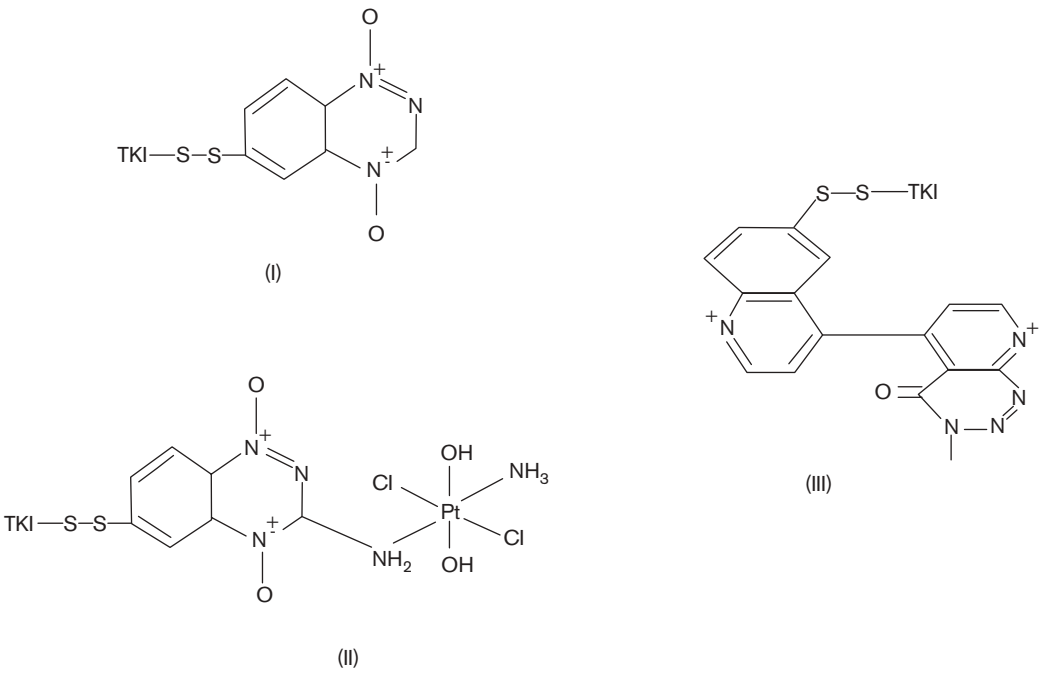
Such a redox cytotoxic entity linked through a disulfide bond that is cleavable by thiols such as GSH to a TKI would (i) selectively target the TKI to tumors, thereby improving safety, decreasing TKI drug resistance and increasing the therapeutic index, (ii) cause a depletion of tumor GSH levels causing redox-mediated apoptosis and/or increased susceptibility to reactive oxygen species (ROS), and (iii) release ROS in the vicinity of tumor cells causing DNA damage.

It has been shown that it is possible to release phenols, amines, thiols and carboxylic acids from appropriate bioreductive indolequinones selectively in hypoxic tissue [51]. Proof-of-concept has been obtained with pro-drugs comprising a bioreducible 'trigger' linked to an 'effector', which fragments off from the intermediate radical [52]. It is therefore reasonable to assume that the release of small-molecule protein kinase inhibitors could be selectively accomplished in hypoxic tumor tissue by linkage with suitable bioreductive agents.

Conclusions

TKIs may be targeted to tumors by linkage with hypoxia-activated pro-drugs. The linker can be a bond that is preferentially degraded by intracellular thiols such as GSH. The redox and physicochemical properties of the hypoxia-activated pro-drug may be more amenable to manipulation in the presence of the TKI, leading to more diffusion into tumor tissue and increased selective hypoxia cytotoxicity. Conversely, the hypoxia-activated pro-drug serves to target the TKI and the linker serves to deplete intracellular GSH, thereby possibly rendering

Fig. 1



Tyrosine kinase inhibitors linked to hypoxia activated bioreductive prodrugs via glutathione depleting disulfide bonds.

the tumor tissue more susceptible to redox-mediated apoptosis and decreasing TKI resistance.

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