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RESEARCH ARTICLE

Inhibition of Cytochrome P450 by Carbon Monoxide: Relevance to Drug Resistance in Human Breast Cancer Therapy

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ABSTRACT

Drug resistance to conventional chemotherapeutics is a great impediment to cancer therapy. A major part of this problem arises from rapid metabolism of the drugs by cytochrome P450 class of enzymes before they reach their targets or at the target itself. Inhibition of such enzymatic deactivation of the drugs could offer partial rescue and make chemotherapy more effective. Site specific delivery of exogenous carbon monoxide has been shown to inhibit cytochrome P450 enzymes and resurrect sensitivity to chemotherapeutics already available in the market. Successful design for application of such CO delivery will thus be extremely desirable to patients particularly in poor countries where the antibody-based or nanodrug therapies, discovered recently, are too expensive for the general population. The potential of such carbon monoxide-induced cytochrome P450 inhibition to improve drug sensitization to conventional chemotherapeutics in breast cancer therapy has been discussed in this account.

Introduction

Chemotherapy has been a major part of the management of cancer along with surgery and radiotherapy for quite some time. The success of anticancer drugs like methotrexate, cisplatin, paclitaxel, and doxorubicin in ameliorating the progress of the disease and specifically thwarting cancer if detected early ushered hope in the past decades and still in practice in countries where expensive alternatives such as immunotherapy is often out-of-reach for the majority of the population. However, cancer cells often develop tolerance to these pharmaceutical treatments over time leading to poor outcome, an observation that has promoted intense research activity to identify mechanisms that promote or enable drug resistance.[1,2] In such effort, several pathways such as drug inactivation, drug target alteration, drug efflux, DNA damage repair, cell death inhibition, and the epithelial-mesenchymal transition have been recognized. Because a majority of chemotherapeutics do exhibit their drug effects following modification (or partial degradation) in *vivo*, mechanisms in which the drugs interact with different proteins have drawn special attention. It is now known that many anticancer drugs undergo metabolic activation in order to acquire clinical efficacy. As a consequence, cancer cells can also develop resistance to such treatments through decreased drug activation. For example, in the treatment of acute myelogenous leukemia with cytarabine (AraC), a nucleoside drug, the chemotherapeutic is activated after multiple phosphorylation events that convert it to AraC-triphosphate. Down-regulation or mutation in this pathway can lead to a decrease in the activation of AraC, and this can lead to AraC drug resistance. Other important examples of drug activation and inactivation include the cytochrome P450 (CYP) system,[3,4] glutathione-*S*-transferase (GST) superfamily,[5] uridine diphospho-glucuronosyltransferase (UGT) superfamily.[6] and drug efflux by membrane-bound transporter proteins.[7]

The CYP class of enzymes play important role in the metabolism of endogenous proteins, hormones, drugs and other xenobiotics in mammalian physiology.[3,4] The various isoforms of this enzyme promote oxidation of substrates and facilitate the excretion of the polar products via the urinary pathway. These enzymes mediate activation and inactivation of anticancer drugs and thus are key players in cancer chemotherapy. For example, hydroxylation of the cancer drug tamoxifen by CYP in the liver affords 4-hydroxytamoxifen, the actual species responsible for its drug action. On the other hand, hydroxylation of letrozole by CYP deactivates its drug action. Drug resistance in cancer cells can therefore be mitigated by amending adverse CYP-mediated pathways.[4,8]

In recent years, three physiologically relevant gases, nitric oxide (NO), hydrogen sulfide (H_2S), and carbon monoxide (CO) have been identified as gaseous signaling molecules, commonly referred to as gasotransmitters.[9] All three gases are endogenously produced in the body and are known to participate in various pathophysiological pathways. Among these CO, endogenously produced by heme oxygenase-1 (HO-1) induced catabolism of heme,[10] holds a special position based on its remarkable chemical stability compared to the other two gasotransmitters. Because of its limited chemical reactivity with biological molecules and potential target sites, and permeability through both endothelial and epithelial membranes, it is possible for CO to exert its effects either locally or systemically by diffusing to remote tissues or organs. Thus, CO can exhibit specific functions by interacting with selected molecular targets and participate in biological processes including the ones involved in drug metabolism. The high affinity of CO toward heme centers in cytochrome P450 immediately raises the question whether inhibition of these enzymes by exogenous CO could be exploited to achieve chemosensitivity in cancer cells.

Application of CO in cancer chemotherapy however raises skepticism and concern to begin with. Nonetheless, in recent years, there have been a lot of studies on the therapeutic effects of CO in treating inflammatory conditions both in animal models and human.[11,12] The results demonstrates that CO has both anti-inflammatory and anti-oxidant capacities.[13] For example, inhalation of 100-125 ppm CO by patients with COPD was found to be safe and feasible and led to trends in reduction of sputum eosinophils and improvement of responsiveness to methacholine.[14] Observation of these kind of therapeutic benefits of CO thus provides intuitive support to the possibility of including this unusual "drug" in cancer chemotherapy following our recent realization that inflammation and cancer are closely related,[15,16] Indeed, research has shown that CO could inhibit mitochondria respiratory effect and glycolysis, two major ATP production pathways in cancer cells.[17] CO has also shown to suppress angiogenesis.[18,19] Recent work has also demonstrated that CO inhibits the activity of cystathionine β-synthase (CBS) that is important in regulating cancer cells redox homeostasis.[20,21] Because a moderately low doses of CO is well-tolerated[22] and CO has been shown

to positively affect several physiological pathways that are closely related to the cancer progression, the concept of application of CO in cancer chemotherapy has now been recognized as viable.

The surprising salutary effect of CO in cancer was first observed in prostate cancer. Otterbein and coworkers reported that both in cell culture and animal models, CO can both prevent tumor growth in prostate and lung cancers through metabolic exhaustion.[17] This group also reported that CO can amplify the effectiveness of chemotherapy 1,000-fold -- while sparing noncancerous tissue from therapy-related debilitating side effects. Significant inhibition of human pancreatic cancer cells and substantial decrease in tumor proliferation and microvascular density of xenotransplanted tumors upon exposure to low doses of CO was subsequently reported by Vitek et. al.[23] Similar suppression of proliferation, migration, and invasion of colorectal cancer cells in *vitro* and *vivo* was noted by Yin's group once again indicating the beneficiary effects of CO in cancer.[24] In a separate study this group also reported that elevated CO level in the blood of colorectal cancer patients affects chemotherapeutic sensitivity.[25] In case of human gastric cancer cells, CO mitigates IL-1β-induced activation of ROS/NF-κB and Erk1/2/AP-1 cascades, blocking IL-8 expression and significantly reduces endothelial cell proliferation in the tumor microenvironment.[26] Taken together, the therapeutic value of CO in cancer chemotherapy and in particular, its ability to enhance chemotherapeutic efficacy in many cases have prompted intense research interest and activity in recent years. The results from such studies in case of human breast cancer (HBC) is reviewed in this account.

CO and Cancer

The therapeutic effect of CO on cancer was originally indicated by the observation that overexpression of heme oxygenase-1 (HO-1) provides cyto-protection against oxidative stress, as demonstrated both in *vitro* and in *vivo* and hence pharmacological modulation may represent a novel target for therapeutic intervention.[27-29] Because HO-1 activation is induced by metabolites such as heme, toxins, cytokines, hormones, and CO (also NO), attention was directed to CO as an agent to intervene cancer progression.[30-32]

Although success in CO inhalation therapy provided success in thwarting inflammation related to oxidative stress in COPD,[14] ischemia-reperfusion (IR) injury,[33] and neointima due to balloon angioplasty,[34] selected delivery of a sustained low doses of CO to a target tissue has posed as a challenge in hospital setting. Motterlini and coworkers, for the first time, reported that metal carbonyl complexes such as lipid-soluble $[Ru(CO)_3Cl_2]_2$ could act as carbon monoxide-releasing molecules or CORMs in biological milieu.[35] Since then, several classes of CORMs that donate CO have been reported in testing the efficacy of CORMs to provide sustained delivery of low doses of CO to biological targets.[36-38] These CORMs release CO via ligand-displacement by solvent molecules (water in most cases). The discovery of photoactive CORMs (photoCORMs) *fac*-$[Re(bpy)(CO)_3(thp)]^+$ (bpy = bipyridine, thp = a phosphine)[39] has expanded the library of CO releasing agents that could now be utilized in site-specific CO delivery to biological targets under the control of visible light.[40-44] Collectively, these CO donors have provided invaluable help in exploring the pathophysiology of CO in *vitro* and in *vivo* during the past two decades.[19,45-47]

The molecular aspects of chemoresistance in cancer therapy have been examined by various groups and several excellent reviews have already been published.[48-54] A close scrutiny of these accounts reveals that chemoresistance in cancer could arise from a number of pathways that include transporter pumps, oncogenes (such as EGFR, Erk, NF-kB), tumor suppressor gene (p53), mitochondrial alteration, DNA repair, autophagy, epithelial-mesenchymal transition (EMT), cancer stemness and exosome. The focus of this article will however be solely on the role of CYP- related drug metabolism in the emergence of chemoresistance in HBC therapy.

Chemoresistance in Cancer, the Present Status

Cancer is one of the major causes of death globally, accounting for 10 million deaths in 2020.[55] The good news is this staggering number of deaths is steadily decreasing due to early diagnosis and several lines of interventions such as surgery, hormone therapy, gene therapy, immunotherapy, radiation therapy, laser therapy, combination therapy, and targeted therapy. In case of chemotherapy, the emergence of drug resistance is often inevitable which eventually becomes a serious obstacle to treat cancer. It is estimated that approximately 90% of cancer treatment failure can be attributed to drug resistance.[56,57] For example, despite establishment of platinum-based chemotherapy as the first line of treatment in case of ovarian cancer, emergence of resistant phenotypes has introduced a major hurdle in curative cancer therapy in recent years. Similarly, success rate of platinum drugs in triple negative

breast cancer has been negatively affected due to drug resistance.[58] Although over time the clinical approaches such as combination chemotherapy, targeted therapy based on various signaling pathways, and introduction of nanocarriers to carry drugs to selected sites have improved this grim outcome, more efforts are clearly needed to develop novel regimens to tackle this critical issue, particularly in poor countries where the antibody-based or nanodrug therapies are too expensive for the general population. Sensitization to conventional chemotherapeutics through manipulation of activity of CYP group of enzymes however could improve the therapy outcome and still offer an effective alternative in this regard.

As mentioned earlier, involvement of CYP enzymes in inflammation and cancer has been studied extensively.[59-61] Because CYP enzymes are responsible for the biotransformation of drugs, xenobiotics, and endogenous substances, inhibition or induction of CYP enzymes by proinflammatory cytokines (such as interleukin-1 (IL-1), IL-6, tumor necrosis factor α (TNF-α), and interferon-γ (IFN-γ) in the tumor microenvironment can promote carcinogenesis and affect chemotherapy, resulting in adverse effects, toxicity, or therapeutic failure. By the same token, careful manipulation of the CYP enzymes can modify the patient's response to medications. Because some CYP enzymes are also selectively expressed in tumors, their role in in the outcome of the chemotherapy is further amplified.

CYP Expression in HBC and Outcome of HBC chemotherapy

HBC is a malignant tumor that occurs in the lobule and the ductal epithelium of the breast. It ranks first in terms of incidence among malignancies in female patients worldwide.[62] In 2005, ~1 million new cases of HBC were reported worldwide and cases were growing at a rate of 5–20% per year. The role of the isoforms of the CYP enzymes in HBC has been indicted by different groups and has been reviewed by several authors in recent years.[63,64]

Expression and overall activity of the CYP enzymes in the breast tissue (and also in liver in general) are two important factors in identifying selected treatment of HBC with limited side effects. A pertinent observation in this regard has been reported by Floriano-Sanchez et. al. who studied the correlation between the expression of a specific member of the CYP family namely CYP3A4, with breast cancer and its association with risk factors in women in Mexico where breast cancer is the leading type of cancer in women.[65] This group analyzed the protein expression in patients with breast cancer and in healthy women (with the aid of immunohistochemical assay) and also assessed its links with some clinic-pathological characteristics. Although the study was confined in one hospital and the number of subjects was modest, a significant CYP3A4 overexpression in the malignant stroma and gland regions was noted in comparison with healthy tissue. In addition, a significant association between protein expression with smoking, alcoholism and hormonal contraceptives use was also observed. Based on these results, the authors suggested that CYP3A4 expression promotes breast cancer development and can be used in the prediction of tumor response to different treatments. Interestingly, this group also alluded that selective blocking of CYP3A4 function may be a therapeutic approach to breast cancer. Profiles of the expressions of CYP enzymes in breast cancer as reported by others also indicate that strong CYP3A4/5 and CYP1B1 expression in general may be features in nonfavorable prognosis.[66,67] Additionally, compared with other members of the large CYP family 4, CYP4Z1 is unique and vital in the development of breast cancer. A 52% increase in CYP4Z1 mRNA expression was identified in breast cancer tissues compared with non-cancerous tissues[68] and later study demonstrated that CYP4Z1 overexpression activates the PI3K/Akt and ERK1/2 signaling pathways and induces HBC angiogenesis and tumor growth.[69]

Among the various members of the CYP family, the roles of CYP3A and CYP2 enzymes along with their unique characteristics in the metabolism of biologically active endogenous compounds and numerous xenobiotics that are important in clinical pharmacology have drawn attention.[70] Certain drugs used to treat advanced stages of HBC such as paclitaxel display little or no toxicity after metabolism by CYP2C8 and CYP3A4. CYP3A4 also metabolizes tamoxifen, etoposide, ifosfamide and vinblastine. Interestingly, the levels of expression of the CYP family enzymes in different types of HBC show significant differences. For example, higher expression of CYP2E1 correlates well with an invasive lobular tumor type and advanced disease compared to the invasive ductal ones.[67] Similarly, expression of CYP1A1 has been found to be high in HBC cells with a positive correlation to tumor grade and menopausal status in newly diagnosed patients with adenocarcinoma of the breast.[71] The expression of CYP enzymes often get elevated during treatment with specific chemotherapeutics. For example, Martinez et al have reported a higher expression of CYP1B1 to be associated with increased drug resistance in HBC patients treated with docetaxel corroborating its

role as a predicator of drug resistance.[72] Similarly, CYP3A4 expression in malignant breast tissues is predictive of resistance to taxane therapy.[73]

It is now evident that the genetic polymorphisms of the CYP enzymes in breast tumors further complicates the drug treatment outcomes of HBC therapy. The various polymorphs are associated with decreased response rates, reduced progression-free survival and shorter overall survival in HBC patients under chemotherapy. For example, CYP2B is a metabolic enzyme for numerous anticancer drugs, including cyclophosphamide, paclitaxel, doxorubicin and tamoxifen. Expression of different alleles of the *CYP2* genes promotes deactivation of different drugs and thus require specific treatment regimen for different patients. Additional complicating factor is the large interindividual differences of the various CYP enzyme levels in patients from different ethnicity, age and regions. For example, CYP3A4 shows the largest interindividual differences, by a factor of several tens to hundreds, in terms of mRNA and protein expression in the liver. Collectively, these observations indicate that targeting specific CYP enzymes with designed drugs will require a formidable number of chemotherapeutics to avoid the drug deactivating effects by the various CYP enzymes during the treatment of HBC patients, an option which is quite undesirable. In contrast, administration of low doses of CO could be a more desirable alternative which will inhibit the CYP enzymes promiscuously and increase the efficacy of a prescribed chemotherapeutic leading to a better outcome irrespective of the CYP expression in the breast tumors.

CO and Breast Cancer

A highly unusual study has recently highlighted the beneficial effect(s) of CO in breast cancer. Huang et. al. examined the impact of CO poisoning on the on the risk of breast cancer in a cohort of female patients who were diagnosed with CO poisoning over the period of 2002 and 2009 as reported by the Nationwide Poisoning Database of Taiwan.[74] After matching two sets of female participants, one who had CO poisoning and one did not, on the index year, age, monthly income, and geographic region of residence, this group followed the participants over five years and noted that CO poisoning was associated with a hazard ratio of 0.67 for breast cancer even after adjustment for comorbidities of hypertension, diabetes, and hyperlipidemia. Clearly exposure to CO exhibited some protective effect against the occurrence of breast cancer. Corroborating this epidemiological observation are the growing number of in *vivo* studies in breast cancer models revealing the prominent role of CO-sensitive enzymes in promoting and maintain cellular malignancy.

To date, several groups have delved into details of the fate of the HBC cells upon interaction with CO in *vitro* to identify the target of CO that leads to cell death. For example, Mascharak and coworkers have utilized light-triggered CO delivery from designed manganese CO complexes (photoCORMs) to HBC cells and demonstrated CO-induced apoptotic death of human breast adenocarcinoma MDA-MB-231 cells in a dose-dependent way.[75] To check whether these manganese-based CO donors deliver CO from outside the cell membrane or not, the group further synthesized fluorescent rhenium analogues of the photoCORMs and showed that these CO donors do enter the cells (with the aid of confocal microscopy) and release CO to cause caspase activation leading to apoptotic death.[76,77] To push the boundaries of such CO delivery to HBC and other cancer cells, Mascharak and coworkers have incorporated the photoCORMs in 70-100 nm mesoporous silica nanoparticles.[78,79] These nanoparticle-based CO donors are readily internalized by the cancer cell by the so-called EPR (Enhanced Permeation and Retention) effect as illustrated by the rhenium-based photoCORMs, and improve the drug uptake process further. With this kind of nanoparticle-based CO donor, apoptotic death of MDA-MB-231 cells could be achieved with much lower concentration of the drug. Interestingly, some of the photoCORMs designed by Mascharak and coworkers exhibit fluorescence "Turn-ON" or "Turn-OFF" effects in MDA-MB-231 cells, and hence the CO delivery process can also be tracked within HBC cells.[80] More recently Kourti et al have employed variations of the ruthenium-based CORM-3 and demonstrated in *vitro* anti-angiogenic behavior against MDA-MB-231 breast cancer cells. As compared to the lead compound, these modified complexes not only could reduce the upregulated VEGF expression from cancer cells as well as inhibit the activation of VEGFR2 and downstream proteins of vascular endothelial cells, but they could also suppress endothelial cell migration and new vessel formation.[81] CO released from CORM-2 on the glycolysis levels (reduction of Warburg effect) was also documented in HBC by this group, where CORM-2 yielded a significant reduction in the glycolysis change rate.[82] Another interesting effect of CO has been reported by Kim et al who noted increase in the expression of Notch-1 and related genes Jagged-1 and Hes1 upon treatment with CO (from CORM-2) followed by mammosphere formation in MBA-MD-231 HBC cells.[83] Significant

upregulation of Notch-1 in triple-negative breast tumors has also been noted upon exposure to CO delivered from a metal-organic framework (MOF) nanoplatform.[84] Elucidation of precise role(s) of CO in activation of these complex signaling pathways however require further research to pin-point its targets. Results from these efforts could then be exploited to overcome drug resistance in HBC chemotherapy via addition of these CO donors in the drug regimen.

During the past few years, a few direct targets of CO that are responsible for the emergence of drug resistance during chemotherapy have been identified. The early report on sensitization of prostate cancer cells (and not normal ones) to chemotherapy upon exposure to CO indicated that mitotic catastrophe is in part responsible for the growth arrest and apoptosis *in vivo*.[17] The higher oxygen consumption, free radical generation, and mitochondrial collapse all strongly suggested that cytochrome c oxidase (a heme-containing protein) is one target of CO in this instance. Indeed, activation of some of the signaling pathways such as caspase-directed apoptosis, reported by Mascharak and coworkers, could be the result of cytochrome c oxidase inhibition by CO. Another target of CO that has been shown to be related to drug response by HBC cells is cystathionine β-synthase (CBS).[20,21] HBC cells overexpress CBS and a much lower cystathionine γ-lyase expression. This truncated transulfuration pathway[85,86] maintains a robust antioxidant capacity by maintaining a high GSH/GSSG ratio (GSH = glutathione, GSSG = oxidized GSH) in HBC cells. With use of controlled CO delivery by a photoCORM, Mascharak group has demonstrated that CO inhibition of CBS perturbs the redox environment of the HBC cells to the point of increasing sensitivity to chemotherapeutics like doxorubicin, a drug that exhibits its antitumor action through generation of a variety of reactive oxygen species (ROS) in cellular milieu.[20,21]

As mentioned before, failure of the cancer therapy often critically depends on the activity of the CYP proteins in a significant way. Recent work by Mascharak group provides strong evidence in support this pathway leading to drug resistance in HBC.[87] This work focused on the rapid development of resistance to paclitaxel, the first-line treatment in HBC treatment.[88] The CYP isoforms 3A4 and 2C8 (CYP3A4 and CYP2C8) are overexpressed in malignant breast tissue, where their activity has been hypothesized to limit the intracellular concentrations of taxanes, including paclitaxel, and impart drug resistance.[4,8] CYP3A4 and CYP2C8 oxidize paclitaxel into hydroxy-paclitaxel, which is 10-fold less active. Despite epidemiological evidences of CYP expression correlating with poor taxane response in breast cancer patients,[65] and the exposure of CO correlating with more favorable prognoses,[73] neither paclitaxel metabolism by CYP3A4/2C8 in breast cancer cells in situ nor the regulation of CYP activity to increase paclitaxel sensitivity had previously been demonstrated in any cancer model. Mascharak and coworkers intended to check whether aberrant expression of two CYP isoforms, CYP3A4 and CYP2C8,[89] metabolically deactivate paclitaxel in three HBC cell lines namely, MCF-7, MDA-MB-231 and MDA-MB-468, and whether CO delivery inhibits such deactivation. Quantitative measurement of the products of these two CYP isoforms with the aid of LCMS/MS-MRM (mass spectrometry-multiple reaction monitoring)[90] showed that CO was capable of dose-dependent inhibition of the formation of hydroxy-paclitaxel in HBC accompanied by an increase in intracellular concentrations of active paclitaxel leading to net 3- to 5-fold drug activation.

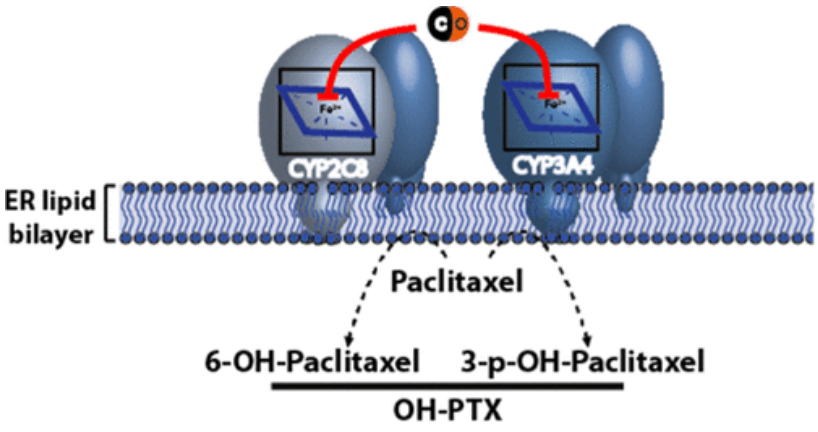

Fig. 1. Schematic of Inhibition of two key CYP enzymes by CO in HBC cells

Cumulative cytotoxic effects were observed when CO and paclitaxel were used together. Also, the malignant cells previously treated with CO showed similar drug activation effect. Co-administration of chloramphenicol, a potent inhibitor of CYP3A4 but not CYP2C8, afforded an estimate of the relative activities of these CYP isoforms and indicated that CYP3A4 is the major partner in the CO-induced metabolic deactivation of paclitaxel in HBC. Significant presence of paclitaxel and its metabolites within the HBC cells in this study indicates low levels of drug efflux by the ATP-dependent membrane-bound multidrug transporter protein MDR-1. Indeed, western blot analysis confirmed very low expression of MDR-1 and breast cancer drug resistance protein (BCRP) in all three HBC cell lines[87] confirming the salutary role of CO in increasing sensitivity to paclitaxel. Parallel binding of CO to cytochrome c oxidase and related mitochondrial failure is also expected to diminish the ATP-dependent MDR-1-related efflux and thus lowers the activity of the drug transporter if present. Given the fact that association of over-expression of MDR-1 with taxane resistance is conflicting,[91] the CYP-induced drug resistance in HBC clearly warrants further investigation.

Conclusion

Heme uptake and biosynthesis is significantly higher in malignant tissues versus normal tissues, suggesting a fundamental role of heme-containing enzymes in cancer cell progression and survival.[92] This up-regulation is known to disrupt tumor suppressors as well as provide the heme-cofactor for enzymes that to play a role in cancer, including CYPs.[93] The broad requirement of the cancer cell for the heme co-factor highlights both the fundamental role of CYPs in cancer cell survival and its candidacy as a therapeutic target. Because CO is a promiscuous inhibitor of heme enzymes, it likely exerts its effects through multiple targets concertedly, though certain CO-sensitive enzymes such as CYP, cytochrome c oxidase, and CBS play a conspicuous role in therapeutic success and cancer-free survival. CYP inhibition by CO binding to the ferrous state of the heme center in CYP is the most fundamental of mechanism of CYP inhibition.[94] The broad sensitivity of CYP isoforms to CO, in light of the prominent role of CYPs in deactivating various cancer drugs thus play a critical role in drug responses. The clinical relevance of extrahepatic metabolism of paclitaxel by CYP in HBC, and its inhibition by CO clearly provide a treatment modality that could mitigate drug resistance in HBC chemotherapy.

During the past few years, a variety of CO-releasing platforms have been designed that can deliver controlled doses of this gaseous drugs precisely to selected targets.[95-103] In recent years, CO has been shown to sensitize cancer cells toward conventional chemotherapeutics. More *in vivo* experiments with convenient CO donors are now required to establish the utility of this unique gaseous "drug" in combination chemotherapy to circumvent drug resistance through CYP inhibition and improve the outcome of the HBC treatment.

Conflict of Interest

The authors have no conflicts of interest to declare.

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