



REVIEW ARTICLE

NO and hepatocellular cancer

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NO has broad and sometimes dichotomous roles in cancer. The effects of NO in tumours depend on the type and localization of NOS isoforms, concentration and duration of NO exposure, and cellular sensitivity to NO. Hepatocellular carcinoma (HCC) is a common and lethal disease for which no effective therapy other than surgical resection exists. Over two decades of research has yielded evidence that NO generated by the inducible NOS (iNOS or NOS2) contributes to HCC progression in at least a subset of patients with HCC. The co-expression of iNOS with COX-2 may portend a particularly aggressive cancer phenotype in HCC and at the same time reveal an opportunity for pharmacological intervention. In this review, we focus on what is known about the influence of NO in HCC neoplastic transformation, proliferation and apoptosis, angiogenesis, invasion, and metastasis, cancer stem cells, and the host immune response against the tumour. We discuss the implications of recent findings for targeting the NO pathways in HCC.

1 | OVERVIEW

While it is now well accepted that endogenously produced gaseous molecules serve as signalling molecules in biological systems, the discovery of the enzymic production of NO as an autocrine and paracrine signalling pathway in mammals in the 1980s came as quite a surprise. Several important milestones in the NO field were reached in 1987. Hibbs, Taintor, and Vavrin (1987), in their quest to identify the mechanisms used by macrophages to kill tumour cells, first showed that arginine was the substrate for nitrogen oxide production by

murine macrophages and that analogues of arginine (including the widely used N^Gmonomethyl-L-arginine [NMA]) could block this newly discovered pathway. Ignarro, Buga, Wood, Byrns, and Chaudhuri (1987) and Palmer, Ferrige, and Moncada (1987) simultaneously identified NO as the endothelium-derived relaxing factor originally described by Furchgott and Zawadzki (1980), providing proof that mammalian cells could produce NO. This also established that NO was the biologically active intermediate in the arginine-dependent production of nitrogen oxides by cells. By 1998, Robert Furchgott, Louis Ignarro, and Ferid Murad would receive the Nobel Prize in Physiology or Medicine for their seminal roles in identifying NO as endothelium-derived relaxing factor and soluble guanylyl cyclase as the intracellular NO receptor (Raju, 2000). The past 30 years of research in the NO field has revealed that NO and its reaction products permeate essentially every realm of biology and can be found to contribute

Abbreviations: APC, adenomatous polyposis coli; CSC, cancer stem cell; eNOS/NOS3, endothelial NOS; HCC, hepatocellular carcinoma; iNOS/NOS2, inducible NOS; LCSC, liver cancer stem cells; NICD, Notch intracellular domain; NMA, N^Gmonomethyl-L-arginine; nNOS/NOS1, neuronal NOS; PDXs, patient-derived xenografts.

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the pathogenesis of many diseases, including cancer (Augsten et al., 2014; Cheng et al., 2014; Thomas & Wink, 2017).

Excessive and/or sustained NO synthesis has been found to produce many of the characteristics of cancer progression such as genomic instability, metastasis, immunosuppression, angiogenesis, cancer cell stemness, chemoresistance, and aberrant proliferation (Fukumura, Kashiwagi, & Jain, 2006; Lala & Chakraborty, 2001). NO is synthesized by a family of enzymes termed NOS. The three isoforms of NOS include neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Because nNOS and eNOS are expressed at baseline by many cells, they have also been referred to as constitutive NOS (Mungrue, Bredt, Stewart, & Husain, 2003). While iNOS can be expressed in a few tissues at baseline, it is more typically up-regulated under cell stress or inflammatory conditions but then produces high and sustained levels of NO. To synthesize NO, all isoforms utilize L-arginine as substrate and depend on the cofactors/coenzymes NADPH, tetrahydrobiopterin, flavin adenine dinucleotide, flavine mononucleotide, oxygen (O₂), and protoporphyrin IX (Menshikova, Zenkov, & Reutov, 2000) to oxidize a guanido nitrogen of arginine to NO with citrulline as co-product.

Expression of NOS has been detected in many cancers such as breast, liver, colon, and CNS cancers (Basudhar et al., 2017; Du et al., 2013; Granados-Principal et al., 2015; Li et al., 2009; Rahman et al., 2001; Wang et al., 2018). While the up-regulation of iNOS or exposure of cancer cells to high levels of NO can prevent tumour cell growth, it remains unclear how important NO may be as part of the host defences against tumour formation (Vannini, Kashfi, & Nath, 2015; Burke, Sullivan, Giles, & Glynn, 2013). In contrast, evidence continues to mount that tumour iNOS expression is associated with an aggressive tumour phenotype (Basudhar et al., 2017; Du et al., 2013; Eyler et al., 2011) and that NO may promote cancer formation and progression (Bing et al., 2001). Therefore, the effect of NO on cancer cannot be easily classified as “pro-cancer” or “anti-cancer” as it may rely on other factors such as the cell type (Ishikawa et al., 2003), NO levels (Le, Wei, Huang, Lancaster, & Xie, 2005; Mocellin, 2009), the redox micro-environment, organs involved (Buijs et al., 2017), or even the type of cancer (Wang et al., 2018). A unique relationship between iNOS and COX-2 in some cancers is now well established with the products of the two enzymes regulating the other (Basudhar et al., 2017).

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most frequent cause of cancer deaths. Its incidence is increasing worldwide because of the dissemination of hepatitis B and C virus infection, increases in the incidence of non-alcoholic steatohepatitis, and the chronic inflammation that is associated with these conditions (Llovet, Burroughs, & Bruix, 2003). Surgical resection or liver transplantation is the only opportunity for cure, but the majority of patients do not qualify for these procedures (Carr, 2004). Moreover, there is a high rate of recurrence after resection or transplantation. Regional liver therapies including ablation, chemoembolization, selective internal radiation with Yttrium90 beads, systemic chemotherapy, and immunotherapy have produced modest results in non-surgical candidates and are not curative approaches.

It is noteworthy that human iNOS expression was initially identified in the liver (hepatocytes; Nussler et al., 1992) and the human iNOS cDNA and gene were originally described and cloned from human hepatocytes (Geller et al., 1993; Geller et al., 1993). Human hepatocytes like many epithelial cell types readily express iNOS, and a dominant effect of iNOS in hepatocytes is to prevent apoptosis under inflammatory conditions (Billiar, 2015; Chen, Zamora, Zuckerbraun, & Billiar, 2003; Collins et al., 2003). Therefore, it may have been predicted that iNOS expression would be associated with worse prognosis in HCC. We first proposed that inflammation-induced iNOS expression, as seen in viral hepatitis, could be part of the pathogenesis of HCC in 1997 (Kane et al., 1997). Increased nitrite and nitrate levels are observed in patients with HCC (Moriyama et al., 1997), and a subset of HCC express iNOS mRNA or protein (Moriyama et al., 2000; Notas, Xidakis, Valatas, Kouroumalis, & Kouroumalis, 2001). Although the association between iNOS expression and decreased survival has been variable, there is mounting evidence that the expression of iNOS with other genes is associated with marked changes in tumour growth and reduced patient survival. Co-expression of iNOS with COX-2 in HCC is associated with a significant reduction in patient survival (Fermor et al., 2002). It is now known that iNOS and COX-2 co-regulate each other in breast cancer and synergize to drive poor outcomes in triple negative breast cancers (Basudhar et al., 2017). In 2018, we showed that iNOS expression is increased in HCC that express the stem cell markers CD24 and CD133 and that this is associated with a highly significant decrease in patient survival (Wang et al., 2018). Many *in vitro* studies have focused on the anti-proliferative and pro-apoptosis effects of NO donors *in vitro* on HCC cell lines, and NO synergizes with sorafenib to kill tumour cells *in vitro* (Ling et al., 2011). However, suppression or inhibition of iNOS in human HCC patient derived xenografts (PDXs) models prevents tumour formation and growth (Wang et al., 2018). While it remains unknown whether administration of exogenous NO will promote or inhibit tumour growth in human HCC, the preponderance of evidence from studies in humans and human tumours is that iNOS associates with a more aggressive HCC phenotype. Below, we summarize the evidence that NO contributes to the pathogenesis and progression of HCC.

2 | NO AND HEPATOCELLULAR CARCINOMA

2.1 | Neoplastic transformation

Neoplastic transformation is a key initial step in cancer. For HCC, this occurs frequently against a backdrop of chronic inflammation where continuous exposure to the sustained NO that is produced by iNOS is thought to promote neoplastic transformation. This may be especially relevant in HCC that occurs in patients with viral hepatitis (Machida et al., 2010; Rahman et al., 2001). NO and NO-derived reactive nitrogen species induce oxidative and nitrosative stress, which results in DNA damage (such as nitrosative deamination of nucleic acid bases, transition and/or transversion of nucleic acids, alkylation, and DNA strand breakage) and inhibition of DNA repair enzymes (such as alkyl

transferase and DNA ligase) through direct or indirect mechanisms (Lala & Chakraborty, 2001; Wink et al., 1998). Some experimental evidence has been provided that increased nitrosative stress promotes HCC formation in a mouse liver tumour model. *S*-Nitrosoglutathione (GSNO) reductase reduces the degree of nitrosative stress in the liver by denitrosylating nitrosoglutathione (GSNO), a low MW nitrosating species that forms when iNOS is expressed in the liver (Liu et al., 2004). Deletion of GSNO reductase, which increases nitrosative stress, has been shown to promote experimental iNOS-dependent HCC formation (Zhang et al., 2019). However, excessive nitrosative stress has yet to be linked to the pathogenesis of HCC in humans.

2.2 | Proliferation and apoptosis

While iNOS/NO have been associated with both the promotion and inhibition of proliferation and apoptosis in experimental cancer models (Gu, Yao, & Sun, 2017; Zhang et al., 2019), the best current evidence suggests that the consequences of iNOS/NO on tumour cell expansion and survival depend on the flux of NO, the redox micro-environment, and cell type. iNOS was significantly higher in HCC with poorer prognosis (as defined by patient survival) and positively correlated with tumour proliferation, genomic instability, and micro-vascularization and negatively with apoptosis (Calvisi et al., 2008). We recently reported that inhibition of iNOS leads to less cell proliferation in a PDX model of human HCC (Wang et al., 2018). Using liver cancer cell lines and liver cancer stem cells (LCSC) isolated from human liver, we also showed that iNOS over-expression promotes human HCC hepatosphere formation, indicating that NO produced at the levels sustained by iNOS expression lead to an expansion of cell mass in vitro (Wang et al., 2018). Thus, these experiments using tumour cells derived from human HCC provide at least some indirect evidence that iNOS/NO can support local tumour expansion.

2.3 | Angiogenesis, invasion, and metastasis

Angiogenesis is the growth of new blood vessels from the original vascular bed and is an essential step in tumour progression and metastasis remodelling (Nannuru, Sharma, Varney, & Singh, 2011). As tumours grow, their original vasculature eventually becomes insufficient to supply the growing tissue mass, and local hypoxia develops. NO can promote angiogenesis by supporting endothelial differentiation, inducing VEGF, inhibiting anti-angiogenic factors, dilating tumour blood vessels, and recruiting bone marrow-derived and perivascular cells (Fukumura et al., 2006). An analysis using samples of both healthy liver and HCC from humans demonstrated that iNOS positively modulates MMP-9 expression, facilitating tumour cell angiogenesis, invasion, and metastasis (Sun et al., 2005). iNOS seemed to play a major role in the proangiogenic effect in multidrug resistance-induced angiogenesis in hepatocellular carcinoma cell lines, and its inhibition by 1400W, an iNOS-selective inhibitor, blunted liver cancer cell proliferation and migration (Lasagna et al., 2006).

2.4 | Cancer stem cells

Growing evidence supports the concept that tumour initiation and maintenance can be driven by cancer stem cell (CSC) subsets that are responsible for tumour relapse, metastasis, and chemotherapy

resistance (Li et al., 2018; Luo et al., 2016; Wang et al., 2016; Wang et al., 2018; Xiong et al., 2018). We recently provided insights into how iNOS/NO promote HCC tumour formation and growth by supporting the stemness of a subset of CD24 and CD133 double positive LCSC (Wang et al., 2018). Important features of this work included the observation that HCC that are CD24+ and CD133+, are associated with iNOS expression and reduced survival. iNOS expressing CD24+, CD133+ LCSC demonstrated greater growth and chemoresistance and lead to more rapid tumour growth in vivo than CSC that did not express iNOS. iNOS in both the LCSC and other cells in the tumour micro-environment were important for in vivo tumour growth resulting from CD24+, CD133+ LCSC indicating that iNOS-derived NO could come from multiple cell sources to support tumour growth. In vitro studies established that iNOS/NO promoted stemness in human LCSC by activating Notch1 signalling. The Notch signalling pathway promotes the self-renewal, differentiation, proliferation, survival, and migration of CSCs in several malignancies. Shedding of the Notch extracellular domain by TACE/ADAM17 provides the signal for γ -secretases to cleave and release the Notch intracellular domain (NICD). NICD then interacts with the DNA-binding protein CSL (CBF-1/RBPJ- κ in *Homo sapiens*/*Mus musculus*, respectively, Suppressor of Hairless in *Drosophila melanogaster*, and Lag-1 in *Caenorhabditis elegans*) and acts as a transcriptional activator for a set of genes that regulate cell proliferation, differentiation, epithelial-to-mesenchymal transition, and cell survival (Wang et al., 2018). We have previously shown that NO can activate the sheddase TACE/ADAM17 in liver cells (Chanthaphavong, Loughran, Lee, Scott, & Billiar, 2012; Deng, Loughran, Zhang, Scott, & Billiar, 2015). Sheddases including ADAM17 are required for Notch activation, and NO-induced Notch activation in LCSC involved cGMP-dependent ADAM17 activation and iRhom2 up-regulation (Figure 1). The link to HCC in humans was made by showing that survival in HCC patients correlated inversely with expression of not only iNOS but also activated ADAM17 and Notch (NICD levels). One implication of this work is that using specific markers such as CD24 and CD133 for stem cells, iNOS, or Notch activation markers might be a useful strategy to target tumours that could be responsive to iNOS inhibitors.

2.5 | Tumour micro-environment and immune response

Tumours consist not only of tumour cells but also of stromal cells such as vascular cells, fibroblasts, and immune cells. Indeed, cancer cells have developed diverse ways to intervene in the production and/or metabolism of NO in tumours and their surrounding tissue to gain an advantage (Fukumura et al., 2006). As mentioned above, we have provided in vivo evidence that iNOS expressed by tumour cells as well as by cells in the micro-environment in a PDX model of human HCC contribute to tumour formation and growth (Wang et al., 2018). While suppression of iNOS expression in LCSC partly limited tumour formation, the combination of LCSC-specific inhibition using siRNA and systemic administration of a highly selective iNOS inhibitor almost completely prevented tumour formation following injection of human

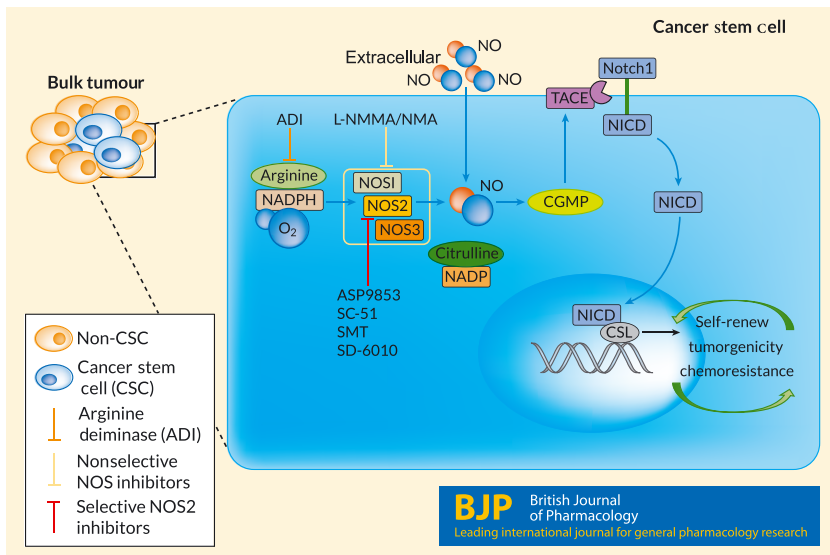


FIGURE 1 In liver cancer stem cells, to synthesize NO, all isoforms of NOS (nNOS/NOS1, iNOS/NOS2, and eNOS/NOS3) utilize L-arginine as substrate and depend on the cofactors/coenzymes NADPH and oxygen (O₂) to oxidize a guanido nitrogen of arginine to NO with citrulline and NADP as co-product. NO-induced Notch activation in LCSC involved cGMP-dependent ADAM17 activation and the cleavage of membrane Notch. NICD then enters the nucleus, activates transcription of target genes, and promotes the self-renewal capacity, tumorigenicity, and chemotherapy resistance. Potential strategies to suppress NO availability in clinic trials include removal of the NO substrate arginine (arginine deiminase), administration of non-selective NOS inhibitors (L-NMMA/NMA), or the use of selective iNOS inhibitor (ASP9853, SC-51, SMT, and SD-6010)

LCSC into the livers of mice. NO is highly diffusible across distances spanning several cell diameters (Bosworth, Toledo, Zmijewski, Li, & Lancaster, 2009; Le et al., 2005). Thus, any cell that produces NO in a tumour has the potential to contribute to the ambient concentration of NO in the micro-environment. The short-lived nature of NO means that it only acts locally and that a high-output source such as iNOS is more likely to lead to a micro-environment that adapts to and even depends on biological effects of NO. Therefore, targeting NOS in tumours such as HCC should not be cell-type specific.

3 | DRIVERS OF ABERRANT iNOS EXPRESSION IN CANCER

3.1 | iNOS/NO and p53

p53 is a tumour suppressor protein that plays an important role in the cellular response to DNA damage from exogenous mutagens. Loss of p53 function, through mutations in p53 itself or perturbations in pathways signalling through p53, is a common feature in the majority of human cancers (Muller & Vousden, 2013). Exposure of human cells to an NO donor resulted in p53 protein accumulation in cancer cells (Forrester et al., 1996). Expression of wild-type p53 down-regulated cytokine-induced iNOS protein and induced NO synthesis, and p53 was shown to inhibit iNOS gene transcription (Forrester et al., 1996). Similar findings were seen in a variety of human and murine cells tested. Co-transfection with a mutant p53 expression vector (175^{his}) abolished this repression, indicating that the human iNOS promoter inhibition is wild-type p53 specific. These data are consistent with a negative feedback loop where endogenous NO-induced DNA damage results in wild-type p53 accumulation that protects against further DNA damage by trans-repression of the human iNOS gene, thus reducing the potential for further NO-induced DNA damage. Another study has shown an up-regulation of iNOS expression in cancer-prone p53 knockout mice (Ambs, Merriam, et al., 1998; Ambs, Ogunfusika, et al., 1998). A further study found that p53 and VEGF regulate

tumour growth of iNOS-expressing human carcinoma cells (Ambs, Merriam, et al., 1998). Hence, NO/p53 interactions have important implications for tumour biology (Hussain, Hofseth, & Harris, 2003).

3.2 | iNOS and Wnt β -catenin/Tcf-4 signalling pathway

Activation of the Wnt receptor complex triggers displacement of the multifunctional kinase GSK-3 β from a regulatory adenomatosis polyposis coli (APC)/Axin/GSK-3 β complex. The conserved Wnt/ β -catenin pathway regulates stem cell pluripotency and cell fate decisions during development (Du et al., 2013; Wang et al., 2016). Many reports have implicated aberrations in the development of human cancers, including HCC (Morin & Weeraratna, 2003; van Es, Barker, & Clevers, 2003). This can result from truncating APC mutations, loss of function axin mutations, or stable dominant β -catenin mutations. Even in the absence of Wnt signalling, APC mutations inhibit the protein's ability to bind β -catenin, so that β -catenin accumulates in the cytosol and translocates to the nucleus. The end result is excessive nuclear β -catenin that activates target genes which promote tumorigenesis. β -Catenin has also been shown to interact with NF- κ B proteins and therefore has the potential to influence tumorigenesis by modulating the apoptotic effects of NF- κ B. With our collaborator Perwez Hussain, we showed that the β -catenin/Tcf-4 signalling cascade regulates human iNOS gene expression by direct binding of Tcf-4 to two binding elements (TBE1 and TBE2) in the 5'-flanking region of the human iNOS gene (Du et al., 2006). Hence, the human iNOS gene is a Wnt β -catenin/Tcf-4 target gene, and excessive Wnt β -catenin/Tcf-4 signalling, as seen in HCC, could contribute to sustained iNOS expression.

4 | TARGETING NO IN HCC

Currently, there are no effective therapies to treat unresectable HCC; therefore, it is reasonable to consider clinical trials for HCC that target

the NO pathway. It is the viewpoint of the authors that there is little evidence that delivering NO through NO donors will be of benefit in the treatment of HCC. While it is possible that subtypes of HCC susceptible to the delivery of NO alone or with other treatments will be identified in the future, we suggest that current efforts should be centred on understanding whether suppressing NO bioavailability will improve outcomes in HCC. Known sources of NO in human HCC include NOS3 (eNOS) and iNOS. While eNOS is almost certainly expressed to some extent in all HCC, as discussed above, iNOS is expressed in only a subset of HCC. Therefore, among the important considerations for targeting NO production HCC will be patient selection, and the pharmacological strategy used reduce NO bioavailability.

Potential strategies to suppress NO availability regardless of the source include removal of the NO substrate arginine, administration of non-selective NOS inhibitors, or the use NO scavenging agents. Arginine-depleting agents are under study as treatment for tumours that are auxotrophic for arginine. For example, arginine deiminase, an enzyme that allows many microorganisms to utilize arginine as an energy source, has been shown to slow disease progression in hepatocellular carcinoma (Izzo et al., 2004). A PEGylated derivative of recombinant human arginase-1 also prevented disease progression in a Phase 1 clinical trial in patients with HCC (Yau et al., 2013). Whether the benefits observed in these early phase trials were through the suppression of NO synthesis is uncertain. Arginine conversion to polyamines is required for cell proliferation and immune responses (Monticelli et al., 2016) and, therefore, arginine depletion could affect tumour growth through multiple mechanisms.

The nonselective NOS inhibitor N^Gmonomethyl-L-arginine (often abbreviated NMA or L-NMMA) has been given to humans in sepsis, where it increased mortality (Watson et al., 2004) and in cardiogenic shock where it had no therapeutic benefit (Dzavik et al., 2007). Like HCC, triple negative breast cancers often express iNOS, and also, like HCC, these cancers are aggressive when iNOS is co-expressed with COX-2. The synergism between iNOS and COX-2 probably involves ability of the products of each enzyme to up-regulate the other. Specifically, NO can increase the expression of COX-2, and PGE₂ acts with cytokines to increase iNOS expression in tumour cells (Basudhar et al., 2017). NMA has shown early promise as rescue therapy in stage 4 triple negative breast cancer (Granados-Principa et al., 2015). Thus, there may be a rationale to test NMA in unresectable HCC with the understanding that this inhibitor would block both iNOS and eNOS. In contrast to the evidence supporting a role for iNOS in tumour progression in HCC, there are no data to establish whether eNOS is also important to tumour progression.

iNOS-selective inhibitors have been developed and tested in clinical trials in humans for asthma (Hansel et al., 2003), migraine headaches (Akerman, Williamson, Kaube, & Goadsby, 2002), and osteoarthritis (Hellio le Graverand et al., 2013). Administration was safe for up to 2 years; however, all trials were negative for therapeutic benefit for these chronic diseases. These trials did establish that iNOS-selective inhibitors can be safely administered to humans for extended periods of time. Trials using a selective iNOS inhibitors could be considered in HCC that are iNOS positive or iNOS/COX-2 positive. Selective iNOS inhibition has the advantage of leaving eNOS

function intact and thereby avoiding the consequences of NOS inhibition on BP. The use of both NOS and COX-2 inhibitors in patients that have double positive tumours could have some merit, especially considering the low toxicity of targeting these pathways in humans. A single study testing ASP9853, an inhibitor of iNOS dimerization, has been reported (Luke et al., 2016). ASP9853 was administered in combination with docetaxel in patients of non-haematological malignancies with late stage cancers (lung, prostate, bladder, oesophagus, bile duct, gastro-oesophageal junction, kidney, pancreas, and skin). Several enrolled patients died before the agents could be administered, and neutropenia was a limiting factor (Luke et al., 2016). The numbers of patients were small, and it was not possible to make conclusions on the effectiveness of the iNOS inhibitor or its role in the neutropenia.

5 | CONCLUDING REMARKS

Several arguments can be made to test NOS inhibitors with or without COX-2 inhibitors in HCC. HCC is common, and no effective therapies currently exist other than surgical resection. Both non-selective and selective NOS inhibitors have been used safely in humans in the absence of sepsis for other diseases; therefore, agents for testing currently exist. Administration of NOS inhibitors significantly limits HCC formation and progression in a PDX model of human HCC (Wang et al., 2018). Finally, tumour markers including iNOS, COX-2, and the stem cell markers CD24 and CD133 have been identified that could be used for patient selection. Major considerations remain in terms of agents to be used and patient selection.

6 | NOMENCLATURE OF TARGETS AND LIGANDS

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

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CONFLICT OF INTEREST

The University of Pittsburgh holds patents on the human NOS cDNA and recombinant protein US Patent serial #5,468,630, #08/265,046, and #5,882,908.

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