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Perspective

Nimbolide, a Neem Limonoid, Is a Promising Candidate for the Anticancer Drug Arsenal

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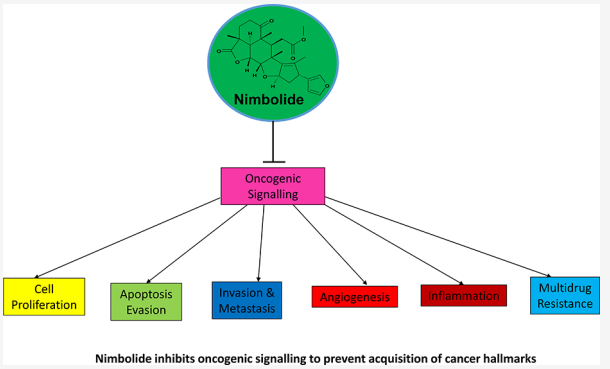
ABSTRACT: Nimbolide, a major limonoid constituent of *Azadirachta indica*, commonly known as neem, has attracted increasing research attention owing to its wide spectrum of pharmacological properties, predominantly anticancer activity. Nimbolide is reported to exert potent antiproliferative effects on a myriad cancer cell lines and chemotherapeutic efficacy in preclinical animal tumor models. The potentiality of nimbolide to circumvent multidrug resistance and aid in targeted protein degradation broaden its utility in enhancing therapeutic modalities and outcome. Accumulating evidence indicates that nimbolide prevents the acquisition of cancer hallmarks such as sustained proliferation, apoptosis evasion, invasion, angiogenesis, metastasis, and inflammation by modulating kinase-driven oncogenic signaling networks. Nimbolide has been demonstrated to abrogate aberrant activation of cellular signaling by influencing the subcellular localization of transcription factors and phosphorylation of kinases in addition to influencing the epigenome. Nimbolide, with its ever-expanding repertoire of molecular targets, is a valuable addition to the anticancer drug arsenal.



Nimbolide inhibits oncogenic signalling to prevent acquisition of cancer hallmarks

1. INTRODUCTION

Cancer is the second most common cause of death worldwide and a major public health issue. Despite recent advances in treatment options, the morbidity and mortality due to cancer continue to increase.[1] Furthermore, toxicity and adverse side effects of chemotherapeutic agents as well as drug resistance pose serious challenges to oncologists. Recent research efforts on cancer drug discovery have therefore focused on natural products that exhibit high therapeutic efficacy with low toxicity and minimal side effects. A large number of phytochemicals from medicinal plants have emerged as promising anticancer drug candidates, and some of them have entered clinical trials.[2] Of late, limonoids from the neem tree have received increasing research attention for their potent antiproliferative effects.[3,4]

Neem (*Azadirachta indica* A. Juss) is an evergreen tree of the Meliaceae family, ubiquitously found in the Indian subcontinent and widely distributed in Asia, Africa, and America. Neem has gained enormous importance for its applications in the agricultural and pharmaceutical industry, as well as for its well-established ethnomedicinal value.[5−7] Neem has been used as a food and folklore medicine to treat a variety of human ailments and has won epithets such as "heal all", "divine tree", "village dispensary", "nature's drug store", and "tree of the 21st century". All parts of the neem tree have been documented to display a wide range of pharmacological properties including antiseptic, antiparasitic, analgesic, antimicrobial, antiulcer, hepatoprotective, antihyperglycaemic, orodental protection, anti-inflammatory, immunomodulatory, and anticancer effects. The antiproliferative effects of neem extracts and constituents have been extensively documented in a wide array of cancer cell lines *in vitro* and in preclinical animal tumor models *in vivo*. Neem is recognized to inhibit the development and progression of cancer by multiple mechanisms ranging from prevention of procarcinogen activation and oxidative DNA damage, upregulation of antioxidant and carcinogen detoxification systems, inhibition of tumor cell proliferation, invasion, and angiogenesis, and induction of apoptosis.[5,8]

The medicinal properties of neem have been attributed to the rich array of over 400 structurally diverse and chemically complex bioactive compounds, one-third of which are tetranortriterpenoids or limonoids.[7,8] The major limonoids present in the neem tree include azadirachtin, azadiradione, epoxyazadiradione, gedunin, 6-desacetyl nimbinene, nimbidin, nimbin, nimolinone, and nimbolide. Nimbolide was found to be the major contributor to the antiproliferative effects of neem.[9] In this review, we provide an overview of the

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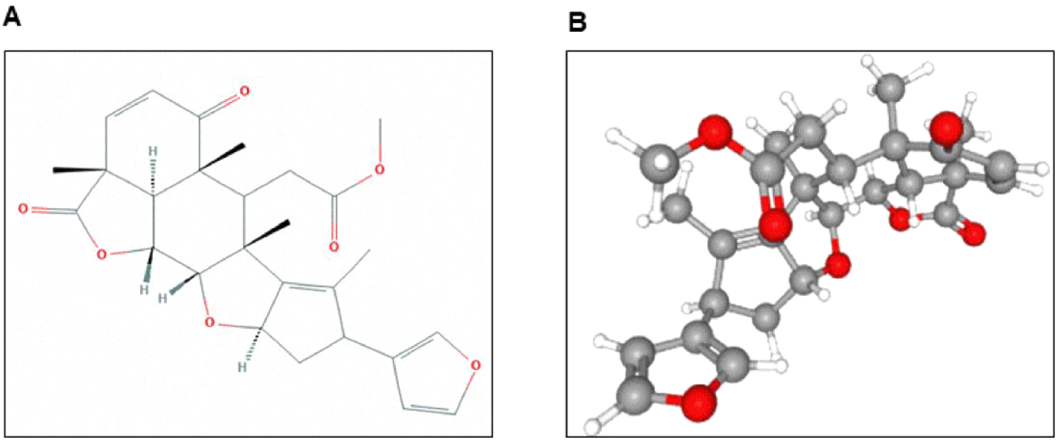

Figure 1. Two-dimensional (A) and three-dimensional structure of nimbolide (IUPAC name: methyl 2-[(1*R*,2*S*,4*R*,9*R*,11*R*,15*R*,18*R*)-6-(furan-3-yl)-7,9,11,15-tetramethyl-12,16-dioxo 3,17dioxapentacyclo [9.6.1.$0^{2,9}$.$0^{4,8}$.$0^{15,18}$] octadeca-7,13-dien-10-yl]acetate). (PubChem CID: 138115244. Molecular Formula: $C_{27}H_{30}O_7$. Molecular Weight: 466.5 g/mol.)

pharmacological properties of nimbolide with particular emphasis on the anticancer effects. We summarize the evidence for the modulatory effects of nimbolide on the acquisition of cancer hallmarks, oncogenic signaling pathways, and epigenetic modifications. Finally, we analyze the efficacy of nimbolide as a chemosensitizer and examine its pharmacokinetic and toxicity profile.

2. NIMBOLIDE STRUCTURAL FEATURES

Nimbolide is a tetranortriterpenoid limonoid (mol formula $C_{27}H_{30}O_7$), abundantly present in the leaves and flowers of the neem tree. Chemically, nimbolide is (4a,5a,6a,7a,15b,17a)-7,15:21,23-diepoxy-6-hydroxy-4,8-dimethyl-1-oxo-18,24-dinor-11,12-secochola-2,13,20,22-tetraene-4,11-dicarboxylic acid γ-lactone methyl ester. Nimbolide contains a decalin skeleton and is a member of the tetranortriterpenoids known as C-secomeliacins. Two important structural features, the α,β-unsaturated ketone structural element and the γ-lactone moiety, are believed to be responsible for the anticancer activity of nimbolide.[10] The 2D and 3D structures of nimbolide are shown in Figure 1. The amide derivatives of nimbolide have been demonstrated to display greater cytotoxicity against various cancer cell lines compared to the parent compound.[11]

3. PHARMACOLOGICAL PROPERTIES OF NIMBOLIDE

A growing body of evidence indicates that nimbolide exhibits a wide spectrum of pharmacological effects including antimalarial, antimicrobial, antifibrotic, anti-inflammatory, antioxidant, hepatoprotective, cardioprotective, and anticancer activities.

3.1. Antiparasitic and Antimicrobial Activities. The antimalarial activity of aqueous and ethanolic extracts of neem was attributed to the presence of nimbolide. Nimbolide was also shown to inhibit *Plasmodium falciparum* in culture with moderate potency.[12] Derivatives of nimbolide exhibited potent antitrypanosomal activity against *Trypanosomiasis brucei rhodesiense*.[13]

Nimbolide exerts antibacterial effects against *Staphylococcus aureus* and *Staphylococcus coagulase*. A study by Sarkar et al.[14] revealed the potential of nimbolide in the treatment of infections caused by multidrug-and meticillin-resistant strains of *Staphylococcus aureus* (MDR MRSA) implicated in nosocomial- and community-acquired infectious diseases. Nimbolide exerted its antibacterial effects by inducing membrane damage and lysis of *S. aureus* cells, disruption of biofilm structure and formation, and by reducing the viability of *S. aureus* in a manner similar to tetracycline and nalidixic acid. Molecular docking studies revealed inhibitory effects of nimbolide against New Delhi metallo-β-lactamase-1 (NDM-1), an enzyme produced by Gram-negative bacteria that hydrolyses β-lactam antibiotics. These findings suggest that nimbolide can be developed as a drug candidate against bacterial infections as well as bacterial resistance to antibiotics.[15] Using computational approaches, Vora et al.[16] suggested that nimbolide is a promising target-specific drug candidate for human immunodeficiency virus (HIV).

3.2. Antifibrotic and Anti-inflammatory Effects. Several studies have demonstrated the antifibrotic and anti-inflammatory potential of nimbolide. In a murine model of bleomycin-induced scleroderma, nimbolide administration induced regression of inflammation-driven fibrosis by abrogating the transforming growth factor-β (TGF-β)/Smad signaling axis and epithelial-to-mesenchymal transition (EMT), two major factors implicated in scleroderma. Furthermore, nimbolide reduced the levels of the collagen cross-linker, lysyl oxidase like-2 as well as extracellular matrix (ECM) deposition.[17] Nimbolide was demonstrated to regulate autophagy signaling in pulmonary fibrosis induced by TGF-β1 *in vitro* and bleomycin *in vivo*. The mechanism involved the down-regulation of mesenchymal and fibrotic markers with increased expression of epithelial markers. Nimbolide regulated autophagy by reducing the expression of LC-3 (microtubule-associated protein 1A/1B-light chain 3) and p62 and enhancing Beclin 1 expression.[18] Annaldas et al.[19] provided evidence for the antifibrotic activity of nimbolide in a mouse model of renal fibrosis induced by unilateral ureteral obstruction. Nimbolide prevented renal fibrogenesis and inflammation by reducing collagen deposition, oxidative stress, and the expression of TGF-β/p-Smad and ECM proteins.

Nimbolide significantly suppressed the expression of the inflammatory cytokines, interleukins IL-6, IL-8, IL-12, and tumor necrosis factor-α (TNF-α) and inhibited nuclear factor-κB (NF-κB) signaling in IEC COLO 205 intestinal epithelial cells and in the murine macrophage cell line RAW 264.7 as well as in peritoneal macrophages from interleukin-10-deficient

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J. Med. Chem. XXXX, XXX, XXX−XXX

(IL-10$^{-/-}$) mice stimulated with TNF-α or lipopolysaccharide (LPS). Additionally, nimbolide alleviated colitis in dextran sulfate sodium-induced acute and chronic colitis in IL-10$^{-/-}$ mice *in vivo* as reflected by disease activity, histopathologic scores, and abrogation of NF-κB signaling.[20] Nimbolide exerted protective effects against cerulein-induced chronic pancreatitis in mice by significantly decreasing α-smooth muscle actin (α-SMA), matrix metalloproteinase-2 (MMP-2), collagen1a, and fibronectin via downregulation of β-catenin/TGF-β1/Smad signaling in a sirtuin1 (SIRT1) dependent manner.[21] Oral administration of nimbolide to arthritis-induced rats significantly reduced rheumatoid arthritic score, paw volume, edema formation, and proinflammatory cytokines.[22]

Pooladanda et al.[23] investigated the protective effects of nimbolide in mitigating the complications associated with acute respiratory distress syndrome (ARDS) induced by bacterial LPS in RAW 264.7, THP-1, MLE-12, A549, and BEAS-2B cells *in vitro* and in C57BL/6 mice *in vivo*. Nimbolide significantly inhibited nitrosative-oxidative stress, as well as the expression of inflammatory cytokines, and chemokines. Nimbolide also suppressed the migration of neutrophils and mast cells, normalized LPS-induced hypothermia, and enhanced antioxidant defenses. Furthermore, nimbolide ameliorated TNF-α-regulated NF-κB and histone deacetylase-3 (HDAC-3) crosstalk by preventing the nuclear translocation of NF-κB and HDAC-3. These findings emphasize the potential use of nimbolide in the treatment of fibrosis and inflammatory diseases, besides underscoring its impact on cancer-related inflammation.

3.3. Cardioprotective and Hepatoprotective Effects. Recently, Li et al.[24] showed that nimbolide supplementation prevented doxorubicin-induced myocardial damage by modulating cardiac markers, ameliorating oxidative stress by enhancing antioxidants, and by suppressing apoptosis and inflammation. Nimbolide was reported to significantly lower the levels of cholesterol, free fatty acids and triglycerides, as well as lipid deposition-induced changes in primary hepatocytes. Additionally, nimbolide also improved hepatocyte function by multiple mechanisms including inhibition of reactive oxygen species (ROS)-induced oxidative DNA damage and lipid peroxidation, enhancement of antioxidant defenses, and by regulating the expression of liver X receptor-α (LXRα), peroxisome proliferator-activated receptor-γ (PPAR-γ), and sterol regulatory element-binding protein-1c (SREBP1c).[25]

3.4. Detoxifying, Antioxidant, and Antigenotoxic Effects. Xenobiotics such as drugs, toxins, and carcinogens are effectively detoxified/metabolized by phase I, II, and III xenobiotic-metabolizing enzymes. Phase I reactions catalyzed by cytochrome P450 (CYP) monooxygenases involve the biotransformation of xenobiotics into more polar intermediates, which are then conjugated by the phase II enzymes such as glutathione S-transferase (GST), and finally excreted with the help of phase III transporters.[26] Nimbolide protects against the deleterious effects of carcinogens by repressing the phase I carcinogen activation enzyme CYP as well as its isoforms CYP1A1 1A2, 2B and CYP1B1, and cytochrome b5, while simultaneously enhancing the activities of the phase II detoxification enzymes- GST and DT diaphorase (NAD(P)H:quinone oxidoreductase).[27] Nimbolide fractionated from neem flowers was shown to enhance the activity of the detoxifying enzyme quinone reductase in mouse hepatoma Hepa 1c1c7 cells.[28]

Nimbolide displayed concentration-dependent radical scavenging activity on a panel of *in vitro* assays and was found to be a more potent antioxidant than ascorbic acid. Administration of nimbolide enhanced the activities of superoxide dismutase, catalase, and glutathione-dependent enzymes in the hamster buccal pouch (HBP) model of oral cancer.[27] Neem leaf fractions containing nimbolide as a primary constituent were found to exert protective effects against H_2O_2-induced oxidative damage to pBR322 DNA and erythrocytes.[29] Nimbolide administration ameliorated oxidative stress, chromosomal aberrations, and formation of micronucleated polychromatic erythrocytes (MnPCEs) induced by the chemotherapeutic drug hydroxyurea in Wistar rats.[30]

3.5. Antiproliferative Effects. The antiproliferative effects of nimbolide have been extensively documented in a myriad of cancer cell lines. These include bladder cancer,[31] breast cancer,[32−36] cervical cancer,[37] choriocarcinoma,[38] colorectal cancer,[39−44] glioblastoma,[45] hematological malignancies,[46−49] hepatocellular carcinoma,[28,50,51] lung cancer,[52] nasopharyngeal cancer,[53] oral squamous cell carcinoma (OSCC),[54,55] osteosarcoma,[56] pancreatic cancer,[57,58] prostate cancer,[59,60] and renal cell carcinoma.[61] Nimbolide was found to be effective both as a single agent as well as in combination with other anticancer agents such as 5-fluorouracil. Several studies have demonstrated the chemotherapeutic effects of nimbolide in preclinical carcinogen-induced animal tumor models[27,62] as well as in tumor-xenografted[41,45,48,51,54,57] and transgenic[59] mouse models. In the 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis model, nimbolide exhibited both chemopreventive and chemotherapeutic effects. In fact, nimbolide exerted significant chemotherapeutic efficacy at concentrations (100 μg/kg bw) five times lower than that used for the reference compound wortmannin.[63] Nimbolide also induced significant tumor regression in the tumor xenografted and transgenic mouse models.

Collectively, these results underscore the protective effects of nimbolide against various disorders. However, more detailed studies on the potential applications of nimbolide as an antimicrobial agent both alone and in combination, the reversal of antibiotic resistance, as well as mechanistic insights on the mode of action, and translational relevance, need to be evaluated. The antifibrotic and anti-inflammatory effects of nimbolide can spur investigations on its efficacy on premalignant conditions such as oral submucous fibrosis and the impact on cancer-related inflammation. The antiproliferative effects of nimbolide may be ascribed to its antioxidant, antigenotoxic, and detoxification activities. The protective effects of nimbolide against different classes of reactive oxygen and nitrogen species and carcinogens as well as its ability to modulate the complex signaling networks orchestrating the DNA damage response (DDR) remain to be elucidated.

The molecular mechanisms of the anticancer effects of nimbolide are discussed in detail in the subsequent sections.

4. NIMBOLIDE PREVENTS THE ACQUISITION OF CANCER HALLMARKS

The development and progression of a malignant tumor involve the acquisition of ten essential attributes, collectively referred to as the hallmarks of cancer. These include sustaining proliferative signaling, insensitivity to growth suppressors,

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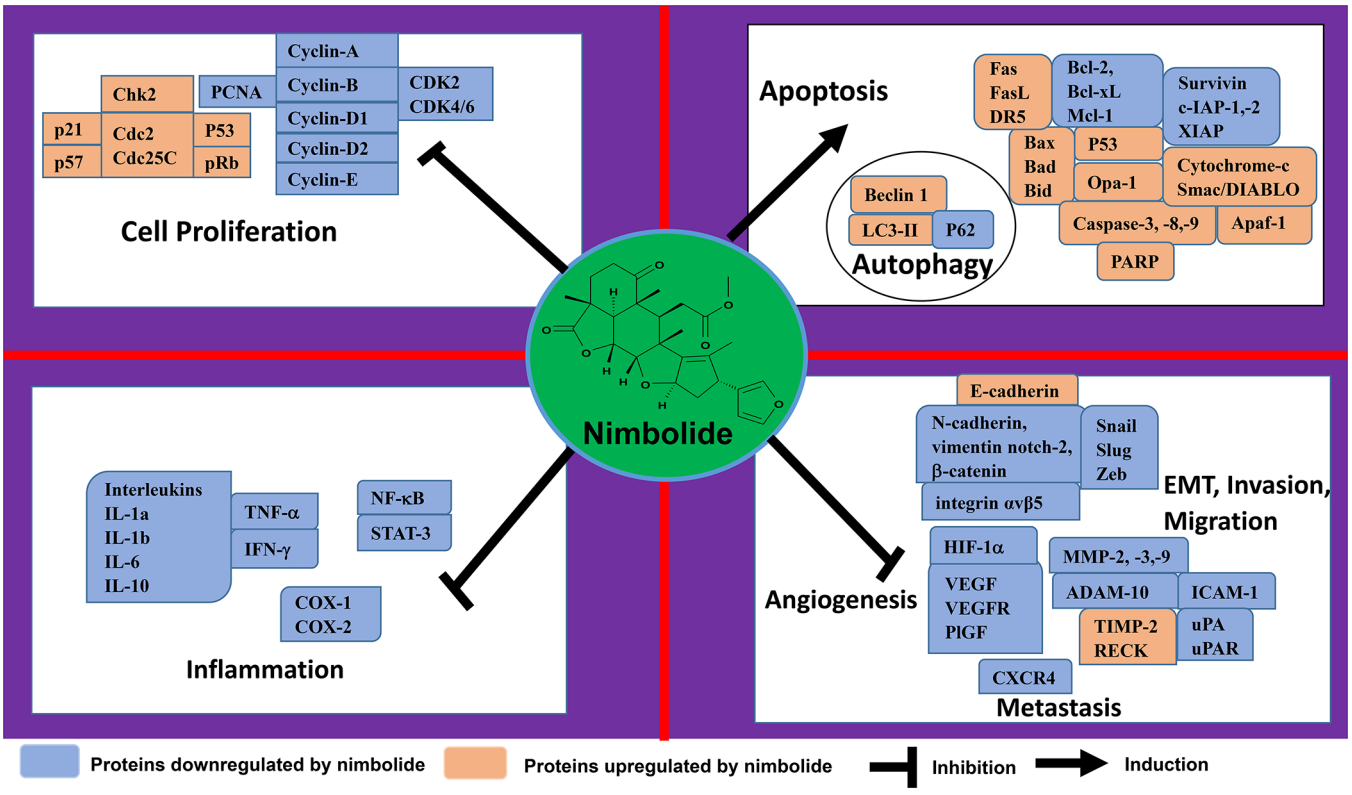

Figure 2. Nimbolide prevents the acquisition of hallmark capabilities of cancer. Nimbolide inhibits cell proliferation, inflammation, EMT, invasion, migration, angiogenesis, and metastasis and induces apoptosis in cancer cells *in vitro* and *in vivo*.

resisting cell death, enabling replicative immortality, evading immune surveillance, inducing angiogenesis, activating tissue invasion and metastasis, reprogramming of energy metabolism, genome instability, and inflammation.[64] Several studies have unraveled the inhibitory influence of nimbolide on the acquisition of cancer hallmarks that are described below and illustrated in Figure 2.

4.1. Nimbolide Halts Cell Cycle Progression and Restrains Cell Proliferation. Dysregulation of the cell cycle with consequent uncontrolled cell proliferation is a defining feature of cancer cells. This is characterized by altered expression as well as phosphorylation of the cell cycle regulators, cyclins, cyclin-dependent kinases (CDKs), proliferating cell nuclear antigen (PCNA), retinoblastoma protein (Rb), p53, and p21.

Nimbolide inhibited the growth of a panel of cancer cell lines in a concentration- and time-dependent manner by modulating the expression of cell cycle regulatory proteins and arresting the cell cycle. Roy et al.[39] demonstrated that nimbolide suppressed the growth of HT-29 colon cancer cells by inducing cell cycle arrest at the G2/M and G0/G1 phases. Further, nimbolide-induced cell cycle arrest at G_2/M phase was accompanied by upregulation of p21, cyclin D2, and Chk-2 with downregulation of cyclin-A, cyclin- E, cdk-2, and Rad-17. In a follow-up of this study, Roy et al.[46] reported growth inhibitory effects of nimbolide on U937 lymphoma, HL-60 leukemia, THP1 leukemia monocytic, and B16 melanoma cell lines. U937 cells treated with nimbolide showed cell cycle disruption initially in S and G2/M phases and subsequently in G0/G1 phase. Exposure of cells to higher concentrations of nimbolide for a longer duration induced DNA damage with an increase in sub-G1 fraction accompanied by a decrease in the number of cells in all other phases of the cell cycle.

Nimbolide was shown to suppress the growth of glioblastoma multiforme (GBM) cells by inhibiting CDK4/6 activity, with consequent hypophosphorylation of Rb and cell cycle arrest at the G1/S phase.[45] Nimbolide was reported to interfere with cell cycle kinetics in WiDr and HCT-116 colon cancer cells by repressing cyclin A/cyclin D1 leading to S phase arrest.[40] Flow cytometric analysis revealed that exposure of renal carcinoma cells to nimbolide induced G2/M phase arrest with increased expression of p53, cdc2, cdc25c and decreased the expression of cyclin A and cyclin B.[61] Treatment with nimbolide inhibited the proliferation of EJ and 5637 bladder cancer cells by inducing G2/M phase arrest through both Chk2-Cdc25C-Cdc2/cyclin B1-Wee1 and Chk2-p21WAF1-Cdc2/cyclin B1-Wee1 pathways.[31]

In BeWo choriocarcinoma cells, the cytotoxic effect of nimbolide was observed within 7 h of incubation accompanied by downregulation of PCNA, a cofactor for DNA polymerase that plays a pivotal role in the cell cycle.[38] Nimbolide suppressed the viability of the human cervical cancer HeLa cells and induced cell cycle arrest at G0/G1 phase associated with p53-dependent accumulation of p21 Cip1/waf1 and downregulation of cyclin B, cyclin D1, and PCNA.[37] In SCC131 and SCC4 oral cancer cells, nimbolide attenuated cell proliferation by increasing the expression of p21 with simultaneous downregulation of cyclin D1 resulting in accumulation of cells in subG0/G1. Notably, nimbolide stimulated glycogen synthase kinase-3β (GSK-3β)-mediated phosphorylation of cyclin D1 at Thr286 with consequent relocalization of cyclin D1 from the nucleus to the cytosol and subsequent degradation. Thus, nimbolide blocks cell cycle

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J. Med. Chem. XXXX, XXX, XXX−XXX

progression and drives cancer cells to apoptotic cell death.[63] Spradlin et al.[36] demonstrated that nimbolide targets the N-terminal cysteine-8 (C8) of the E3 ubiquitin ligase RNF114 leading to impaired ubiquitination of RNF114 substrates such as p21 and p57, resulting in their accumulation with consequent inhibition of breast cancer cell proliferation.

4.2. Nimbolide Induces Cell Death by Apoptosis. One of the major hallmarks of cancer is the ability to resist the death of cells that have undergone DNA damage. Accumulating evidence indicates that apoptosis evasion by malignant tumors enables tumor progression besides blunting therapeutic response. Reinstating apoptosis in tumor cells is a promising approach for cancer prevention and therapeutics.[65] An overwhelming body of evidence indicates that nimbolide exerts its antiproliferative effects by inducing apoptosis. The mechanism of apoptosis induction by nimbolide has been reviewed by us earlier.[66] Here, we summarize the literature on the effect of nimbolide on apoptosis pathways as well as the crosstalk between apoptosis and autophagy.

Exposure of cancer cells to nimbolide induced stereotypical changes characteristic of apoptosis including alterations in nuclear morphology such as fragmentation and condensation, DNA fragmentation, subdiploid G1 peak, and annexin-V staining. Several studies by us and others have revealed that nimbolide transduces apoptosis via both the extrinsic (death receptor) and intrinsic (mitochondrial) pathways. Nimbolide also facilitates crosstalk between the two pathways of apoptosis by stimulating procaspase-8-catalyzed cleavage of BH3 interacting-domain death agonist (BID). The major mechanisms by which nimbolide induces apoptosis include some or a combination of the following: upregulation of death receptors, a shift in the expression of BCL2 family members to a proapoptotic phenotype, increased expression of p53, and downregulation of inhibitors of apoptosis (IAP) proteins, eventually culminating in the activation of caspases.[66−68]

Nimbolide-induced mitochondria-mediated apoptotic cell death has attracted the major focus of research attention. Nimbolide has been demonstrated to induce the generation of ROS with consequent mitochondrial outer membrane permeabilization (MOMP), associated with increased expression of Opa-1, a dynamin-related mitochondrial protein involved in cristae remodeling and release of apoptogenic molecules such as cytochrome c and second mitochondria-derived activator of caspase (SMAC)/direct IAP binding protein with low pI (DIABLO) from the mitochondria into the cytosol resulting in activation of the caspase cascade and cleavage of poly(ADP ribose) polymerase (PARP).[37,38,57,62] Kumar et al.[58] provided evidence to show that nimbolide induced mitochondrial dysfunction, caspase activation, and apoptosis of pancreatic cancer cells with greater efficacy than the chemotherapeutic agents, gemcitabine and sorafenib. Nimbolide also reduced mutant p53 and enhanced mitochondrial ROS. In human Huh-7 and PLC/PRF/5 hepatocellular carcinoma cells, nimbolide induced mitochondrial dysfunction with downregulation of MCL-1 and BCL-2 and increased expression of BAX and activation of caspases.[51] In osteosarcoma cells, nimbolide induced apoptosis by multiple mechanisms including increased ROS generation, mitochondrial dysfunction, endoplasmic reticulum stress, and caspase activation.[56]

Nimbolide has been reported to induce apoptosis in both T- and B-cell lymphomas. Recently, Jaiswara et al.[69] demonstrated that nimbolide induced apoptosis of Dalton's, HuT-78, and J6 lymphoma cells by enhancing ROS, p53, and Bax with cleavage of caspase-3. Interestingly, apoptosis induction by nimbolide was associated with the reversal of dysregulated cellular metabolism as revealed by downregulation of hypoxia-inducible factor-1α, glucose transporter-3, hexokinase II, and pyruvate dehydrogenase kinase-1, with consequent suppression of glycolysis and activation of oxidative phosphorylation. Nimbolide promotes apoptosis in B-cell cancer models such as multiple myeloma and non-Hodgkin lymphoma. Waldenströms macroglobulinemia (WM), an indolent, non-Hodgkin B-cell lymphoma, was found to be highly sensitive to the cytotoxicity of nimbolide. WM cells including those that are drug-resistant (bortezomib or ibrutinib) undergo mitochondria-mediated apoptosis when exposed to nimbolide. Using cheminformatics-based approaches, BCL2 was identified as the preferential binding partner of nimbolide. This was validated *in vitro* wherein nimbolide-induced apoptosis in BCL2-dependent RS4;11 tumor cells was significantly higher than in BCL2 mutated Jurkat BCL2(Ser70-Ala) cells as well as *in vivo* in WM tumor xenografted mice.[48]

Nimbolide is believed to potentiate apoptosis by inhibiting the IAPs, IAP-1, IAP-2, neuronal apoptosis inhibitory protein (NAIP), I-FLICE, XIAP, and survivin in a wide variety of cancer cell lines by releasing the potent IAP inhibitors cytochrome c and SMAC/DIABLO into the cytosol. In addition, nimbolide also induced trafficking of survivin from the cytosol where it exerts IAP function, to the nucleus where it favors apoptosis by activation of proapoptotic BAX and p53.[62,66] Using apoptosis antibody array, the IAP family proteins XIAP, c-IAP1, and c-IAP2, which are potent inhibitors of caspases as well as SMAC/DIABLO, were identified as the major targets of nimbolide.[51]

Nimbolide induced caspase-mediated apoptosis by both the extrinsic and intrinsic pathways in 786-O and A-498 renal cell carcinoma cells, human nasopharyngeal carcinoma HONE-1 cells as well as in DMBA-induced HBP carcinomas as reflected by increased activities of caspase-3, −8, and −9, associated with enhanced expression of death receptor 5 (DR5), C/EBP homologous protein (CHOP), Fas, FasL, and proapoptotic members of the BCL2 family.[53,62] Likewise, the growth inhibitory effects of nimbolide on estrogen-dependent (MCF-7) and -independent (MDA-MB-231) human breast cancer cell lines were ascribed to both the intrinsic and extrinsic pathways.[32] Nimbolide, when administered in combination with TNF-α, significantly enhanced apoptosis of HT-29 colon adenocarcinoma cells by upregulating the expression of Bid and DR5 and caspase-3 via activation of the c-Jun N-terminal kinase (JNK) pathway.[42] Nimbolide-mediated apoptosis was associated with cleavage of PARP, a caspase-3 substrate. Additionally, treatment with caspase inhibitors rescued cancer cells from the cytotoxicity of nimbolide, confirming that nimbolide-mediated apoptosis is caspase-dependent.[37,38,50]

4.3. Nimbolide Regulates Autophagy to Induce Apoptosis. Although apoptosis is the major mechanism by which chemotherapeutic agents induce cell death, regulation of autophagy, a cytoprotective survival mechanism that tumor cells use to evade apoptosis has recently emerged as a promising strategy to improve therapeutic outcome.[70] Apoptosis and autophagy are intricately interconnected through protein networks mediated chiefly by ATG5, Bcl-2, and Beclin-1.[71,72]

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J. Med. Chem. XXXX, XXX, XXX−XXX

Nimbolide has been documented to potentiate functional crosstalk between apoptosis and autophagy. In pancreatic cancer cells, nimbolide stimulates both apoptosis and autophagy. While autophagy serves as an adaptive stress response to prolong cell survival, mitochondria-mediated apoptosis induced by ROS generation is believed to be responsible for nimbolide-induced death of pancreatic cancer cells.[57] Nimbolide was shown to promote apoptosis via autophagy as evidenced by overexpression of Beclin 1 and LC3B with downregulation of p62, mechanistic target of rapamycin (mTOR), and BCL-2 in MCF-7 and MDA-MB-231 breast cancer cells.[35] Nimbolide was found to regulate autophagy signaling by enhancing the expression of Beclin 1 while simultaneously reducing the expression of LC-3 and p62 in TGF-β1-induced *in vitro* and bleomycin-induced *in vivo* models of pulmonary fibrosis.[18]

Studies from this laboratory provided proof-of-concept that nimbolide toggles between apoptosis and autophagy using SCC131 and SCC4 oral cancer cells and the HBP model of oral oncogenesis. Nimbolide induced classic hallmarks of autophagy such as accumulation of acidic vesicles, enhanced expression of Beclin-1, LC3-II, and truncated ATG5 associated with degradation of p62. Time-course experiments revealed that nimbolide initially promotes cytoprotective autophagy and subsequently switches over to apoptosis via modulation of the phosphatidylinositol 3-kinase (PI3K)/Akt/GSK3β signaling cascade. Pharmacological inhibition and siRNA ablation of ATG5 and Beclin-1 augmented apoptosis, further confirming that nimbolide stimulates autophagy-dependent apoptosis.[55]

4.4. Nimbolide Inhibits Tumor Invasion, Angiogenesis, and Metastasis. Tumor metastasis, the major cause of cancer mortality, encompasses a complex series of alterations termed the metastatic cascade by which the tumor cells are disseminated from the primary site via circulation to distant sites or organs to establish secondary tumors. Invasion, a crucial step in metastasis involves EMT, followed by degradation of the ECM catalyzed by a family of zinc-dependent endopeptidases known as matrix metalloproteinases (MMPs) that are regulated by tissue inhibitors of MMPs (TIMPs) and reversion-inducing cysteine-rich protein with Kazal motifs (RECK). During ECM processing by MMPs, various proangiogenic molecules are released, predominantly the vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α), which promote angiogenesis, an essential prerequisite for tumor progression.[73−75]

Babykutty et al.[40] demonstrated that nimbolide inhibits invasion, migration, and angiogenesis in WiDr and HCT-116 colon cancer cells by decreasing the expression of MMP-2 and −9 and VEGF via blockade of ERK1/2 and NF-κB signaling. Nimbolide treatment suppressed the growth of colorectal cancer xenografts by downregulating the expression of proteins involved in invasion (MMP-9, ICAM-1), angiogenesis (VEGF), and metastasis (CXCR4) and modulated the proinflammatory microenvironment.[41] Nimbolide-treated non-small cell lung carcinoma (NSCLC) cells exhibited a significant decrease in the expression of MMP-3 and Snail with an increase in the expression of E-cadherin.[52]

Using the scratch assay, the migratory abilities of MIAPaCa-2, PANC-1, and HPAC pancreatic cancer cells were shown to be suppressed. Nimbolide prevented EMT in pancreatic cancer cells by increasing the expression of E-cadherin, an epithelial marker, while simultaneously decreasing the expression of the mesenchymal markers, vimentin, β-catenin, notch-2, N-cadherin, and the transcription factors Snail, Slug, and Zeb. Additionally, nimbolide also inhibited EMT and metastasis in a pancreatic cancer xenograft model.[57] Nimbolide inhibited the migration of osteosarcoma cells by decreasing the expression of integrin $\alpha v\beta 5$ via modulation of PI3K/Akt and NF-κB signaling.[56] Nimbolide blocked tumor invasion and metastasis by downregulating the expression of MMP-9, intercellular adhesion molecule-1 (ICAM-1), CXCR4 (chemokine receptor type 4), and VEGF via abrogation of NF-κB signaling.[41,47] The inhibitory effect of nimbolide on the invasion and migration of breast cancer cells was demonstrated by transwell invasion and wound healing assays. Nimbolide was found to downregulate the expression of urokinase plasminogen activator (uPA) and uPA receptor (uPAR) genes and chemokines as well as MMPs, VEGF, and NF-κB signaling.[34] Nimbolide hampered wound healing, migration, and invasion in bladder cancer EJ and 5637 cells by inhibiting MMP-9 as well as the binding activity of the transcription factors NF-κB, Sp-1, and AP-1, which regulate MMP-9.[31]

Extensive studies from this laboratory have provided compelling evidence for the anti-invasive, and antiangiogenic potential of nimbolide as reflected by the downregulation of MMPs, placental growth factor, VEGF, VEGF receptors, and HIF-1α accompanied by increased expression of TIMP-2 and RECK and reduced microvascular density, an independent marker of angiogenesis in the HBP carcinogenesis model.[27,76] Nimbolide administration reversed "RECKlessness", a hallmark of cancer and abrogated VEGF and Notch signaling in oral cancer cells *in vitro* and in HBP carcinomas *in vivo*. Upregulation of RECK, a common negative target of oncogenic signaling pathways, resulted in diminished activities of MMPs and a disintegrin and metalloproteinase-10 (ADAM-10). Nimbolide was found to activate RECK and inhibit proinvasive and proangiogenic molecules in SCC131 and SCC4 oral cancer and EAhy926 endothelial cells under hypoxic conditions. Transfection with RECK plasmid/siRNA indicated that nimbolide activated RECK by targeting HIF-1α and miR-21. HIF-1α knockdown and nimbolide administration led to RECK overexpression. Furthermore, molecular docking studies revealed that nimbolide interacts with Arg 33, His 98, Thr 99, and Ser 34 in the PAS-A domain of HIF-1α, suggesting HIF-1α as a likely target of nimbolide besides underscoring its antiangiogenic potential.[76]

4.5. Nimbolide Inhibits Cancer-Related Inflammation. Chronic inflammation is recognized to potentiate tumorigenesis by releasing growth factors, cytokines, chemokines, chemokine receptors, and MMPs that promote a pro-growth microenvironment and facilitate the acquisition of cancer hallmarks. Increased synthesis and secretion of cytokines also activates the activity of transcription factors such as NF-κB and signal transducer and activator of transcription3 (STAT3), triggering downstream signaling cascades that stimulate tumor development and progression.[77]

Treatment of OSCC xenografted mouse models with a highly pure super critical CO_2 neem leaf extract (SCNE) as well as nimbolide, a primary component of SCNE, significantly reduced the serum levels of pro-tumor inflammatory cytokines involved in OSCC development and progression such as IL-1β, IL-1α, IL-6, IL-10, TNFα, and IFNγ. This was associated with inhibition of pro-tumor inflammatory signaling cascade as reflected by blockade of NFκB, STAT3, and cyclooxygenase2 (COX2) expression and/or activity in OSCC cell lines.[54] Likewise, treatment of HCT116 and HT29 human colon

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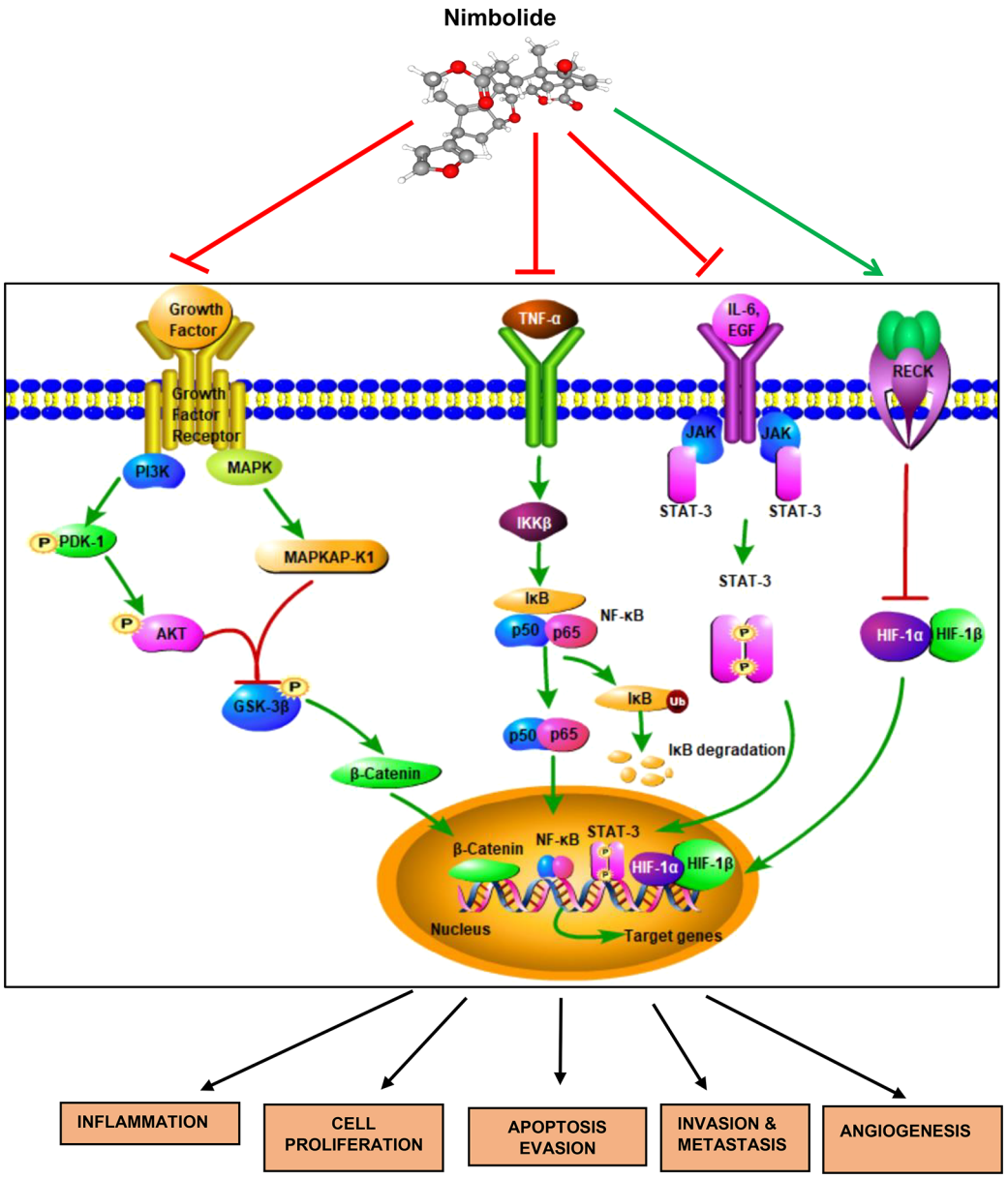

Figure 3. Nimbolide abrogates key oncogenic signaling pathways. Nimbolide inhibits PI3K/Akt/MAPK signaling to activate GSK-3β with consequent trafficking of β-catenin away from the nucleus. Nimbolide inhibits multiple steps in the NF-κB signaling cascade including IKKβ activation, IκB degradation, phosphorylation of IKKα/β, IκBα and NF-κB subunits, nuclear translocation, and DNA binding activity of NF-κB. Nimbolide impedes aberrant activation of the JAK/STAT3 pathway by preventing phosphorylation of STAT3, a prerequisite for its nuclear translocation. Likewise, by activating RECK, nimbolide represses HIF-1α, preventing its nuclear translocation. Thus, nimbolide blocks DNA binding activity of oncogenic transcription factors such as β-catenin, NF-κB, STAT3, and HIF1-α and transactivation of genes implicated in the acquisition of hallmark capabilities of cancer.

cancer cells with SCNE or nimbolide reduced the expression of the transcriptional factors, STAT3 and NF-κB associated with downregulation of markers of inflammation (COX1, IL-6, and TNF-α) and invasion (MMP2 and MMP9).[43] The impact of nimbolide treatment on NF-κB and STAT3 signaling are discussed in the next section.

The foregoing findings provide substantial evidence for the modulatory effects of nimbolide on major hallmarks of cancer. There is ample evidence in the literature to support the antiproliferative and apoptosis augmenting effects of nimbolide in diverse malignant cells *in vitro* as well as in animal tumor models. Given that apoptosis evasion enables cancer cell survival that facilitates the accumulation of mutations leading to tumor progression, the apoptosis-inducing potential of nimbolide can create a suitable environment to inhibit tumor invasion and angiogenesis, major drivers of metastasis, besides overcoming resistance to chemotherapeutic agents. There is, however, paucity of information on the effect of nimbolide on DNA repair systems that could mitigate DNA damage, a critical event in neoplastic transformation. It is also important to investigate whether nimbolide orchestrates other types of cell death; primarily immunogenic cell death that has received increasing attention as a useful therapeutic strategy.[78] More systematic studies on the effect of nimbolide on the regulation of immune and inflammatory cells in the tumor microenvironment from a mechanistic perspective are warranted.

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J. Med. Chem. XXXX, XXX, XXX−XXX

5. NIMBOLIDE ABROGATES ONCOGENIC SIGNALING

Cellular signaling pathways play a pivotal role in regulating various cellular processes such as cell division, differentiation, apoptosis, angiogenesis, among several others. These pathways are interconnected networks whose components include growth factors, growth factor receptors, kinases, cytoplasmic proteins, nuclear proteins, and transcription factors. Aberrant activation of signaling pathways enables the acquisition of cancer hallmarks.[79,80] Nimbolide exerts modulatory effects on oncogenic signaling, thereby preventing the acquisition of hallmark capabilities of cancer (Figure 3).

5.1. Nimbolide Modulates PI3K/Akt/MAPK Signaling Networks. GSK-3β, a serine/threonine kinase, is a central hub in cellular signaling that elicits regulatory influences on metabolism, insulin signaling, differentiation, cell proliferation, apoptosis, EMT, cancer stemness, and chemoresistance. GSK3β, a key suppressor of the Wnt/β-catenin pathway, undergoes activation/inactivation by site-specific phosphorylation at Tyr216/Ser9 residues. Several oncogenic signaling kinases inactivate GSK-3β including phosphatidylinositol 3-kinase (PI3K/Akt) and mitogen-activated protein kinase (MAPK)/extracellular signal-related kinases (ERK). GSK-3β interacts with multiple signaling pathways and noncoding RNAs (ncRNAs) to influence various hallmarks of cancer. Dysregulation of GSK-3β has been documented in diverse malignancies. GSK-3β is regarded as a potential therapeutic target in cancer.[81]

Molecular docking studies revealed that nimbolide docks to key signaling kinases in the PI3K/Akt/MAPK/GSK3β signaling axis. Nimbolide forms hydrogen bonds with Tyr-134 and Gln-185 residues on the kinase domain of GSK-3β and with Gly 34, Lys 54, Arg 67, Glu 71, and Ser 153 in the kinase domain of ERK2, underscoring its kinase inhibitory effects. Nimbolide interacts with Gln 231 in the RAS binding domain of PI3Kγ that may inhibit RAS binding vital for activation of PI3Kγ and downstream signaling. Interaction of nimbolide with Glu 193, Asp 275, and Gly 295 residues in the kinase domain of Akt 2 may hinder translocation of Akt from the cytoplasm to the plasma membrane, thereby preventing PDK-1-catalyzed Ser473 phosphorylation essential for its activation.[63]

Experimental studies *in vitro* and *in vivo* validated the *in silico* findings. In oral cancer cell lines and in HBP carcinomas, nimbolide inhibited cell proliferation and induced apoptosis by abrogating PI3K/Akt signaling with consequent activation of GSK-3β. Additionally, nimbolide stimulated GSK-3β activity by decreasing p-GSK-3βSer9, the inactive form of the enzyme, and upregulating miR-126 and let-7, well-known inducers of GSK-3β. Nimbolide also induced cytoplasmic accumulation of β-catenin, facilitating its phosphorylation and degradation. PI3K overexpression promoted autophagy that was alleviated by nimbolide administration. Nimbolide was found to inhibit phosphorylation of Akt at Ser473 with a consequent increase in p-GSK3βTyr216, the active form of GSK3β that inhibits autophagy and induces apoptosis.[55,63] Subramani et al.[57] demonstrated that nimbolide inhibited proliferation and metastasis of pancreatic cancer cells via blockade of PI3K/AKT/mTOR and ERK signaling. Further, using the autophagy inhibitors 3-methyladenine and chloroquine as well as the apoptosis inhibitor z-VAD-fmk, nimbolide was shown to mediate these effects through ROS-induced mitochondrial apoptosis but not via autophagy.

Nimbolide suppressed the phosphorylation of the p85 subunit of PI3K and Akt at Ser473 in osteosarcoma cells that was blocked by pretreatment with inhibitors of PI3K (Ly294002) and Akt (Akti).[56] Similarly, in GBM cells and in tumor xenografts, nimbolide was reported to impede growth factor-induced phosphorylation of Akt.[45] Nimbolide was shown to inhibit cell proliferation and induce apoptosis of breast and prostate cancer cell lines by restraining insulin growth factor (IGF) signaling via the PI3K/Akt and MAPK pathways.[33,60,82,83]

Nimbolide exerted modulatory effects on the MAPK signaling pathway comprising ERK1/2, JNK1/2/3, and p38-MAPK. Nimbolide induced caspase-mediated apoptosis by inhibiting ERK1/2 and activating p38 and JNK1/2 in colon cancer cells.[40] Nimbolide inhibited TNF-α-mediated apoptosis by upregulation of DR5 expression via the JNK pathway in colon cancer. Chien et al.[53] demonstrated that the apoptosis-inducing potential of nimbolide in HONE-1 nasopharyngeal cancer cells is mediated through ERK1/2 inhibition. Incubation with nimbolide prevented the invasion and migration of NSCLC cells by inactivation of ERK1/2. Knockdown of dual-specificity protein phosphatase 4 (DUSP4), a negative regulator of ERK1/2 attenuated nimbolide-mediated inhibition of NSCLC invasion and migration, confirming that nimbolide targets ERK1/2.[52] In hepatocellular carcinoma cells, nimbolide effectively inhibited c-IAP1 expression and induced apoptosis via targeting the ERK pathway.[51] In bladder cancer EJ and 5637 cells, nimbolide was shown to increase JNK phosphorylation and simultaneously decrease the phosphorylation of p38MAPK and AKT.[31]

5.2. Nimbolide Abrogates NF-κB Signaling. The Rel family transcription factor, nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), is present as an inactive heterodimer of p50 and p65 subunits complexed with the inhibitor of κB (IκB) in the cytoplasm. Aberrant activation of NF-κB documented in diverse malignant neoplasms, involves the release of the heterodimer from IκB, followed by nuclear translocation and transactivation of genes involved in the acquisition of cancer hallmarks such as cell proliferation, apoptosis avoidance, inflammation, invasion, angiogenesis, metastasis, and therapy resistance.[84]

Experiments by different research groups established that nimbolide inhibits multiple steps in the NF-κB signaling cascade by blocking the activation of inhibitor of κB kinaseβ (IKKβ), IκB degradation, phosphorylation of IKKα/β, IκBα and NF-κB subunits, nuclear translocation, and DNA binding activity of NF-κB.[37,40,41,56,62] Nimbolide induced mitochondria-mediated apoptosis by blocking both constitutive and TNF-α-induced NFκB activation in HepG2 hepatocellular carcinoma cells transfected with NFκB-responsive luciferase reporter plasmid.[50] Nimbolide treatment decreased the phosphorylation of IKKα/β, IκBα and the p65 subunit of NF-κB in osteosarcoma cells. Further, nimbolide-induced decrease in NF-κB activity was confirmed using a κB-luciferase activity assay. Pretreatment with the NF-κB inhibitor TPCK blocked the effects of nimbolide in a migration assay and restored activity of the NF-κB luciferase reporter, illustrating the modulatory effects of nimbolide on NF-κB signaling.[56] Gupta et al.[47] provided evidence for direct interaction of nimbolide with the Cys 179 of IKK-β that could prevent phosphorylation and degradation of IκBα, leading to inactivation of NF-κB and transactivation of NF-κB responsive genes.

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J. Med. Chem. XXXX, XXX, XXX−XXX

5.3. Nimbolide Targets the JAK/STAT-3 Pathway. The transcription factor, STAT3 when activated by cytokines and growth factors, undergoes phosphorylation by Janus kinase (JAK) followed by homodimerization and translocation to the nucleus, where it regulates the expression of genes vital for various cellular processes such as cell differentiation, proliferation, angiogenesis, and immune function. Aberrant activation of JAK/STAT3 signaling reported in diverse malignant tumors also occurs concurrently with other kinase-driven pathways.[85]

The anticancer effects of nimbolide on human PCa DU145 and LNCaP prostate cancer cells and in the transgenic adenocarcinoma of mouse prostate (TRAMP) model were found to be mediated via ROS-induced inhibition of both constitutive and IL-6 inducible STAT3 signaling.[59] An ethanol-soluble fraction of *A. indica* leaves containing nimbolide as the principal cytotoxic constituent suppressed the proliferation of GBM cells *in vitro* and in tumor xenografts by downregulating BCL2 and blocking growth factor-induced phosphorylation of Akt, ERK1/2, and STAT3.[45] Morris et al.[54] reported the anticancer effects of nimbolide in SCC4, Cal27, and HSC3 oral cancer cell lines and in xenografted nude mice generated from the three cell lines. Nimbolide displayed greater cytotoxicity on OSCC cell lines than the nonsteroidal anti-inflammatory drug (NSAID) celecoxib. Treatment with nimbolide decreased the expression of COX2 and nuclear NFκBp65 as well as pSTAT3, pAKT, and pERK1/2 in all three cell lines. In addition, cell migration was significantly reduced associated with down-regulation of MMP-2 and MMP-9.

5.4. Nimbolide Pins Down Pin1. Peptidyl-prolyl cis−trans isomerase NIMA-interacting 1 (Pin1), an enzyme that catalyzes the isomerization of phosphorylated serine/threonine-proline (pSer/Thr-Pro) motifs, activates a number of oncogenic proteins and signaling pathways involved in the acquisition of cancer hallmarks. Pin1 is aberrantly activated in diverse malignant tumors and has emerged as an attractive therapeutic target. da Costa et al.[86] used various computational approaches such as molecular docking, pharmacophore filtering, and structural clustering allied to molecular dynamics simulations and binding free energy calculations to show that nimbolide interacts with high affinity with the Pin1 substrate peptide and destabilizes Pin1 structure.

Taken together, nimbolide apparently targets transcription factors that are the ultimate executors of oncogenic signaling. Transcription factors are vital components of complex signaling networks that regulate a plethora of diverse cellular processes including cell division, differentiation, migration, cell death, and homeostasis. The human transcription factor repertoire of over 1600 proteins acts individually or as an ensemble to recognize specific DNA sequences and regulate gene expression.[87] Aberrant expression of transcription factors contributes to dysregulated signaling and acquisition of hallmark capabilities of cancer. There is mounting evidence to indicate that transcription factors such as NF-κB, β-catenin, HIF-1α, and STAT-3 are potential druggable targets of nimbolide. The use of three-dimensional cultures, cancer stem cells and cell-based reporter assays, and protein−protein interactions can shed light on the effects on specific signaling pathways and molecular targets. Studies that provide insights on the molecular mechanisms by which nimbolide targets transcription factors could impact cancer therapeutics as well as provide scope for designing analogues with higher efficacy.

6. NIMBOLIDE MODULATES THE EPIGENOME

Epigenetic alterations that play a critical role in tumor progression include DNA methylation, histone modifications, and changes in ncRNAs. While aberrant promoter hypermethylation can cause silencing of TSGs, global hypomethylation can activate protooncogenes leading to genomic instability. Likewise, histone acetylation enhances gene transcription, whereas deacetylation leads to gene repression. The ncRNAs, primarily microRNAs (miRs), and the long non-coding RNAs (lncRNAS) have gained increasing attention as important modulators of oncogenic signaling pathways and acquisition of cancer hallmarks.[88]

Several studies have documented the modulatory effects of nimbolide on the epigenome. Nimbolide suppressed the proliferation of HCT116 and HT29 colon cancer cells by inhibiting the expression and activities of DNA methyltransferases (DNMT) and histone deacetylases (HDACs), thereby reducing methylation and stimulating the acetylation of the p16 gene promoter with consequent reexpression of the p16 tumor suppressor protein.[44] Nimbolide administration inhibited HDAC1 that plays a vital role in cell proliferation, apoptosis avoidance, and chemoresistance in a hamster model of oral carcinogenesis.[27] Nimbolide promoted acetylation of H3K27 by inhibiting HDAC2 to induce autophagy-driven apoptotic cell death of MDA-MB-231 and MCF-7 breast cancer cells.[35]

Nimbolide effectively modulated the expression of ncRNAs in oral cancer cells *in vitro* and in the HBP model *in vivo*. Administration of nimbolide significantly increased the expression of miR-126 and Let-7, which are known to induce GSK-3β.[63] The addition of nimbolide to SCC4 cells overexpressing miR-126 upregulated the expression of GSK-3β accompanied by downregulation of Homeobox transcript antisense intergenic RNA (HOTAIR), a lncRNA that sponges miR-126, indicating that nimbolide induces activation of GSK-3β via modulation of ncRNAs.[55] Nimbolide was shown to function as an antagonist of miR-21, an oncomiR that inhibits RECK expression to inhibit invasion and angiogenesis.[76]

To sum up, epigenetic remodelling by phytochemicals has opened up new opportunities and challenges in cancer therapy. There are very few reports on the epigenetic modifications induced by nimbolide. More intensive studies on global epigenetic changes, especially on those that influence oncogenic signaling networks, cancer hallmarks, and chemoresistance, are warranted to expand the molecular target repertoire of nimbolide.

7. NIMBOLIDE IS AN EFFECTIVE CHEMOSENSITIZER

Resistance to chemotherapeutic drugs remains a major challenge in cancer treatment. In particular, the development of multidrug resistance (MDR) has become a major contributor to chemotherapy failure and cancer mortality. Among the several mechanisms implicated in MDR, enhanced efflux of drugs by the ATP-binding cassette (ABC) transporters has assumed increased significance. Natural products that are able to reverse or overcome MDR have received growing attention in recent years as chemosensitizers.[89]

Mahmoud et al.[49] explored the cytotoxicity of nimbolide against the well-established MDR mechanisms ABCB1, ABCG2, ABCB5, TP53, and epidermal growth factor receptor. P-glycoprotein (ABCB1/MDR1)-overexpressing CEM/ADR5000 multidrug-resistant leukemic cells exhibited hyper-

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J. Med. Chem. XXXX, XXX, XXX−XXX

sensitivity to nimbolide compared to the CCRF-CEM drug-sensitive parental leukemia cells, a phenomenon known as collateral sensitivity. Nimbolide circumvented MDR by modulating the expressions of the tumor suppressor protein phosphatase and tensin homologue (PTEN), and the transcription factors, HIF1α and forkhead box O transcription factor1 (FOXO1). Upregulation of PTEN was suggested to inhibit PI3K/AKT/mTOR signaling with consequent negative regulation of IKK/NFκB signaling, degradation of HIF1α, and suppression of P-glycoprotein. Additionally, inhibition of PI3K/AKT/mTOR signaling can also lead to activation of FOXO1, which is known to antagonize MYC and reduce intracellular ROS levels. Alam et al.[90] demonstrated that nimbolide in combination with Akt Inhibitor VIII synergistically induced Bax-dependent cell death by targeting the Akt pathway in SCC9/SCC4-cisplatin-resistant oral cancer cells that could be beneficial for chemosensitizing cisplatin-resistant human OSCC.

Cancer stem cells (CSCs) that have the ability to self-renew and differentiate into malignant cells are believed to be responsible for drug resistance as well as cancer relapse. Selective targeting of CSCs has emerged as a promising strategy to overcome drug resistance.[91] In pancreatic cancer cells, nimbolide reduced the population of $CD44^+$ cells, thereby inhibiting the CSC population.[58] However, more in-depth studies are required to establish the signaling pathways and cell surface markers through which nimbolide targets CSCs in a variety of malignant tumors.

8. PATENTS ON NIMBOLIDE

Several patents have been granted for the anticancer activities of neem extracts. A US patent (US 7.179.927 B2) was granted for a method of extraction of neem components including azadirachtin and nimbolide. Patents specifically pertaining to nimbolide, although few are significant. A pharmaceutical formulation comprising nimbolide, nimbandiol, 2′,3′dihydronimbolide, and 28-dihydronimbolide was reported to modulate at least one of integrin βi activity, calreticulin activity, and focal adhesion kinase activity in cancer cells (WO/2015/035199A1). A recent patent publication relates to the synthesis and anticancer activities of nimbolide analogues (20190211027). One of the analogues, IM-1372-Kn-13 (A4), was found to inhibit the growth of pancreatic cells *in vitro* and in a xenograft model *in vivo*. Acute toxicity studies failed to show toxic effects up to 50 mg/kg.

9. PHARMACOKINETIC PROFILE OF NIMBOLIDE

Pharmacokinetics (PK) plays a vital role in various stages of the drug development process including generation of lead compounds, target identification and validation, preclinical pharmacological investigations and clinical trials. Most importantly, PK studies enable the determination of the bioavailability of a drug which facilitates optimization of dosage and route of administration to achieve therapeutic efficacy. Baira et al.[92] reported the complete PK profile of oral as well as intravenously administered nimbolide in Sprague–Dawley rats. The absolute bioavailability was found to be 2.72%, 1.76%, and 3.06% after oral administration of 10, 30, and 50 mg/kg nimbolide, respectively. The kinetic properties of nimbolide were characterized as slow absorption, middle-speed elimination, and very poor absolute bioavailability that could be attributed to its high lipophilicity. However, plasma levels were significantly elevated following intravenous administration of 10 mg/kg nimbolide.

Wang et al.[93] developed a sensitive and robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantitative analysis of nimbolide in mouse serum and for validation of preclinical PK studies. They demonstrated good linearity in the range 5−1000 ng/mL, with accuracy and precision in accordance with the Food and Drug Administration (FDA) guidelines of <15%. In the preclinical PK study, the peak concentration (C_{max}) of nimbolide (0.78 μmol/L) was reached approximately 2 h after oral administration of 3 mg/kg indicating that nimbolide is rapidly and readily absorbed. Although the C_{max} of nimbolide was lower than the IC_{50} values reported in breast and colorectal cancer,[32,59] it was well within the *in vitro* effective concentration range of 0.5−10 μmol/L. These results imply that it is possible to achieve effective therapeutic levels of nimbolide *in vivo*. In colorectal cancer xenografted mice administered 5 and 20 mg/kg intraperitoneal (ip) nimbolide, the bioavailability of nimbolide in blood plasma was found to be 222 and 409 ng/mL, whereas, in the tumor tissues, the bioavailability was 345 and 868 ng/g, respectively.[41]

Recently, nimbolide was demonstrated to fulfill Lipinski's drug-likeness "rule of five" that includes criteria of molecular mass less than 500 Da molecular weight, less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors, and less than 5 partition coefficient (log*P*).[94] Although predictions for ADMET properties indicated blood−brain barrier penetrating potential and inhibition of CYP3A4, rigorous laboratory investigations are essential. More robust and sensitive bioanalytical methods are required for estimating the effective plasma and tissue concentrations of nimbolide to evaluate bioavailability. Additionally, there is a gap in complete knowledge of other critical parameters including clearance, half-life, dose optimization, formulation, as well as route and schedule of administration that are roadblocks to initiating clinical trials.

10. TOXICITY PROFILE OF NIMBOLIDE

A large number of potential drug candidates fail to progress to clinical trials due to toxicity-based attrition. Acute toxicity studies revealed higher toxicity of nimbolide in mice compared to rats and hamsters. Nimbolide was found to be toxic to mice when administered by the ip and intravenous (iv) routes with median lethal dose (LD50) values of 225 mg/kg and 24 mg/kg, respectively. Lethal doses of nimbolide caused death in mice as a result of necrosis of the kidneys, liver, and pancreas as well as due to a sudden drop of arterial blood pressure. However, intragastric, oral, or subcutaneous administration of nimbolide was nontoxic with LD50 values greater than 600 mg/kg body weight. Furthermore, in rats and hamsters, nimbolide was well tolerated with LD50 values of 600 and 500 mg/kg body weight, respectively.[95]

Nimbolide was not mutagenic in six strains of *Salmonella typhi*.[96] Studies on the spermicidal effects remain inconclusive. While one study failed to show significant spermicidal effects following *in vitro* exposure to 50 μM nimbolide, in another study, significant depletion of antioxidants associated with oxidative stress was observed in rat epididymal sperms exposed to 0.5−2 μM of nimbolide.[97] In an *in vivo* study on Wistar rats, subcutaneous administration of graded doses of nimbolide reduced sperm functional parameters.[98]

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J. Med. Chem. XXXX, XXX, XXX−XXX

Table 1. Mechanisms Underlying the Anticancer Effects of Nimbolide

Study type	Cancer Type	Cell line/Animal model	Mechanisms	References
In vitro	Bladder	EJ &5637	• Induces G2/M phase cell cycle arrest • Increases p-JNK, Decreases p-p38MAPK &p-AKT • Represses wound healing, migration and invasion by suppressing MMP-9 via inhibition of transcription factor (NF-κB, Sp-1, and AP-1) binding activity	31
		231MFP	• Inhibits cell proliferation by disrupting RNF114-mediated ubiquitination and degradation of p21and p57.	36
	Breast	MCF-7 & MDA-MB-231	• Induces autophagy mediated apoptosis (increasedBeclin 1 and LC3B; decreased p62, mTOR, and BCL-2) • Modulates HDAC-2 and H3K27Ac expression	35
		MCF-7 & MDA-MB-231	• Inhibits IGF-I mediated PI3K/Akt and MAPK signalling	33
		MCF-7 & MDA-MB-231	• Inhibits invasion and migration by downregulating expression of uPAR chemokine genes, VEGF, MMPs & NF-κB	34
		MCF-7 & MDA-MB-231	• Induces apoptosis by intrinsic and extrinsic pathways	32
	Cervical	HeLa	• Induces cell cycle arrest at G0/G1 phase • Promotes mitochondria-mediated apoptosis	37
	Choriocarcinoma	BeWo	• Inhibits cell proliferation • Induces ROS-mediated intrinsic apoptosis	38
	Colorectal	HCT116 and HT29	• Inhibits DNMT and HDACs with re-expression of p16	44
		HCT116 and HT29	• Modulates pro-inflammatory pathways by reducing expression of NF-κB and STAT3	43
		HT29	• Enhances apoptosis via JNK-mediated DR5 upregulation	42
		HCT-116, HT-29, & Caco-2	• Inhibits proliferation and induces apoptosis • Downregulates expression of proteins involved in invasion, metastasis and angiogenesis (MMP-9, ICAM-1, CXCR4, VEGF) • Inhibits NF-κB activation by suppressing IKK, IκBα phosphorylation, and p65 nuclear translocation.	41
		WiDr& HCT-116	• Induces S-phase arrest by inhibiting cyclin A • Induces apoptosis by inhibiting ERK1/2 and activating p38 and JNK1/2 • Retards migration, invasion, and angiogenesis by downregulating MMP-2/-9 through inhibition of ERK1/2 and DNA binding activity of NF-kB	40
		HT-29	• Induces cell cycle arrest at G2/M, G0/G1 phases and upregulates p21	39
	Glioblastoma	T98G, U87, U87 EGFRVIII	• Induces cell cycle arrest at G1/S phase by inhibition of CDK4/6 and consequent Rb hypophosphorylation • Blocks growth factor-induced phosphorylation of Akt, ERK1/2, and STAT3	45
	Haematological malignancies (lymphoma, leukaemia, multiple myeloma, Waldenströms macroglobulinemia)	U937, HL-60, THP1	• Induces cell cycle arrest and apoptosis	46
		U266, U266/ BR, KMS, Raji, OPM2, OPM2/ BR, BCWM.1, BCWM.1/BR	• Induces mitochondrial-mediated apoptosis • Targets BCL2, HSP90 and PI3K.	48
		CEM/ ADR5000 leukaemia	• Circumvents MDR by upregulating PTEN, HIF1α and FOXO1.	49
		K562 leukaemia	• Selectively degrades oncogenic BCR-ABL • E3 ligase recruiter for PROTACS	102
	Liver	Huh-7 and PLC/PRF/5	• Induces apoptosis by activating ERK-mediated c-IAP1 inhibition	51
		HepG2	• Induces mitochondrial-mediated apoptosis by blocking constitutive and TNF-α-induced NFκB activation	50

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J. Med. Chem. XXXX, XXX, XXX−XXX

Table 1. continued

Study type	Cancer Type	Cell line/Animal model	Mechanisms	References
	Liver	Hepa 1c1c7	• Enhances quinone reductase activity	28
	Lung	A549 & H1650	• Suppresses invasion and migration by upregulating DUSP4 expression and inhibiting ERK1/2 activation	52
	Nasopharyngeal	HONE-1	• Induces apoptosis by inhibiting ERK1/2 pathway	53
		SCC9/SCC4-CisR	• Overcomes cisplatin resistance by inducing Bax-dependent cell death via targeting the Akt pathway in combination with Akt inhibitor VIII	90
	Oral	SCC4, Cal27, and HSC3	• Disrupts pro-tumour inflammatory cytokines NFκB, COX2, IL-1, IL-6, TNFα, and IFNγ and downregulates STAT3 and AKT to inhibit proliferation, migration, and inflammation.	54
		SCC131, SCC4	• Inhibits autophagy to induce apoptosis by abrogating PI3K/Akt pathway via down-regulation of HOTAIR that sponges miR-126	55
		SCC131, SCC4	• Upregulates RECK by targeting miR-21 and HIF-1a leading to reduced MMP activity and blockade of VEGF and Notch signalling thereby regulating invasion and angiogenesis.	76
	Osteosarcoma	MG63, U2OS, & HOS	• Induces apoptosis by ROS generation, mitochondrial dysfunction, ER stress, and caspase activation • Suppresses cell migration by decreasing integrin αvβ5 expression via abrogation of PI3K/Akt and NF-κB signalling	56
	Pancreatic	MIA PaCa-2 and BX-PC3	• Reduces CD44+ and CSC population • Induces mitochondrial apoptosis • Reduces mutant P53 and mitochondrial ROS	58
In vivo		HPAC, MIAPaCa-2, & PANC-1	• Regulates apoptosis and autophagy by ROS generation • Inhibits proliferation and metastasis via blockade of PI3K/AKT/mTOR and ERK signalling • Prevents EMT by increasing E-cadherin expression and decreasing expression of vimentin, β-catenin, notch-2, N-cadherin, Snail, Slug, and Zeb	57
	Prostate	PC-3	• Inhibits cell survival and proliferation via suppression of NF-κB, IGF, PI3K/Akt and MAPK pathways	60,83
		DU145 & LNCaP	• Abrogates constitutive and IL-6 inducible STAT3 activation and JAK1/2 via ROS generation by inducing GSH/GSSG imbalance	59
	Renal	786-O and A-498	• Induces cell cycle arrest at G2/M phase • Promotes intrinsic and extrinsic apoptosis	61
	Glioblastoma	U87EGFRvIII xenografted mouse model	• Inhibits cell proliferation and induces apoptosis • Blocks phosphorylation of Akt, ERK1/2, and STAT3	45
	Liver	Huh-7 xenografted mouse model	• Inhibits cell proliferation (low Ki-67 expression) • Induces apoptosis (increased cleaved PARP)	51
		HCT-116-derived mouse xenograft	• Inhibits proliferation and induces apoptosis • Downregulates proteins involved in invasion, metastasis and angiogenesis (MMP-9, ICAM-1, CXCR4, VEGF) • Inhibits constitutive activation of NF-kB	41
	Oral	DMBA-induced hamster buccal pouch (HBP) carcinogenesis model	• Inhibits PI3K/Akt to activate GSK3b with attenuation of cell proliferation and stimulation of apoptosis	63
			• Inhibits cell proliferation • Transduces apoptosis by intrinsic and extrinsic pathways	62
			• Prevents procarcinogen activation and DNA damage • Enhances antioxidants and detoxification enzymes • Inhibits tumour invasion and angiogenesis	27

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J. Med. Chem. XXXX, XXX, XXX−XXX

Table 1. continued

Study type	Cancer Type	Cell line/Animal model	Mechanisms	References
In vivo	Oral	SCC4, Cal27, and HSC3-xenografted mouse models	• Disrupts pro-tumour inflammatory cytokines NFκB, COX2, IL-1, IL-6, TNFα, and IFNγ and downregulates STAT3 and AKT to inhibit proliferation, migration, and inflammation	54
	Pancreatic	HPAC-xenografted mouse model	• Induces autophagy by increasing LC3A/B and decreasing p62 expression • Inhibits proliferation and metastasis by inducing caspase-mediated apoptosis • Inhibits EMT by increasing E-cadherin and decreasing vimentin, β-catenin, notch-2, slug, and N-cadherin • Suppresses expression of p-AKT, p-PI3K, and p-mTOR, enhances PTEN.	57
	Prostate	Transgenic adenocarcinoma of mouse prostate (TRAMP)	• Abrogates constitutive and IL-6 inducible JAK/STAT3 activation by ROS arising from GSH/GSSG imbalance	59
	Waldenströms macroglobulinemia	RPCI-WM1-xenografted mouse model	• Decreases cell proliferation (Ki-67 stain) • Increases apoptosis (cleaved caspase-3 stain, low BCL2)	48

Comprehensive, well-designed long-term toxicity studies on nimbolide in nonrodent species, dose−response relationship, and repeated-dose toxicity studies under different routes of administration are essential to understand the complete toxicology profile of nimbolide as well as to identify the no observed adverse effect level (NOAEL).

11. CHALLENGES IN THE COMMERCIALIZATION OF NIMBOLIDE

Natural products have been the single largest source of anticancer drugs and novel lead compounds. However, there are several obstacles in the commercialization of phytochemicals such as nimbolide. These include extraction and characterization, bioavailability, analysis of ADMET, and high-throughput screening. Although simple and rapid methods have been devised for the isolation and purification of nimbolide from neem leaves, high yield, purity, and cost-effectiveness are major factors that influence product manufacture. In addition, procurement, authentication, quality assurance, and scale-up are time-consuming and labor-intensive. Recently, nimbolide was extracted using a simple, fast, and efficient microwave-assisted extraction method coupled with chromatography that yields 0.0336 g of nimbolide from 5 g of neem leaves within 3 h.[99] More such technical initiatives are necessary to promote the potential pharmaceutical applications of nimbolide.

Evaluation of different routes of administration, solubility, gastrointestinal absorption, and bioavailability are critical parameters to be considered in drug development from natural sources. Nimbolide is known to be only sparingly soluble in aqueous solutions. The poor bioavailability and high lipophilicity of nimbolide are major impediments in translating its anticancer potential to the clinical setting. Strategies such as combination with compounds that increase solubility and absorption as well as novel delivery systems such as microemulsions, phospholipid complexes, nanoparticles, or polymer micelles can enhance bioavailability. In this connection, poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles of nimbolide that exhibit sustained release and enhanced cytotoxicity in breast and pancreatic cancer cell lines relative to free nimbolide raise hope for targeted delivery by conjugation with polyethylene glycol or receptor-binding ligands.[100] Conjugation of nimbolide liposomes conjugated with iRGD peptide (iRGD-NIMLip) was found to be a promising novel drug delivery system to target lung inflammation based on the significant inhibition of oxidative stress and cytokine storm associated with acute respiratory distress syndrome and COVID-19.[101] Further studies on targeted delivery will open up new possibilities for therapeutic intervention.

The paucity of information on the ADMET of nimbolide is another bottleneck in the drug development pipeline. Moreover, a large majority of the investigations on the anticancer effects of nimbolide have been conducted in cellular models. Although some studies have been conducted in a few cell-line-derived tumor xenograft mouse models, chemotherapeutic efficacy needs to be firmly established on a range of malignant tumors as well as in patient-derived tumor xenograft (PDTX) models.

Commercialization of nimbolide requires validation of pharmacological, pharmacokinetic, toxicicological, and clinical studies. Although Gupta et al.[41] confirmed the presence of nimbolide in plasma and tumor tissues of colorectal cancer xenografted mice, the method has not been fully validated by the FDA. The ability of nimbolide to cross the blood−brain barrier based on *in silico* predictions[94] and studies on GBM xenografted mice[45] needs further validation. Nimbolide must be thoroughly investigated in preclinical settings for safety, efficacy, and druggability standards before approval for clinical trials. High-throughput screening using a range of relevant cell-based and molecular bioassays, target validation, and examination of effects on specific physiological pathways can accelerate the evaluation of small molecules such as nimbolide for drug development.

12. PERSPECTIVES AND CONCLUSIONS

Nimbolide that displays remarkable antiproliferative effects against numerous human cancer cells *in vitro* as well as in animal tumor models *in vivo* can be regarded as a "broad-spectrum" anticancer agent. Nimbolide influences all the steps in multistage carcinogenesis ranging from carcinogen metabolism, through genotoxicity, cell cycle progression, apoptosis evasion, angiogenesis, invasion, to metastasis as well as

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J. Med. Chem. XXXX, XXX, XXX−XXX

epigenetic modifications. Accumulating evidence has convincingly demonstrated that nimbolide targets a plethora of molecules and kinase-driven oncogenic signaling pathways to effectively prevent the acquisition of hallmark capabilities of cancer. Compelling evidence from various studies indicates that nimbolide abrogates oncogenic signaling by influencing the subcellular localization of transcription factors and phosphorylation of signaling kinases. Nimbolide also exerts modulatory effects on the epigenome and effectively overcomes MDR. The recent discovery of nimbolide as a covalent recruiter for the E3 ligase RNF114 underscores its broad utility for targeted protein degradation. Interestingly, a proteolysis-targeting chimera (PROTAC) linking nimbolide to the kinase and BCR-ABL fusion oncogene inhibitor dasatinib, BT1, was demonstrated to selectively degrade oncogenic BCR-ABL over c-ABL in leukemic cells.[102] This has paved the way for the identification of EN219, a fully synthetic RNF114-based recruiter that mimics the mode of action of nimbolide.[103] These findings further expand the arsenal of nimbolide and reinforce its potential as a promising anticancer drug candidate. It is noteworthy that nimbolide is listed among the ten potential compounds for oral cancer treatment based on large-scale mining as well as annotation of reliable compounds and bioactivity databases.[104] A recent review by Agarwal et al.[105] based on an extensive search using various literature databases revealed the preventive and therapeutic potential of neem in general and nimbolide in particular against oral cancer. Table 1 summarizes the mechanisms underlying the anticancer effects of nimbolide.

Over the past several decades, anticancer drug design and discovery have focused on single-target agents that selectively target a single biological molecule, usually a protein. However, this approach is beset with certain drawbacks including low therapeutic efficacy and resistance. Given the multifactorial etiology and complexity of cancer, the use of multitarget drugs has emerged as a more pragmatic strategy resulting in the rational design of a large number of novel small molecule multitarget drugs in recent years with translational success in the clinic. The development of these drugs involves the integration of pharmacophores and is a daunting task.[106] Natural products like nimbolide, a small molecule with broad-spectrum and multitargeted activity, are attractive options for anticancer drug development. Extensive investigations on soluble nimbolide analogues, efficient delivery systems and nanoformulations that can enhance the bioavailability, as well as studies on subacute, acute, and chronic toxicity are, however, warranted before translating preclinical findings to the clinic.

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Rajakishore Mishra is presently working as an Assistant Professor in the Department of Life Sciences at the Central University of Jharkhand, India. He obtained his Ph.D. from the Institute of Life Sciences, India, in 2005 and soon after his Postdoctoral studies at Texas A&M University, USA (2005−2007), and Loyola University, Chicago (2007−2010). His doctoral and postdoctoral work mainly focused on understanding the underlying molecular mechanisms of oral and breast cancers. His current research interests include the identification of suitable biomarkers and designing novel therapeutics for effective treatment of oral cancer using natural products.

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J. Med. Chem. XXXX, XXX, XXX−XXX

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■ ABBREVIATIONS USED

ABC, ATP-binding cassette; ADMET, absorption, distribution, metabolism, elimination, and toxicity; Bid, BH3 interacting-domain death agonist; CDK, cyclin-dependent kinase; CYP, cytochrome P450; DMBA, 7,12-dimethylbenz[*a*]anthracene; DNMT, DNA methyltransferase; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; ERK, extracellular signal-related kinase; FOXO1, forkhead box O transcription factor1; GBM, glioblastoma multiforme; GSK-3β, glycogen synthase kinase-3β; GST, glutathione S-transferase; HBP, hamster buccal pouch; HDAC, histone deacetylase; HIF-1α, hypoxia-inducible factor-1α; IAP, inhibitor of apoptosis protein; IKKβ, inhibitor of kappa B kinaseβ; IκBα, inhibitor of κBα; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; LC3, microtubule-associated protein 1A/1B-light chain 3; LD50, median lethal dose; lncRNA, long noncoding RNA; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MDR, multidrug resistance; MMP, matrix metalloproteinase; MOMP, mitochondrial outer membrane permeabilization; mTOR, mammalian target of rapamycin; ncRNA, noncoding RNA; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NSCLC, nonsmall cell lung carcinoma; OSCC, oral squamous cell carcinoma; PARP, poly(ADP ribose) polymerase; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase; Pin1, peptidyl-prolyl cis–trans isomerase NIMA-interacting 1; PK, pharmacokinetics; PTEN, phosphatase and Tensin homologue; Rb, retinoblastoma protein; RECK, reversion-inducing cysteine-rich protein with Kazal motifs; ROS, reactive oxygen species; Smac/DIABLO, second mitochondria-derived activator of caspase/direct IAP binding protein with low pI; STAT, signal transducer and activator of transcription; TGF-β, transforming growth factor-β; TIMPs, tissue inhibitors of MMPs; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; WM, Waldenströms macroglobulinemia

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