Chapter 20

Strategies for Improving Photodynamic Therapy Through Pharmacological Modulation of the Immediate Early Stress Response

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Abstract

Photodynamic therapy (PDT) is a minimally to noninvasive treatment modality that has emerged as a promising alternative to conventional cancer treatments. PDT induces hyperoxidative stress and disrupts cellular homeostasis in photosensitized cancer cells, resulting in cell death and ultimately removal of the tumor. However, various survival pathways can be activated in sublethally afflicted cancer cells following PDT. The acute stress response is one of the known survival pathways in PDT, which is activated by reactive oxygen species and signals via ASK-1 (directly) or via TNFR (indirectly). The acute stress response can activate various other survival pathways that may entail antioxidant, pro-inflammatory, angiogenic, and proteotoxic stress responses that culminate in the cancer cell's ability to cope with redox stress and oxidative damage. This review provides an overview of the immediate early stress response in the context of PDT, mechanisms of activation by PDT, and molecular intervention strategies aimed at inhibiting survival signaling and improving PDT outcome.

Key words Cancer cell survival, Tumor recalcitrance, ASK-1, p38 and JNK, Therapy resistance, Photosensitizer, Pharmacological intervention

Abbreviations

$^1O_2$	Singlet oxygen
ABC	ATP-binding cassette
AhR	Aryl hydrocarbon receptor
ALA	5-Aminolevulinic acid
AP-1	Activator protein 1
APE	Apurinic/apyrimidinic endonuclease

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ASK1	Apoptosis signal-regulating kinase 1
ASV	Avian sarcoma virus
ATF1/2/3/4/6/7/8	Activating transcription factor 1, 2, 3, 4, 6, 7, 8
ATG	Autophagy-related genes
ATP	Adenosine triphosphate
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2-associated X
BCL-2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
BMP2	Bone morphogenic protein 2
bZIP	Basic leucine zipper
cAMP	Cyclic adenosine monophosphate
CDH11	Cadherin 11
C/EPB	CCAAT-enhancer-binding protein
CMPD1	2'-Fluoro-*N*-(4-hydroxyphenyl)-[1,10-biphenyl]-4-butanamide
COX-2	Cyclo-oxygenase 2
CRE	Cyclic AMP response element
CREB	ATF/CRE-binding protein
CRP	C-reactive protein
DAMPs	Damage-associated molecular patterns
DPI	Diphenylene iodonium
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELK-1	ETS like-1 protein
Em	Emission
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EP2	Prostaglandin E2 receptor 2
Ex	Excitation
FGF-2	Fibroblast growth factor 2
FHPI	4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole
FOXO	Forkhead box O
FRA1/2	FOS-related antigen 1 and 2
GADD153/CHOP	Growth arrest- and DNA damage-inducible gene 153
HDAC	Histone deacetylase
HIF-1	Hypoxia-inducible factor 1
HO-1	Heme oxygenase 1
HSF1	Heat-shock factor 1
HSP27	Heat-shock protein 27
HUVECs	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis
IC_{50}	Half maximum inhibitory concentration
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor alpha
IKKβ	Nuclear factor kappa-B kinase subunit beta
IL-1β/6/8/10	Interleukin 1β, 6, 8, and 10

iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal (administration route)
i.v.	Intravenous (administration route)
JDP-1/2	JUN dimerization protein 1 and 2
JNK	c-JUN terminal kinase
KRAS	Kirsten rat sarcoma virus
LC-3 II	Microtubule-associated protein 1A/1B-light chain 3, phosphatidylethanolamine conjugate
LC_{50}	Half maximum lethal concentration
LD_{50}	Half maximum lethal dose
LKB1	Liver kinase B1
MAF	Musculoaponeurotic fibrosarcoma protein
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MEF2	Myocyte enhancer factor 2
MEK-1	MAPK/ERK kinase 1
miRNA	microRNA
MK2/5	MAPK-activated protein kinase 2 and 5
MKK3/4/6/7	MAPK kinases 3, 4, 6, and 7
MMP3/9	Matrix metalloproteinase-3 and 9
MNK1/2	MAPK-interacting serine/threonine-protein kinase 1 and 2
MPPa	Pyropheophorbide-α methyl ester
MSK1	Mitogen- and stress-activated protein kinase 1
MSV	Murine osteogenic sarcoma virus
mTOR	Mammalian target of rapamycin
MW	Molecular weight
NA	Not available
NAC	*N*-acetylcysteine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLK	Nemo-like kinase
NOX	NADPH oxidase
NQDI-1	2,7-Dihydro-2,7-dioxo-3H-naphtho[1,2,3-de]quinoline-1-carboxylic acid ethyl ester
NRF2	Nuclear factor erythroid 2-related factor 2
$O_2{\bullet}^-$	Superoxide
p300HAT	Histone acetyltransferase p300
PARP-1	Poly(ADP-ribose) polymerase 1
PDK-1	3-Phosphoinositide-dependent kinase 1
PDT	Photodynamic therapy
PDTC	Pyrrolidine dithiocarbamate
PGE_2	Prostaglandin E2
P-gp	P-glycoprotein
PI3K	Phosphatidylinositol 3-kinase
PP5	Protein phosphatase 5
PS	Photosensitizer
Rac3	Rac family small GTPase 3
ROS	Reactive oxygen species
SAPK1/2	Stress-activated protein kinase 1 and 2
s.c.	Subcutaneous (administration)
SCR-1/2/3	Steroid receptor coactivator 1, 2, and 3

siRNA	Small interfering RNA
$t_{1/2}$	Circulation half-life
TDLO	Lowest dose causing a toxic effect
TF	Transcription factor
TFE3	Transcription factor binding to IGHM enhancer 3
TFEB	Transcription factor EB
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor alpha receptor
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
TPPS2a	Meso-tetraphenyl porphyrin disulfonate
TRAF	TNF-α receptor-associated factor
TRE	TPA (12-0-tetradecanoylphorbol-13-acetate) responsive element
Trp53	Transformation-related protein 53
TRX	Thioredoxin
UPR	Unfolded protein response
USP7/21	Ubiquitin-specific protease 7 and 21
USP9X	Ubiquitin-specific peptidase 9 X-linked
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
XBP1	X-box-binding protein 1
XIAP	X-linked inhibitor of apoptosis protein
ZEB2	Zinc finger E-box-binding homeobox 2
ZnPC	Zinc phthalocyanine

1 Introduction

Photodynamic therapy (PDT) is a minimally to noninvasive treatment modality that has emerged as a promising alternative to conventional cancer therapies such as surgery, radiotherapy, and chemotherapy. PDT was first approved for clinical use in 1993 in Canada for the treatment of papillary bladder cancer and has since been approved for various oncological indications in many countries [1].

PDT is based on the photosensitization of tumor tissue with light-sensitive molecules called photosensitizers (PSs) that are administered intravenously or topically, depending on the location of the tumor. Following intratumoral PS accumulation, the tumor is illuminated at a wavelength that is absorbed by the PS. Absorption of resonant light causes PS excitation from a ground state to a singlet state, after which the singlet-state electron undergoes intersystem crossing to a more stable but less energetic triplet state [2]. Triplet-state PSs can undergo two types of photochemical reactions with molecular oxygen and biomolecules. Type I photochemical reactions result in the transfer of the triplet-state electron to oxygen, yielding superoxide anion ($O_2^{\bullet -}$), or to other

molecules to produce radicals. The primary ROS and radicals may in turn react with other substrates to form secondary and tertiary derivatives in a biological environment [3]. Type II photochemical reactions involve the transfer of energy to molecular oxygen, causing its transition from triplet state (native state of oxygen) to singlet state, thereby generating the highly reactive singlet oxygen ($^1O_2$) [4, 5].

The photogenerated ROS and radical transients damage tumor tissue via three distinct routes that collectively account for a therapeutic effect. First, ROS and non-oxygen radicals oxidize biomolecules and inflict oxidative damage and consequent oxidative stress. Excessive oxidative damage following PDT culminates in cell death, which is predominantly but not exclusively apoptotic and necrotic in nature [6–13]. Second, PDT affects photosensitized endothelium that lines intratumoral vasculature, leading to thrombosis-mediated vascular shutdown, tumor hypoxia, and metabolic catastrophe [14, 15]. Third, PDT-treated damaged and dying cells release an armament of damage-associated molecular patterns (DAMPs) and tumor-associated antigens that prime innate and adaptive immune cells, which in turn mount an antitumor immune response [16]. The antitumor immune response is required for long-term tumor control [17, 18].

Despite the profound lethality of PDT toward parenchymal cells in solid tumors, clinical accounts on PDT outcomes report tumor recurrence in different types of cancer [19–23]. Tumor recurrence may be attributed to the activation of survival pathways in sublethally afflicted cancer cells following PDT [8–10, 12, 24, 25]. As exemplified in Fig. 1, cancer cells that are more distally located from the light source are most prone to insufficient damage from PDT, especially in bulkier tumors. Although wavelengths are used in clinical PDT that do not have competing natural chromophores, laser-tissue interactions still abide by the Lambert-Beer law that dictates an attenuating photon density with tissue depth. Inasmuch as photochemical ROS production is directly proportional to fluence rate, and the degree of oxidative stress is negatively correlated with cell survival, the cancer cells that are exposed to subcritical fluence rates will be able to cope with the lower levels of oxidative stress. These cells will have ample biological reserves to activate survival cascades that ultimately ensure their post-PDT viability. In time, surviving cancer cells will proliferate and restore the parenchymal matrix that clinically will manifest as tumor recurrence. In some instances, post-PDT tumor control is managed by the adaptive immune system (also via abscopal effects) that could offset cancer cell survival [17, 18], but this mechanism is by no means fail-safe.

To date, five main survival pathways that are activated by PDT have been described [24]. The pathways include (1) the antioxidant response mediated by nuclear factor erythroid 2-related factor

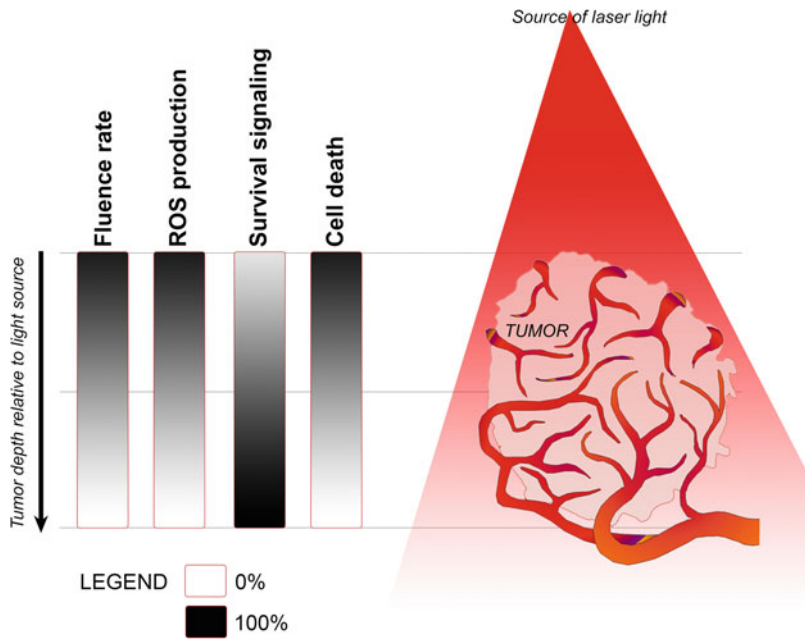

Fig. 1 Key interrelated photophysical, biochemical, and biological variables that influence PDT outcome. Light is attenuated with tumor depth relative to the light source (Beer-Lambert law), accounting for lower fluence rates in the more distal tumor regions. A reduction in photon density translates to a lower probability that electrons absorb the photons and are raised to a singlet and subsequently triplet energy state, from which ROS are produced. Lower ROS production yields less damage and redox stress in cancer cells, allowing sublethally afflicted cells to cope with the PDT-induced injury and survive by activating and executing survival pathways. Cancer cells that have survived ultimately proliferate and reform tumors, which is a likely explanation for the tumor recurrence that is observed in PDT-treated patients

2 (NRF2); (2) the pro-inflammatory and angiogenic response mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB); (3) the survival response to hypoxia driven by hypoxia-inducible factor 1 (HIF-1); (4) an immediate early stress response triggered by apoptosis signal-regulating kinase 1 (ASK-1), which activates activator protein 1 (AP-1) via the mitogen-activated protein kinases (MAPKs) c-Jun N-terminal kinase (JNK) and p38 MAPK (from here onward referred to as p38); and (5) the proteotoxic stress response comprising the unfolded protein response (UPR) and the heat-shock response that signal through activating transcription factor (ATF) 4, ATF6, heat shock factor 1 (HSF1), and X-box binding protein 1 (XBP1) (*see* Fig. 2) [24]. ROS constitute the overarching trigger for the survival pathways, although some of the pathways can be induced by other means related to PDT. For example, NF-κB and HIF-1 can be activated by hypoxia, whereas the UPR and the heat-shock response require redox modifications of the cell's proteome. Other pathways activated by PDT but not listed here also have interacting and overlapping segments with the survival pathways that may lead to activation of the latter.

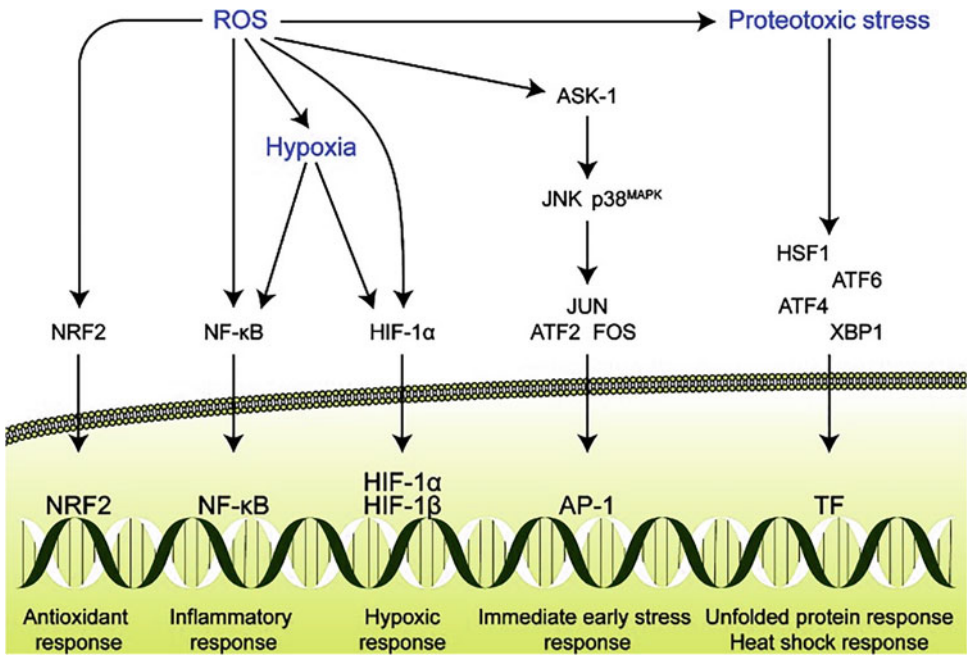

Fig. 2 The 5 main survival pathways responsible for therapeutic recalcitrance to PDT

The principal activator molecules (NRF2, NF-κB, HIF-1α, ASK-1, HSF1, ATF4/6, and XBP1) are also capable of transactivating or modulating the activity and progression of the other pathways through downstream signaling.

PDT considerably alters the biological and biochemical landscape of cells [12, 26–28], where sublethally afflicted cells mobilize a maximum number of functional resources to sustain viability. The survival pathways occupy a central position in the cell's resource carousel [24, 29, 30]. It is therefore arguable that pharmacological inhibition of the survival pathways will lead to improved PDT outcomes [24], which has been empirically demonstrated for the HIF-1 survival pathway using the selective HIF-1α inhibitor acriflavine [9, 10]. The hypoxic cytotoxin tirapazamine also imparted an adjuvant effect in PDT-treated cells post-therapeutically that were exposed to hypoxic conditions [11]. A non-exhaustive list of other eligible HIF-1 pathway inhibitors is provided in Chapter 19. This chapter addresses potential immediate early stress response pathway inhibitors against a backdrop of the ASK-1 signaling cascade and its role in PDT.

2 ASK-1 Pathway

The acute stress response is generally induced by immediate stress factors such as ROS, endoplasmic reticulum (ER) stress, or paracrine signals from cells damaged by PDT. The stress factors enable

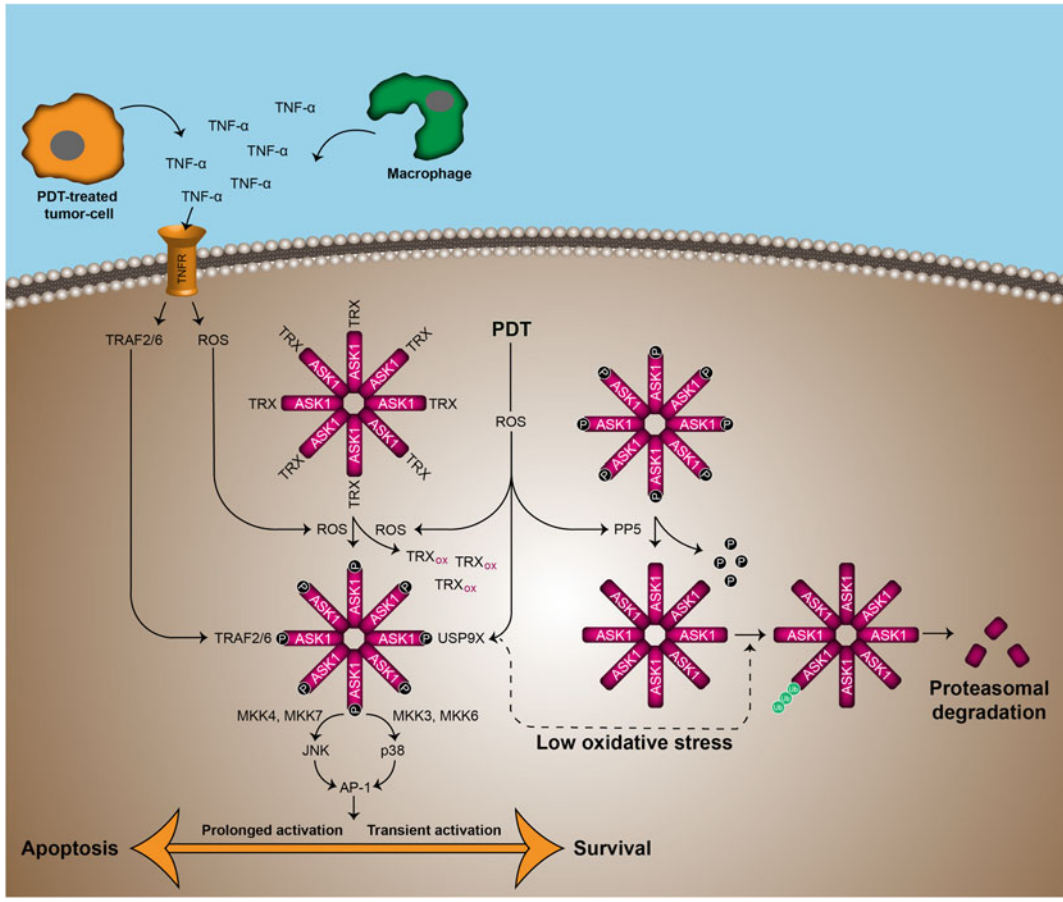

Fig. 3 Summary of the immediate early stress response, where ASK-1 occupies a central role. The ASK-1 signalosome requires autophosphorylation to become activated, a process inhibited by thioredoxin (TRX) in the absence of oxidative stress. TRX dissociates when oxidized by ROS that are generated by either PDT or tumor necrosis factor alpha receptor (TNFR) signaling. Phosphorylated ASK-1 relays downstream signals via MKK4 and MKK7 as well as MKK3 and MKK6, which phosphorylate JNK and p38, respectively. These subsequently activate components of the AP-1 complex and associated proteins that in turn activate transcription at DNA loci that contain an AP-1 promoter region. The biological ramifications of AP-1 signaling depend on activation time. Transient activation induces survival by inflammatory, proliferative, and angiogenic processes, whereas prolonged activation culminates in apoptosis

signal-receiving cells to sustain cell viability and to engage other downstream survival processes, such as the antioxidant, pro-inflammatory, angiogenic, and proteotoxic stress responses [24]. ASK-1 is the prime orchestrator of the immediate early stress response, summarized in Fig. 3.

In its inactive state, ASK-1 forms homo-oligomers through its C-terminal coiled-coil domain, establishing a complex that is referred to as the signalosome [31, 32]. To become activated, the ASK-1 subunits need to be autophosphorylated. However, in a homeostatic redox environment the autophosphorylation of

ASK-1 is prevented by reduced thioredoxin (TRX) molecules that bind to the N-terminal region of ASK-1 subunits [31]. ASK-1 activation is triggered via several mechanisms. First, ROS generated either by PDT or via tumor necrosis factor alpha (TNF-α) receptor (TNFR) signaling oxidize TRX, resulting in its dissociation from the signalosome, autophosphorylation of Thr845 in the kinase domain of the ASK-1 subunits, and subsequent activation of the signalosome [33, 34]. There are 2 TNF-α-associated receptors; TNF-R1 (p55) and TNF-R2 (p75). Second, the ubiquitin-specific peptidase 9 X-linked (USP9X) deubiquitination enzyme binds to ASK-1 upon the manifestation of oxidative stress. In the absence of ROS, ASK-1 is ubiquitinated and proteasomally degraded as a control mechanism. USP9X therefore positively regulates ASK-1 signaling by deterring proteasomal removal [31]. Third, TNF-α receptor-associated factor (TRAF) 2 and 6 associate with the ASK-1 signalosome adjacent to the C-terminal coiled-coil domain during redox stress and positively regulate ASK-1 activity [35]. Fourth, the binding of TNF-α to its cognate receptor promotes ASK-1-mediated cell death and pro-inflammatory signaling through TRAF2. This is not a trivial mechanism inasmuch as PDT stimulates TNF-α production and secretion by macrophages in the tumor microenvironment [36–38] and by photosensitized cancer cells [7], further feeding signals into the ASK-1 cascade via TNFR [39–41]. Negative feedback regulation of consecutively activated ASK-1 is provided by protein phosphatase 5 (PP5) through ROS-dependent dephosphorylation of the activating phosphorylation site on ASK-1 [42].

Phosphorylated ASK-1 activates two different subgroups of MAPK kinases (MKK) [43], including MKK4 and MKK7 that activate c-JUN N-terminal kinase (JNK) MAPK [44, 45], and MKK3 and MKK6 that activate p38 [46, 47] (*see* Subheading 2.1). Additionally, MKK4 has been shown to activate p38 [48].

2.1 JNK and p38

There are three genes of JNK (also known as the stress-activated protein kinase (SAPK or SAPK1)): *JNK1*, *JNK2*, and *JNK3*. Alternative splicing of the *JNK* transcripts produces at least ten different isoforms. *JNK1* and *JNK2* give rise to four isoforms: α1, α2, β1, and β2. *JNK3* can produce an α1 and β1 isoform [49, 50]. There are four known isoforms of p38: p38α (SAPK2a), p38β (SAPK2b), p38γ (SAPK3), and p38δ (SAPK4) [51, 52].

The JNK isoforms are variably distributed in the body. JNK3 isoforms are mainly expressed in the brain, heart, and testes and share many functional elements. JNK1 and JNK2 isoforms are found throughout the entire body and have functional differences [53], albeit somewhat elusive. JNK splicing variants differ in size, where α1 and β1 isoforms have a molecular weight of 46 kDa and the α2 and β2 isoforms have a molecular weight of 54 kDa [50]. JNK splicing variants also differ in binding specificity toward

downstream targets [49], suggesting that the isoform expression pattern governs the downstream effects.

Upstream activation of JNK does not exclusively occur via the ASK-1 pathway, as various other pro-inflammatory signaling pathways induce MAPK activity that can also lead to the activation of JNK [54]. Both JNK1 and JNK2 seem to be primarily involved in the regulation of tissue growth, development, and regeneration, being particularly important in the differentiation of neural cells and the regulation of apoptosis in many cell types, including cancer cells [55–58]. JNK-regulated apoptosis seems to be key in tissue remodeling and regeneration, and in the growth and differentiation of nervous tissue [59]. Finally, JNK can act as a tumor suppressor [56, 60].

The p38 kinase isoforms also exhibit differential expression patterns. The p38α and p38β isoforms are found ubiquitously in virtually all tissues, whereas the p38γ and p38δ isoforms are expressed in a tissue-specific manner in skeletal muscle and various organs (lungs, kidneys, testes, pancreas, and small intestine) [61–63]. Evidence also points toward divergence in both normal and disease-related functions (including different forms of cancer) between the isoforms [64–68]. In general, p38 plays a role in inflammation, apoptosis, cell cycle regulation, and differentiation and can also be activated by various upstream regulators other than ASK-1 [69].

Both JNK and p38 have tumor suppressor and pro-oncogenic attributes as evidenced by their role in apoptosis and inflammatory signaling, and both may act as negative regulators of each other [65, 67, 70–72]. JNK has been implicated in inflammatory signaling linked to cancer development, while p38 has been shown to activate downstream targets that are involved in invasion and metastasis [24, 72, 73]. JNK inhibits, among others, some of the anti-apoptotic members of the B-cell lymphoma 2 (BCL-2) protein family, including BCL-2 and BCL-xL, while activating pro-apoptotic members of the BCL-2 family such as Bcl-2-associated X (BAX) and Bcl-2 homologous antagonist/killer (BAK), the cell cycle regulator and tumor suppressor p53, and the oncoprotein c-MYC. JNK can also act as a positive regulator of autophagy through forkhead box O (FOXO)-dependent transcription of autophagy-related genes (ATG) and post-translational modification of BCL-2 [74, 75]. P38 can activate the FOS and CCAAT-enhancer-binding protein (C/EBP) family of proteins as well as the cell cycle regulators MAP kinase-activated protein kinase 2 (MK2), MK5, and myocyte enhancer factor-2 (MEF2), which are involved in cell division and differentiation. P38 further activates mitogen- and stress-activated protein kinase-1 (MSK1) as well as MAP kinase-interacting serine/threonine-protein kinase 1 (MNK1) and MNK2 that regulate the translation of newly transcribed genes. JNK and p38 both stimulate inflammation through activation of

the JUN and ATF2 protein families and ETS like-1 protein (ELK-1) as well as the antioxidant response through NRF2 (*see* Fig. 2). Additionally, p38 activates the HIF-1 pathway [76] that is further addressed in Chapter 19. Given the aforementioned downstream effects, p38 inhibitors have been proposed as anti-inflammatory drugs as well as adjuvant agents for chemotherapy to offset drug resistance [77–79].

2.2 AP-1 Transcription Factor

AP-1 is involved in several key cellular processes in the progression of cancer, including proliferation, survival, growth, differentiation, cell migration, and transformation. AP-1 plays a regulatory role in redox-responsive gene expression [80] and inflammatory signaling [81–83]. Ambivalently, AP-1 is linked to both tumorigenesis and tumor suppression and is capable of inducing both pro-apoptotic and anti-apoptotic effects [24, 81].

AP-1 is a superfamily of dimeric basic leucine zipper (bZIP) transcription factors that bind to the AP-1 sequence motif (TGACTCA), also known as the TPA (12–0-tetradecanoyl-phorbol-13-acetate) responsive element (TRE), and the AP-1-binding site on DNA to activate transcription [84]. Additionally, AP-1 binds to the ATF/CRE (cyclic-AMP response element) sequence motif (TGACGTCA) [85]. AP-1 homodimers and heterodimers are composed of JUN (c-JUN, JUNB, and JUND), FOS (c-FOS, FOSB, and FOS-related antigen 1 and 2 (FRA1/2)), ATF/CRE-binding protein (CREB) (ATF1, ATF2, ATF3, ATF4, ATF6, ATF7, and ATF8), and musculoaponeurotic fibrosarcoma (MAF) protein families [81, 85–89].

All AP-1 proteins share a common DNA-binding bZIP motif that allows the formation of dimers with other AP-1 proteins, although not all proteins share the same dimerization partners [85, 89–91]. Moreover, the DNA-binding site specificity of the AP-1 complex is dimer-dependent. For example, ATF7 dimerizes with proteins from the JUN and FOS family, where ATF7/c-JUN dimers show efficient binding to the DNA-binding sites of ATF, CRE, and AP-1, while ATF7/c-FOS dimers do not [92]. The JUN/FOS dimers have at least a 25-fold higher binding affinity toward TRE domains compared to JUN/JUN dimers [93]. In general, AP-1 complexes that contain ATF family protein members have a higher affinity for the ATF/CRE sequence motif, whereas complexes that exclusively consist of FOS and/or JUN family proteins exhibit greater affinity for the AP-1-binding site [85].

2.2.1 JUN

The JUN transcription factor gene family consists of known proto-oncogenes that are susceptible to gain-of-function mutations (thus transgressing to oncogenes), leading to increased protein levels. Accordingly, the oncogenic properties of JUN proteins have been extensively researched. c-JUN was found to be instrumental in malignant melanoma and its overexpression has been observed in

a large number of other cancer types [94]. Experimental data have not been unequivocal regarding the oncogene properties of JUN. For example, c-JUN upregulated the expression of the tumor suppressor $p14^{ARF}/p19^{ARF}$. Moreover, c-JUN and JUNB jointly elevated transcription of the tumor-suppressor gene *DMP1* and regulated the redistribution of $p19^{ARF}$ from the nucleolus to the nucleoplasm [95], which increases p53 stabilization [96]. Under oxidative stress, c-JUN/ATF2 dimers mediate the activation of apurinic/apyrimidinic endonuclease (APE), an enzyme that is involved in DNA repair, redox regulation of transcription factors, and initiation of an adaptive response to oxidative stress [97]. In esophageal cancer (TE7) cells, c-JUN/ATF2 dimers were implicated in cell cycle progression, as knockdown of ATF2 resulted in downregulation of c-JUN, prevention of G2/M cell cycle arrest, and increased apoptotic signaling [98], suggesting that both ATF2 and c-JUN protect against oxidative stress.

JUNB was first purported to be a tumor suppressor through antagonistic action on c-JUN [99]. However, a study involving the knock-in of *JunB* in c-JUN double-knockout (*Jun*-null) mouse embryos showed that JUNB may substitute for c-JUN, disputing that JUNB exclusively acts as a c-JUN antagonist [100]. In fact, JUNB exhibits tumor-suppressive activity [81] as well as oncogenic properties in some tumor types [101].

JUND and c-FOS jointly regulate hydrogen peroxide levels in cells and can mediate the transcriptional response to remediate redox- and UV-radiation-induced stress [102], suggesting a cytoprotective role during oxidative stress.

2.2.2 FOS

FOS proteins are critically involved in the development of the immune system, central nervous system, and bone structures [103]. c-FOS also exerts oncogenic effects, as overexpression in chimeric mice incites the development of chondrogenic tumors [104]. In humans, upregulated c-FOS expression has been reported in several types of cancer, and a positive correlation between c-FOS protein levels and malignant progression and poor prognosis has been reported in multiple studies [103, 105, 106]. However, in epithelial ovarian carcinoma, low c-FOS expression was associated with unfavourable progression-free survival and reduced overall survival [107]. Furthermore, in rhabdomyosarcoma cells established from transformation related protein 53 (*Trp53)/Fos* double-knockout murine rhabdomyosarcoma cell lines, re-expression of *c-Fos* through retroviral gene transfer increased apoptosis [108], suggesting that c-FOS might also play an anti-oncogenic role. Corroboratively, under an oxidative stress impetus, c-FOS induces the expression of heme oxygenase-1 (HO-1), a stress-responsive enzyme that protects against cell damage [109].

Wild-type FOSB displays pro- and antitumor characteristics. Low expression of FOSB in gastric cancer is linked to increased

tumor progression and poor prognosis, while induced overexpression limits cell proliferation [110]. Similar results have been reported in pancreatic cancer [111]. In epithelial ovarian cancer the inverse appears to hold, where overexpression of FOSB correlates with reduced progression-free survival. FOSB actually steers chronic stress-mediated cancer progression in ovarian cancer [112, 113]. FOS-related antigen (FRA)1 also exhibits this dualistic phenotype, while FRA2 is primarily associated with pro-oncogenic properties [114–119]. In summary, the particular role that FOS proteins occupy seems to rely on each particular type of cancer as well as the prevailing biological milieu and molecular landscape.

Direct p38- or JNK-mediated phosphorylation of FOS proteins has not been observed to date. Nevertheless, multiple studies have reported downregulation of FOS proteins upon inhibition of p38 [120–124]. Knockout of MSK1 and MSK2, which are both targets of p38 [125], resulted in a 50% decrease in *c-Fos* transcription in primary mouse cell lines [126]. As alluded to in Subheading 2.1, all FOS proteins can dimerize with all JUN proteins, but FOS proteins do not form dimers among themselves. However, FOS proteins could possibly substitute for each other, as one study demonstrated that inhibition of a single member of the FOS family has no effect on cell cycle progression, whereas simultaneous inhibition of all four FOS proteins profoundly prevents cell cycle progression [127].

2.2.3 ATF/CREB

The ATF/CREB protein family members are able to dimerize with some of the other AP-1 proteins [85, 87, 89, 128]. The ATF/-CREB family consists of over 10 different proteins, which often have multiple alternatively spliced variants that are typified by rather complex nomenclature [88]. CREB2, for example, can refer to an alternatively spliced CREB, CRE-BP1, and ATF4, while some of the other proteins have up to five different designations. As a result, some articles do not list the same proteins as members of the ATF/CREB family. Moreover, not every member of the ATF family is necessarily an AP-1 protein, and some ATF proteins that are classified as AP-1 proteins *de facto* inhibit AP-1-mediated transcription. Examples include B-ATF and JUN dimerization protein-1 and -2 (JDP-1/2), which all dimerize with JUN to form transcriptionally inactive AP-1 complexes [129, 130]. ATF5 has been reported to be the exact same protein as c-FOS [88]. The ATF/-CREB proteins that are considered AP-1 transcription factors are CREB, ATF1, ATF2, ATF3, ATF4, ATF6, ATF7, and ATF8.

ATF1 and CREB are different from most of the other mentioned ATF/CREB family members insomuch that they do not dimerize with any other of the ATF/CREB proteins [89] nor with any FOS or JUN family members, putting into question their status as AP-1 proteins. However, since both proteins are activated by MAPK-activated protein kinase (MK)2, a downstream

target of p38 [131], ATF1 and CREB are part of the ASK-1 pathway. CREB is a pivotal regulator of neuroprotective signaling against ROS-mediated cell death [132] and is required for the activation of APE under oxidative stress [97]. ATF1 upregulates the transcription of *HSF1* that codes for HSF-1, which is the master regulator of heat-shock response proteins and associated with cancer aggressiveness, cell proliferation, and suppression of apoptosis [133, 134]. Inhibition of ATF1 reduced cell proliferation and migration in 2 different esophageal cancer cell lines (EC1 and Kyse450) [135].

ATF2 is the most extensively researched member of the ATF/-CREB family. In tumorigenesis, both oncogenic and tumor-suppressive roles have been reported for ATF2 [136, 137]. In the yeast species *Schizosaccharomyces pombe*, Atf1 (also known as Gad7 and Mts1), the functional homolog of human ATF2 [88], is one of the central players in the general stress response, of which the oxidative stress response is a subdivision [138]. In humans, under oxidative stress, c-JUN/ATF2 dimers mediate the transcriptional activation of APE (*see* Subheading 2.2.1) aimed at restoring the redox balance [97]. Another study found that ATF2 both upregulates and dimerizes with c-JUN in esophageal cancer (TE7) cells and is responsible for regulating cell cycle progression under oxidative stress [98]. Knockdown of ATF2 under these circumstances reinforced oxidative stress-induced apoptosis. ATF2 can be phosphorylated by both JNK and p38, though it has been reported that phosphorylation by the MAPK extracellular signal-regulated kinase (ERK) is also necessary for the activation of ATF2 [139, 140].

ATF3 is mainly activated by cell damage [141] downstream of JNK and p38 [142–145]. As a homodimer it mainly functions as a transcriptional repressor [87], negatively regulating pro-inflammatory cytokine production [146]. ATF3 is also able to dimerize with c-JUN and JUND proteins to form transcription-activating AP-1 complexes [87]. ATF3/ATF2 and ATF3/JUNB dimers possess both transcription repression and activation functions [88]. ATF3 double-knockout mice do not present with developmental abnormalities but become "over-inflamed" upon being exposed to pro-inflammatory stimuli, in line with ATF3's repressive role in pro-inflammatory cytokine production [146]. ATF3 possesses both oncogenic and tumor-suppressive qualities [146] in that it regulates genes that are involved in cell cycle progression and apoptosis signaling. One study reported that stress-induced ATF3 in normal cells leads to the activation of a set of pro-apoptotic genes, whereas in cancer cells ATF3 represses those same genes [147]. Under oxidative stress, ATF3 contributes to the cytoprotective and antioxidant functions of NRF2 [148].

ATF4 is one of the mediators of the proteotoxic stress/UPR response [149–151], another PDT-triggered survival pathway (*see* Subheading 1 and Fig. 2), that is also a downstream target of both

JNK and p38 [152–155]. Like the ASK-1 pathway, the UPR pathway is not strictly a survival pathway as it can promote apoptosis too, depending on the cellular context [150, 156]. Apart from playing a role in the UPR, ATF4 confers resistance against oxidative stress through the activation of the glutathione-redox cycle (in cardiomyocytes that have a mutation in aldehyde dehydrogenase 2) [157] and through the upregulation of vascular endothelial growth factor (VEGF) (following arsenite-induced oxidative stress) [158]. ATF4 has been proven to dimerize with c-FOS, c-JUN, and JUND but not with other ATF/CREB family members [87, 88].

ATF6 is another AP-1 protein that is heavily involved in the UPR [87, 150]. In fact, it is one of the three proximal sensors located in the ER that initiate the UPR [150]. ATF6 is directly phosphorylated by p38 [159], a process that was shown to be central to the UPR [160], suggesting possible cross-talk between the ASK-1 pathway and the UPR. Since no evidence supports ATF6 acting outside of the UPR, and since ATF6 was shown to preferentially dimerize with itself and not with other AP-1 proteins [161], ATF6 will be considered as part of the UPR and not necessarily the ASK-1 pathway. This protein will therefore not be further discussed in the review.

The last two ATF/CREB family members are ATF7 (formerly known as ATFa [162]) and ATF8. These are the most recently discovered ATF proteins and have not been extensively studied. ATF7 is structurally very similar to ATF2 [136] and is phosphorylated by p38 during stress related to diet, social isolation, UVB radiation, or aging in mice [163–166]. One study showed that ATF7 was involved in cytoprotection against oxidative stress in *C. elegans* [167], while no data on the potential role in oxidative stress recovery was retrieved for ATF8.

3 ASK-1 Pathway in PDT

The direct activation of ASK-1 by PDT has hitherto not been experimentally confirmed. Only ancillary proof using downstream targets is available to attest to the role of ASK-1 in PDT. Particularly JNK and p38 have been implicated, most often as drivers of apoptosis and inflammation. The majority of studies have been performed in vitro, providing the lowest level of evidence for the involvement of ASK-1 as a survival pathway (as opposed to animal and clinical studies).

3.1 ASK-1 Signaling: Apoptosis

In terms of apoptosis as outcome category, Shi et al. [168] demonstrated the activation of JNK and p38 by photoproduced ROS using sinoporphyrin sodium-PDT (semiconductor laser, 650 nm, cumulative radiant exposure of 3 J/cm^2) in human esophageal cancer (Eca-109) cells. Similarly, Tu et al. [169] demonstrated the

activation of JNK by PDT in human osteosarcoma (MG-63) cells using aloe emodin as PS (LED light, 430 nm, cumulative radiant exposure of 2.4–6.4 J/cm^2). Aloe emodin is an anthraquinone found in aloe vera that in itself possesses anticancer properties [170]. PDT with aloe emodin (10 μM) resulted in apoptosis and autophagy. JNK activity and degree of apoptosis were reduced by the ROS scavenger *N*-acetylcysteine (NAC, Subheading 4.3), indicating that the photoproduced ROS activated JNK signaling and suggesting that the ASK-1-JNK pathway may have accounted for the apoptotic response. The conclusions of both previous studies were supported by another investigation that showed that apoptosis was mediated by activated JNK and p38 in response to 5-aminolevulinic acid (ALA) and hexyl-ALA-PDT (halogen light, irradiance of 14 mW/cm^2, cumulative radiant exposure of 2 J/cm^2) in human lung carcinoma (H460) cells [171]. JNK activation and corollary caspase activation were also observed in human leukemia (K562) cells treated with hypericin-PDT (0.4 μg/mL, LED light, 595 nm, irradiance of 0.3 mW/cm^2, illumination time of 4 min) [172].

Although activation of p38 is more often implicated in tumor cell survival than JNK, both JNK and p38 can confer a pro-death or survival signal. Several reports have provided evidence that JNK-mediated survival signaling and/or p38-mediated cell death signaling can occur following PDT. For example, Xu et al. [173] observed that p38 was activated in human breast cancer (MDA-MB-231) cells exposed to evodiagenine-PDT (365 nm, irradiance of 23 mW/cm^2, 20-s illumination), resulting in cell death. Experiments in human melanoma (A375) cells treated with the zinc phthalocyanine (ZnPC) variant Pc13 (halogen lamp, 675 ± 15 nm, cumulative radiant exposure of 340 mJ/cm^2) showed the manifestation of apoptosis as a result of p38-induced poly (ADP-ribose) polymerase 1 (PARP-1) cleavage, whereas JNK conferred cytoprotection through autophagic signaling [174]. The signaling relationship between p38 and PARP-1 was reinforced by Zhang et al. in human lung carcinoma (A549) cells subjected to gallium (III) tris(ethoxycarbonyl)corrole-PDT (625 nm, 5-W output power, 30-min illumination) using in vitro and in vivo test models (BALB/c nude mouse xenografts) [175]. Comparable results were also obtained in the study of Salmerón et al. [176], who demonstrated an interaction between p38 and caspase-8 acting as an inducer of apoptosis in acute promyelocytic leukemia (HL60) cells treated with perinaphthenone-PDT (broadband white light, cumulative radiant exposure of 5–20 J/cm^2). Here, JNK mediated cell survival entailed phosphatidylinositol 3-kinase (PI3K)/Akt signaling. Weyergang et al. [177] revealed that inhibition of p38 in rat ovarian cancer (NuTu-19) cells with SB203580 (*see* Subheading 4.2.6) increased cell viability from <5% to 50% following PDT with meso-tetraphenyl porphyrin disulphonate (TPPS2a; 0.2 μg/mL,

435 nm, irradiance of 13.5 mW/cm^2), while inhibition of JNK with SP600125 (*see* Subheading 4.3) enhanced phototoxicity. Finally, combination treatment entailing cisplatin and berberine-PDT (LED light, 420 nm, cumulative radiant exposure of 5.4 J/cm^2) in melanoma (A375, M8, SK-Mel-19) cells and cisplatin-resistant variants resulted in p38-induced apoptosis that could be blocked with SB203580 (*see* Subheading 4.2.6) and NAC (*see* Subheading 4.3) [178].

In other instances, blockade of both p38 and JNK results in increased cell death. Ge et al. [179] reported that inhibition of p38 or JNK reduced cell viability in cultured skin squamous carcinoma (SCL-1) cells subjected to 5-ALA-PDT (40 mg/mL, 632 nm, irradiance of 23 mW/cm^2, illumination time of 30 min). Similarly, mouse embryonic fibroblasts could be sensitized to hypericin-PDT (white light, irradiance of 4.5 mW/cm^2, cumulative radiant exposure of 0.8–1.9 J/cm^2) by antagonizing p38 using molecular inhibitors, short hairpin RNA, and knockout cell lines. In this case, p38 steered cell survival through autophagy and redox stress management via activation of the NRF2 pathway [180]. Likewise, therapeutic recalcitrance was reversed in chlorin e6-PDT-treated human colorectal cancer (SW620) cells (650 nm, cumulative radiant exposure of 3 J/cm^2) by knockdown of p38 with siRNA [181].

3.2 ASK-1 Signaling: Inflammation

With respect to inflammation as outcome category, which is considered pro-tumorigenic in nature, JNK and p38 have been shown to activate AP-1 in response to PDT [86], leading to an increase in pro-inflammatory signaling owing to treatment-induced upregulation of interleukin 6 (IL-6) [182], IL-10 [183], and C-reactive protein (CRP) [184]. Corroboratively, Weijer et al. [8] reported the upregulation of AP-1-related genes (*JUN*, *FOS*, *FOSB*, *ATF2*, and *ATF3*) in PDT-treated human perihilar cholangiocarcinoma (SK-ChA-1) cells following photosensitization with ZnPC-encapsulating DPPC-DSPE-PEG liposomes (1.5 μM PS concentration, diode laser, 671 nm, irradiance of 50 and 500 mW/cm^2, cumulative radiant exposure of 15 J/cm^2). Several upstream regulators of ASK-1 such as *TRAF2* and *TRAF6* were also upregulated, implying an ASK-1 signal feed from upstream inducers (*see* Fig. 3). ASK-1 activation by paracrine signaling was also suggested with increased transcript levels of *TNF* in the 50 mW treatment group, as explained in Subheading 2. The data were largely reproducible in SK-ChA-1 cells, human epidermoid carcinoma (A431) cells, human umbilical vein endothelial cells (HUVECs), and mouse (RAW 264.7) macrophages treated with ZnPC-PDT at half maximum lethal concentration (LC_{50}) and LC_{90} using PEGylated cationic liposomes as PS delivery system (30-750 nM PS concentration, 500 mW/cm^2, cumulative radiant exposure of 15 J/cm^2). *JUN*, *FOS*, and *JUNB* were upregulated in all cell lines except the HUVECs, which exhibited downregulation of *JUNB* [12].

On a protein level, it was demonstrated that HO-1 (*see* Subheading 2.2.2) protected human cervix carcinoma (HeLa) cells and human urothelial carcinoma (T24) cells that had been subjected to hypericin-PDT (L18W30 fluorescent lamps, emission between 530 and 620 nm, irradiance of 4.5 mW/cm^2 with a cumulative radiant exposure of 4 J/cm^2) [185]. The HO-1 cytoprotection was mediated by PDT-activated p38 and PI3K, as inhibition of p38 and PI3K using PD 169316 (*see* Subheading 4.2.2) and LY294002 (*see* Subheading 4.3), respectively, resulted in increased susceptibility to apoptotic cell death following PDT. Post-PDT pro-inflammatory signaling has also been corroborated based on the cyclooxygenase 2 (COX-2) signaling axis. Hendrickx et al. [186] demonstrated that JNK1 and p38 were activated in human cervix carcinoma (HeLa) and human urothelial carcinoma (T24) cells as soon as 0.5 h after PDT with hypericin (L18W30 fluorescent lamps, 530–620 nm maximum intensity, irradiance of 4.5 mW/cm^2). Activation of JNK1 and P38 coincided with COX-2 upregulation at 3 h post-PDT and lasted for at least 21 h in both cell types. The COX-2 upregulation was p38 specific. Furthermore, PDT activated NF-κB signaling at 24 h post-PDT, reflecting an interplay between the ASK-1 and the NF-κB survival pathways (*see* Subheading 1 and Fig. 2). P38 and COX-2 inhibition with PD 169316 (1 μM) and NS-398 (50 μM; Subheading 4.3), respectively, increased the extent of apoptosis in HeLa cells in the order of PD 169316 > NS-398, confirming an adjuvant effect on cell death induction by survival signaling blockade at various levels in the ASK-1-p38 pathway. Similarly, Song et al. [187] reported that sublethal Photofrin-PDT (5 μg/mL, 635 nm, irradiance of 2 mW/cm^2, cumulative radiant exposure of 0–360 mJ/cm^2) resulted in COX-2 induction and corollary release of prostaglandin E_2 (PGE_2), which was mediated by p38. Both COX-2 and PGE_2 were suppressed by pretreatment with NAC (Subheading 4.3), PD98059 (20 μM, MAPK/ERK kinase (MEK)-1 inhibitor; Subheading 4.3), SB203580 (10 μM, p38 inhibitor; Subheading 4.2.6), LY294002 (10 μM, PI3K inhibitor), diphenyleneiodonium (5 μM, NADPH oxidase inhibitor), rotenone (5 μM, mitochondrial complex I inhibitor), pyrrolidine dithiocarbamate (PDTC; 10 μM, NF-κB inhibitor), and N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK; 10 μM, IκB protease inhibitor). Furthermore, PDT activated p53, an effect that was blocked by PD98059 and SB203580 as well as COX-2 siRNA. COX-2 siRNA also increased Noxa (downstream effector of p38) and reduced Bax protein levels that in turn translated to exacerbated caspase-3 activation (occurs downstream of p38 [188]) and consequent apoptosis, altogether implicating the immediate early stress response pathway at the level of p38 and below. In PDT-treated mice bearing murine breast cancer (EMT6) xenografts, COX-2 inhibition by celecoxib reduced COX-2, PGE_2, VEGF, TNF-α, and IL-1β

protein levels, which were all considerably elevated after PDT. p38-regulated COX-2 signaling can also activate histone acetyltransferase p300 (p300HAT) that, when directly inhibited with anacardic acid or short hairpin RNAs or indirectly with upstream inhibitors of COX-2 (NS-398), sensitized human melanoma (A375) cells to PEGylated liposomal chlorin e6 PDT (diode laser, 662 nm, irradiance of 95 mW/cm^2) [189]. Furthermore, Solban et al. showed that p38 could regulate VEGF expression independently of HIF-1α in prostate cancer cells (LNCaP) following sublethal benzoporphyrin-derivative PDT (140 nmol/L, 690 nm diode laser, 0.5 J/cm^2) [190]. The increased expression of VEGF could be abrogated using p38 inhibitor SB202190. Similarly, another study succeeded in suppressing hypericin-PDT-induced migration of endothelial cells by inhibiting either p38 using PD 169316 or COX-2 using NS-398 in T24 cells (L18W30 fluorescent lamps, maximum emission between 530 and 620 nm, cumulative light dose of 4 J/cm^2) [191]. The inhibition of p38 in this study also further sensitized cells to PDT. These data suggest that pro-inflammatory signaling, which may translate to tumor survival and recurrence if left unchecked, occurs after PDT and can be pharmacologically controlled.

In the final analysis, the discrepancy between pro-survival versus pro-death signal relay can be ascribed to prolonged or transient activation of the ASK-1/p38/JNK signaling cascade, the particular PS used and its localization preference [13, 25], and cell type and genotype [24, 177]. Furthermore, particular inhibitors for the same target can have different effects on PDT outcome. For example, the p38 inhibitor PD 169316 reduced apoptosis while SB203580 (another p38 inhibitor) increased apoptosis [192], indicating that there could be variation in downstream effects imparted by different inhibitors of the same target or that other (unknown/pharmacodynamic) factors are at play. All these should be taken into consideration when designing combinatorial PDT modalities encompassing small molecular inhibitors of survival pathways.

4 Inhibition Strategies for the ASK-1 Pathway

Given the role of ASK-1 in survival signaling under conditions of oxidative stress, inhibition of the ASK-1 pathway is expected to improve PDT outcome, although some anticancer treatments that mechanistically rely on ROS generation in fact require ASK-1 for therapeutic potency [193–195]. In those cases, inhibition would be ill advised. In the previous section it has become clear that p38 and JNK exhibit pleiotropic divergence after PDT, signaling either pro-apoptosis or pro-survival and therefore making it difficult to pharmacodynamically prognosticate the net effect of pathway inhibition. On the other hand, some cancer types are known to rely on

ASK-1 for its oncogenic properties [196], which in itself could provide cues as to the possible end result of pathway inhibition. All these elements should be carefully considered when implementing ASK-1 inhibitors as adjuvants for PDT. In the following sections a summary is given of ASK-1 and p38 inhibitors that could be used as adjuvants in PDT.

Inhibitors of JNK were omitted given that JNK is a key regulator of apoptosis (inhibits anti-apoptotic BCL-2 family members while activating pro-apoptotic BCL-2 family members) and executes tumor suppressor and pro-oncogenic functions (*see* Subheading 2.1). Its downregulation may be deleterious to PDT outcome, although in some particular cases pharmacological intervention could be fruitful for reasons provided above. Those cases did not, however, warrant inclusion of and elaboration on the respective inhibitors.

4.1 ASK-1 Inhibitors

Most ASK-1 inhibitors are small molecules that have been primarily investigated as anti-inflammatory drugs for the treatment of immune disorders, neurodegenerative diseases, and surgery-induced fibrosis [197–204]. A selection of ASK-1 inhibitors that could be explored for use in PDT is presented in Fig. 4 that outlines their molecular structure, chemical attributes, spectral properties, and pharmacodynamic properties. An overview of the mechanism of action, pharmacological and biological effects, test systems, and application in PDT is provided Table 1. A select number of the listed inhibitors is addressed in the text.

4.1.1 Gilead Science's ASK-1 Inhibitors (GS-4997/ Selonsertib, GS-444217, GS-459679)

Gilead's synthetic ASK-1 inhibitors are linear amide backbone polycyclic compounds that constitute interesting pharmacological candidates for PDT due to their low 50% inhibitory concentration (IC_{50}) values toward ASK-1, namely in the 3–10 nM range [210, 231]. To date, none of the compounds have been tested in combination with PDT and no studies have been performed in oxidatively (hyper)stressed cancer cells.

Selonsertib (GS-4997) is a selective inhibitor of ASK-1 that has been indicated for the treatment of nonalcoholic steatohepatitis owing to its anti-inflammatory and anti-fibrotic properties [228]. Selonsertib binds the catalytic kinase domain (serine/threonine) of ASK-1 in an adenosine triphosphate (ATP)-competitive manner and prevents phosphorylation of downstream kinases such as JNK and p38 [224, 227]. Although late-stage clinical trials for various liver indications failed [229], selonsertib is being considered as a cancer therapeutic because of its ability to reverse ATP-binding cassette (ABC) transporter-facilitated multidrug resistance (MDR) through ABCB1 or ABCG2 [230]. It should be noted that selonsertib attenuated apoptosis induced by ecliptasaponin A in human non-small cell lung cancer (H460 and H1975) cells, which was mediated by p-ASK-1, p-JNK, and cleaved caspase-

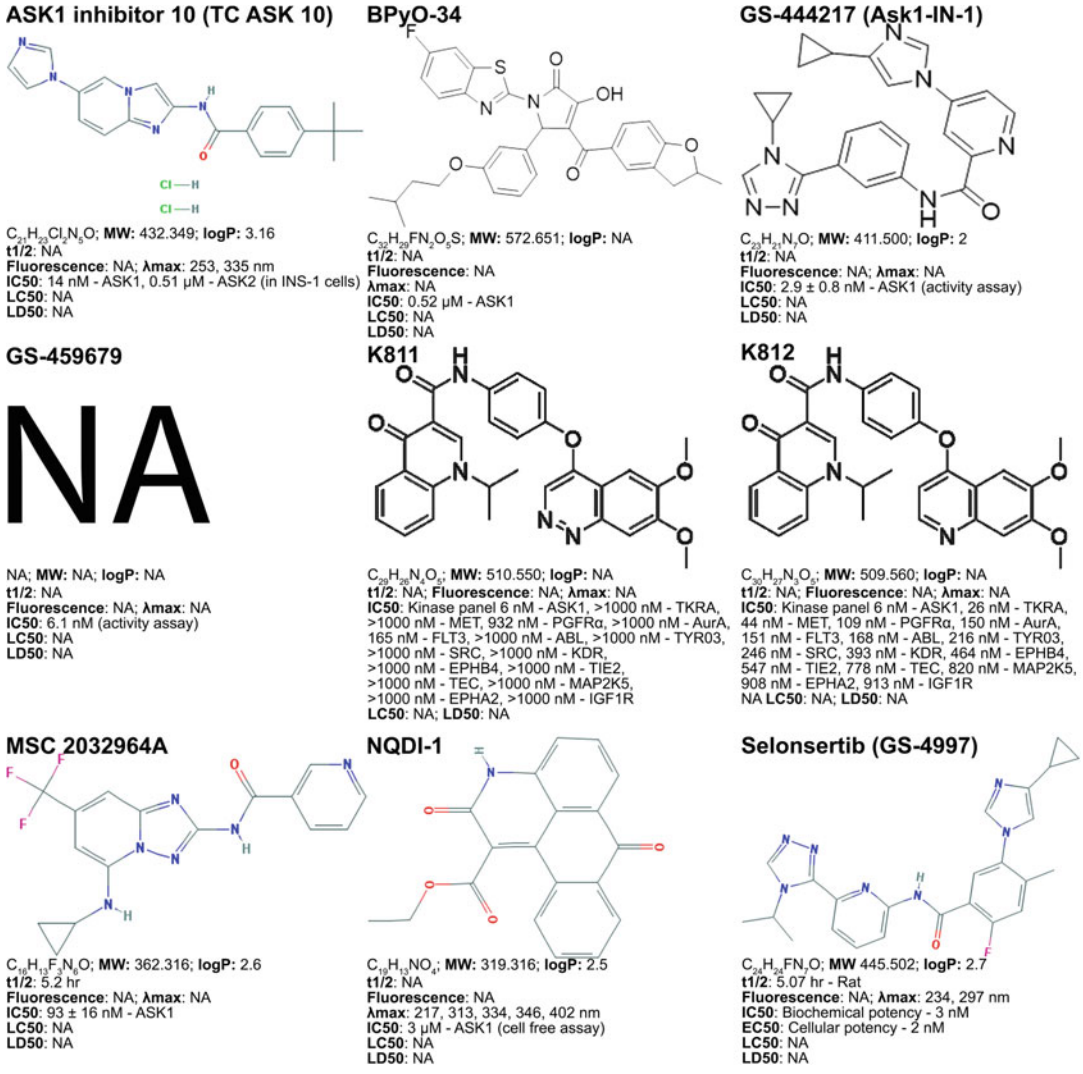

Fig. 4 Overview of ASK-1 inhibitors that are eligible candidates for use as adjuvants in PDT. LogP (octanol: water partition coefficient) values were retrieved from PubChem and were predicted with XLogP2 or XlogP3 software. The half maximum inhibitory concentration (IC_{50}, enzymes), half maximum lethal concentration (LC_{50}, in vitro), half maximum lethal dose (LD_{50}, in vivo), $t_{1/2}$ (circulation half-life), and spectral properties were obtained from the material safety data sheets (retrieved from the Cayman Chemicals, MedKoo, and Spectrum Chemical websites) and PubChem. The half maximum inhibitory concentration (IC_{50}, used for proliferation and enzymes) and half maximum cellular potency (EC_{50}) were obtained from literature. This also applies to LC_{50}, LD_{50}, and $t_{1/2}$ data that were missing from or inconsistent in the abovementioned databases. Abbreviations: *Em* emission, *Ex* excitation, *ip* intraperitoneal, *iv* intravenous, *MW* molecular weight, *NA* information not available, *sc* subcutaneous, λ_{max} the wavelength at which there is an absorption maximum (may be multiple absorption bands)

3 [226]. However, ASK-1 was not activated by ROS per se, as ROS do not seem to be the driver behind ecliptasaponin A-induced ASK-1 activation.

Table 1
Overview of ASK-1 inhibitors and their pharmacological and biological characteristics

Name	Mechanism	Pharmacological effect	Biological effect	Tested in	Tested in PDT	Ref.
ASK-1 inhibitor 10 (TC ASK-10)	Selective inhibition of ASK-1	Reduced phosphorylation of JNK and p38	Inhibition of growth and migration	*In vitro*: INS-1, primary airway smooth muscle cells. *In vivo*: rats	No	[205, 206]
BPyO-34	Inhibition of ASK-1	NA	NA	NA	No	[207]
GS-444217 (ASK-1-IN-1)	Selective inhibition of ASK-1	Inhibition of ASK-1 by kinase domain binding; reduced phosphorylation of p38 and JNK; reduced expression of TNF-α	Anti-inflammatory; anti-fibrotic	*In vitro*: human pulmonary adventitial fibroblasts, neonatal rat ventricular cardiomyocytes, mouse primary Kupffer cells, mouse hepatic stellate cells, mouse hepatocytes, mouse cardiac fibroblasts, HEK293T cells *In vivo*: Nos3-deficient C57BL/6, Tg26, *Nlrp3*-KI mice, Sprague-Dawley and Wistar-Kyoto rats	No	[197, 204, 208–212]
GS-459679	NA	Downregulation of ASK-1 and p-ASK-1; inhibition of JNK activation; inhibition of TNF-α, IL-6, and IL-1β; inhibition of caspase-3 activation	Inhibition of apoptosis; anti-inflammatory; oxidative stress reduction	*In vivo*: CD1 mice, C57BL/6 mice, mouse model of APAP hepatotoxicity	No	[202, 213, 214]

K811	Inhibition of ASK-1	Downregulation of p-JNK, p-p38, and cyclin D1 protein levels; inhibition of glial activation	Antiproliferative; inhibition of tumor growth; neuroprotective	*In vitro*: AGS, NCI-N87, 293 T, HeLa, MKN45, MKN74, MKN, HuG1-N, SCH gastric cancer cells, NSC34 cells *In vivo*: SH101 and MKN45 xenografts in BALB/cAJcl-nu/nu mice, C57B6 WT and ASK-1 KO mice, ALS $SOD1^{G93A}$ transgenic mice	No	[215–218]
K812	Inhibition of ASK-1	Inhibition of glial activation	Neuroprotective	*In vitro*: NSC34 cells *In vivo*: ALS $SOD1^{G93A}$ transgenic mice	No	[216, 218]
MSC 2032964A	Selective inhibition of ASK-1	Inhibition of LPS-induced ASK-1 and p38 phosphorylation; reduction of TNFα and iNOS protein levels	Anti-inflammatory; neuroprotective	*In vivo*: Sprague-Dawley rats, C57BL/6 J WT and ASK-1 KO mice	No	[198, 219]
NQDI-1	Selective inhibition of ASK-1	Inhibition of ASK-1 through competitive binding in the catalytic domain; reduction of JNK, c-Jun, p53, caspase-3 phosphorylation; downregulation of inflammatory cytokines (TNF-α, IL-6, IL-10); upregulation of miR-1, miR-27a, and miR-133a	Anti-inflammatory; inhibition of growth	*In vitro*: BV2, HL60, RAW 264.7, THP-1, SKOV3 cells *In vivo*: Sprague-Dawley rats, SKOV3 xenografts in athymic BALB/c nude mice	No	[199, 201, 220–223]
Selonsertib (GS4997)	Selective inhibition of ASK-1	Inhibition of ASK-1 catalytic kinase domain (serine/threonine) in ATP-competitive manner; reduction of downstream kinase phosphorylation (JNK	Anti-inflammatory; anti-fibrotic; reversal of ABC transporter-mediated MDR	*In vitro*: HSC-T6, LX-2, primary mouse hepatic macrophages, RAW 264.7, KB-3-1, KB-C2, SW620, SW620/Ad300, NCI-H460, NCI-H460/MX20, S1,	No	[200, 224–230]

(continued)

Table 1
(continued)

Name	Mechanism	Pharmacological effect	Biological effect	Tested in	Tested in PDT	Ref.
		and p38); inhibition of MMP expression		S1-M1–80, HEK293, HEK293/ABCG2–482-R2, HEK293/ABCG2–482-G2, HEK293/ABCG2–482-T7, HEK293/ABCB1, HEK293/ABCC1, HEK293/ABCC10, H460, H1975 cells *In vivo*: Sprague-Dawley rats, C57BL/6 mice *Clinical*: adults with normal and impaired hepatic function, nonalcoholic steatohepatitis, and pulmonary arterial hypertension		

GS-444217 is a selective ASK-1 inhibitor that targets to the ASK-1 kinase domain with an IC_{50} of 2.9 nM [209]. Gilead has studied the compound in the framework of nephropathies [204, 208, 209, 211], pulmonary diseases [197, 232], and hepatic fibrosis [212] but not for oncological indications. ASK-1 inhibition by GS-444217 has been sufficiently demonstrated in the cited studies with downstream ramifications on p38 and JNK [208]. The advantage of this compound is that it can be administered orally and that it enjoys high oral bioavailability (>89% in rats) [210].

GS-459679 is the most elusive selective ASK-1 inhibitor of the three, with no publicly available information on binding site(s) and molecular structure. The IC_{50} with respect to ASK-1 is 6.1 nM [214]. The compound has been studied in conditions of hypoxia/reoxygenation, which have ROS generation, oxidative/nitrosative stress, and sterile inflammation at their helm [3, 233–238]. GS-459679 ameliorated infarct size, reduced cardiomyocyte apoptosis, and preserved left ventricular function in a mouse model of myocardial ischemia-reperfusion [202]. In vivo ASK-1 inhibition by GS-459679 was confirmed in a mouse model of acetaminophen-induced liver toxicity, where GS-459679 was shown to reduce p-ASK-1 and p-JNK protein levels, liver damage, and inflammation as well as improve antioxidative capacity [213].

Taken together, these data suggest that ASK-1 blockade by these GS inhibitors may cause the downstream biological responses to veer into an undesired direction (i.e., cytoprotection) when juxtaposed to the goals of cancer drugs, with the exception of sensitization of cancer cells to chemotherapeutics. Nevertheless, the conditions in which these inhibitors have been tested do not emulate the redox landscape created by PDT, which embodies substantial oxidative damage by ROS that above all are not produced under pathophysiological circumstances (i.e., $^1O_2$). The testing of these inhibitors as well as the many others detailed in [210, 231] in cancer cells as adjuvants for PDT is therefore still warranted, even if these inhibitors ultimately fulfill their function as negative controls in assays.

4.1.2 ASK-1 Inhibitor 10

ASK-1 inhibitor 10, also known as TC ASK 10, is a synthetic imidazo[1,2-a]pyridine compound that selectively inhibits ASK-1 at an IC_{50} of 14 nM [205, 210]. ASK-1 inhibitor 10 has been tested in neither cancer cells nor PDT. The compound was used in growth assays with ASK-1-overexpressing airway smooth muscle cells isolated from patients with chronic obstructive pulmonary disease. ASK-1 inhibitor 10 reduced mitogen-activated cell growth and migration in a concentration-dependent manner, which were mediated by p38 and JNK [206]. ASK-1 inhibitor 10 was also found to inhibit streptozotocin-induced JNK in INS-1 pancreatic β cells, whereby phosphorylation of p38 was dose-dependently

inhibited [205]. Finally, ASK-1 inhibitor 10 exhibited good bioavailability when dosed orally in rats [205]. Oral dosing is easy and practical and generally has preference over intravenous delivery, which has to be performed in a clinical setting.

4.1.3 MSC2032964A

MSC2032964A is a synthetic compound identified by high-throughput screening that exhibits selectivity and inhibitory potency toward ASK-1 (IC_{50} = 93 nM) [198]. The compound has a favorable pharmacokinetics profile; the good metabolic stability, enterocytic permeability, and moderate plasma protein-binding affinity account for excellent oral bioavailability (82%), moderate clearance (1.1 L/kg/h), long circulation half-life (5.2 h), and a high distribution volume (1.0 L/kg) in rats. Comparable results were obtained in mice. MSC2032964A also possesses brain-penetrating capabilities [198]. In follow-up studies, MSC2032964A blocked lipopolysaccharide-induced ASK-1 and p38 phosphorylation in cultured mouse astrocytes and was able to almost completely blunt ASK-1 enzymatic activity in mice with experimental autoimmune encephalomyelitis during an 18-d oral gavage regimen (30 mg/kg), which translated to reduced spinal cord demyelination, decreased astrocyte and microglial activation, and partial prevention of optic nerve demyelination [198]. In an optic nerve injury mouse model, p38 activation and retinal ganglion cell death were suppressed by MSC2032964A, which concurred with a reduction in TNF-α and inducible nitric oxide synthase (iNOS) protein levels [219]. Despite its inhibitory activity toward ASK-1 and downstream p38-suppressive effects, MSC2032964A has not been investigated in cancer cells or oncological PDT.

4.1.4 NQDI-1

NQDI-1 (2,7-dihydro-2,7-dioxo-3H-naphtho[1,2,3-de]quinoline-1-carboxylic acid ethyl ester) is a synthetic aminoanthraquinone derivative [239] that selectively and reversibly inhibits ASK-1 at an IC_{50} of 3 μM by competitively targeting the ATP-binding site in the catalytic domain [222]. ASK-1 inhibition by NQDI-1 was confirmed in a Langendorff Sprague-Dawley rat heart perfusion experiment, where the roles of ASK-1, JNK, and MKK7 were investigated in a setting of sunitinib-induced cardiotoxicity. Compared to untreated hearts, NQDI-1 reduced protein levels of p-ASK-1 and its downstream targets p-JNK and p-MKK7, in addition to increasing levels of microRNA (miR)-1, miR-27a, and miR133a [221]. Implementation of NQDI-1 in differentiated human monocytic leukemia (THP-1) cells that had been primed with the quercetin derivative quercetin 3-oxyloside showed that (1) NQDI-1 was not toxic to cells up to a 50 μM concentration; (2) NQDI-1 reduced TNF-α protein levels in a concentration-dependent fashion; and (3) NQDI-1 blocked the activation of ASK-1 and its downstream target p38, altogether reversing the

inductive effects of quercetin 3-oxyloside on these proteins [223]. The cytotoxicity of NQDI-1 was contested in [221], where the compound caused a concentration-dependent reduction in the viability of cultured human acute myeloid leukemia (HL60) cells and exacerbated the potency of sunitinib [221]. Finally, in a study on the role of glucose in metformin cytotoxicity toward human ovarian cancer (SKOV3) cells, Ma et al. [220] demonstrated in monolayer cultures that NQDI-1 reduced protein levels of p-JNK and growth arrest- and DNA damage-inducible gene 153 (GADD153/CHOP), and cleaved caspase-4 under conditions of hypoglycemia. The hypoglycemia in itself triggered activation of ASK-1 (but not JNK) and reduced Bcl-2 protein levels, whereby inhibition of ASK-1 by siRNA translated to increased apoptosis via the intrinsic pathway. In euglycemic mice bearing SKOV3 xenografts, NQDI-1 reduced tumor volume by completely abrogating intratumoral ASK-1 activation but compromised the therapeutic efficacy of metformin.

NQDI-1 has not been investigated in relation to PDT. Readers should note that numerous other potent ASK-1-inhibiting aminoanthraquinone derivatives are available, some of which have shown antitumor efficacy in mouse models of human cancer [239] and could be considered as adjuvants for PDT.

4.2 p38 Inhibitors

As described in Subheading 3, p38 is a kinase downstream of ASK-1 involved in survival signaling, albeit one that can also act as a pro-death signal inducer. A selection of p38 inhibitors that could be explored for use in PDT is presented in Fig. 5. An overview of the mechanism of action, pharmacological and biological effects, test systems, and application in PDT is provided in Table 2. Some of the included inhibitors are addressed in the text.

4.2.1 CMPD1

CMPD1 (2′-fluoro-N-(4-hydroxyphenyl)-[1,1′-biphenyl]-4-butanamide) is a selective noncompetitive inhibitor of p38α that blocks the binding of its substrate, mitogen-activated protein kinase-activated protein kinase 2 (MK2a), which post-translationally regulates TNF-α [341]. Currently CMPD1 is not known to inhibit any other substrate of p38α.

CMPD1 has been tested as a therapeutic agent against several types of cancer. In gastric cancer (MKN-45 and SGC7901) cells CMPD1 inhibited cell proliferation in a dose-dependent manner by inducing G_2/M cell cycle arrest and apoptosis [244]. Apoptosis was accompanied by downregulation of BCL-2 and c-MYC and upregulation of BAX and cytochrome c release and cleavage of PARP. In the treatment of human cervix carcinoma (HeLa) cells, CMPD1 inhibited the phosphorylation of heat-shock protein 27 (HSP27), a downstream target of p38/MK2a, that protected cells from TNF-α-induced apoptosis [241]. This study also revealed that the pro-apoptotic proteins cleaved PARP and that cleaved caspase-3

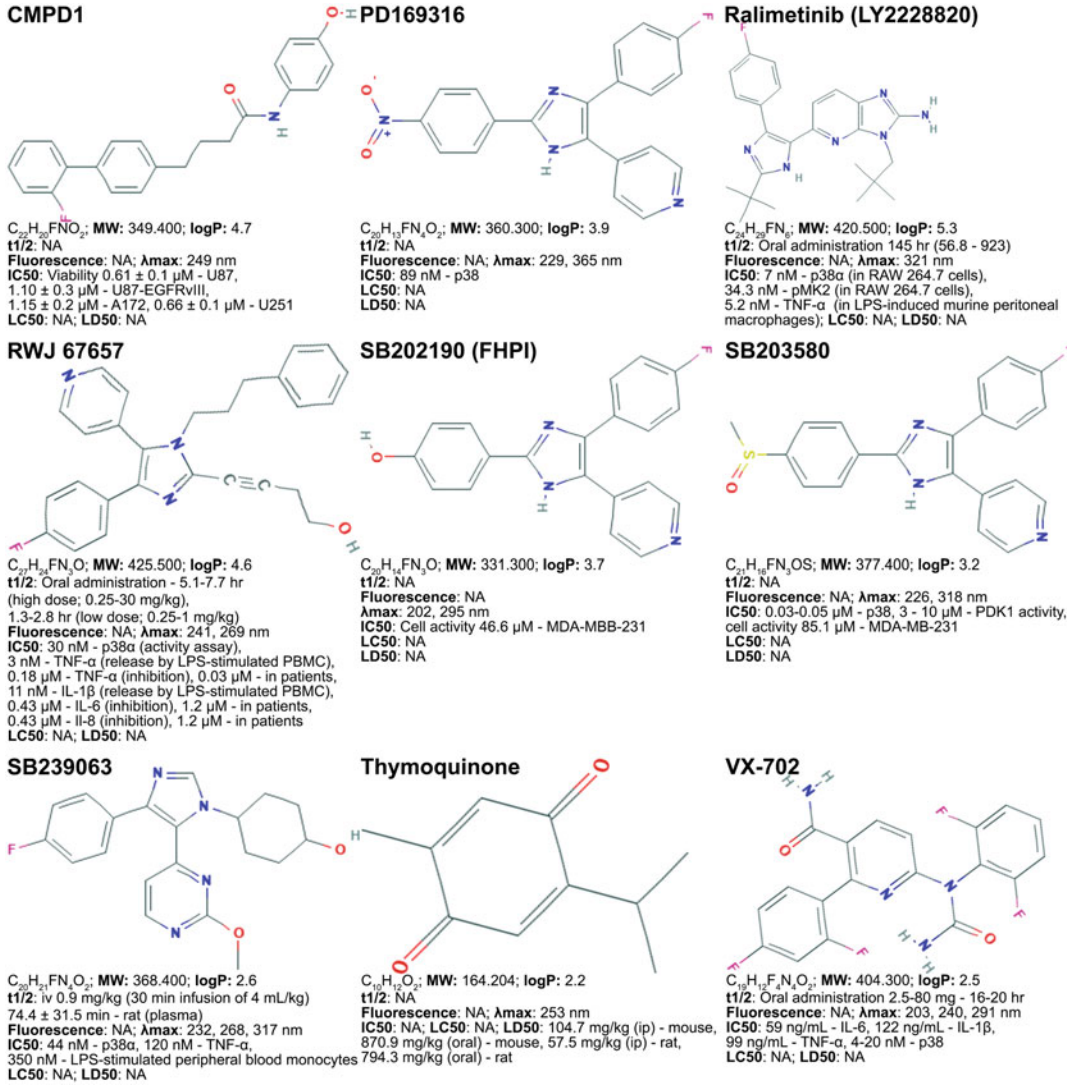

Fig. 5 Overview of p38 inhibitors that are eligible candidates for use as adjuvants in PDT. LogP (octanol:water partition coefficient) values were retrieved from PubChem and were predicted with XLogP2 or XlogP3 software. The half maximum inhibitory concentration (IC_{50}, enzymes), half maximum lethal concentration (LC_{50}, in vitro), half maximum lethal dose (LD_{50}, in vivo), $t_{1/2}$ (circulation half-life), and spectral properties were obtained from the material safety data sheets (retrieved from the Cayman Chemicals, and Spectrum Chemical websites), PubChem, and Selleckchem. The half maximum inhibitory concentration (IC_{50}, used for proliferation and enzymes) was obtained from available literature. This also applies to LC_{50}, LD_{50}, and $t_{1/2}$ data that were missing from or inconsistent in the abovementioned databases. Abbreviations: *Em* emission, *Ex* excitation, *ip* intraperitoneal, *iv* intravenous, *MW* molecular weight, *NA* information not available, *sc* subcutaneous, λ_{max} the wavelength at which there is an absorption maximum (may be multiple absorption bands)

Table 2
Overview of p38 inhibitors and their pharmacological and biological characteristics

Name	Mechanism	Pharmacological effect	Biological effect	Tested in	Tested in PDT	References
CMPD-1	Inhibition of p38α; depolymerization of microtubules	P38α inhibition by blocking the binding of MK2a (substrate); downregulation of BCL-2 and c-MYC proteins; upregulation of BAX, cytochrome c release, and cleavage of PARP; inhibition of HSP27	Antiproliferative; cell cycle arrest (G_2/M); apoptosis	*In vitro*: MKN-45, SGC7901, CN/AML patient-derived blast and induced pluripotent stem cells (iPSC), Kasumi-1, HeLa, HepG2, MCF-7, U87, U87-EGFRvIII, A172, U251 cells, BV-2, primary glioblastoma cells, primary human astrocytes, mouse embryonic fibroblasts	No	[240–245]
PD 169316	Inhibition of p38 and TGFβ signaling	Inhibition of TGFβ- and activin A-induced signaling; reduced SMAD2 and SMAD3 phosphorylation and nuclear translocation, and upregulation of SMAD7	Tumor growth inhibition; apoptosis induction/ suppression	*In vitro*: T47D, HeLa, T24, CaOV3, U-937, PC12, Rat-1, rat cerebellar granule neuron cells	Yes	[186, 188, 246–250]
Ralimetinib (LY222 8820)	Inhibition of p38α and p38β	P38 inhibition by ATP-competitive and selective binding; inhibition of HSP27 phosphorylation; dose-dependent inhibition of MK2; inactivation of HSP27	Anti-angiogenic; anti-inflammatory; anti-migratory; anti-invasive; anti-apoptotic; inhibition of EMT	*In vitro*: U-87MG, HeLa, MDA-MB-468, 786-O, OPM-2, A2780, B16-F10, RAW 264.7, HC116, LXFA-629, MDA-MB-231, NCL-H1650, PC-3, PMC42ET, MDA-MB-436, BT549, A549, H129, NCI-H23,	No	[251–263]

(continued)

Table 2
(continued)

Name	Mechanism	Pharmacological effect	Biological effect	Tested in	Tested in PDT	References
				NCI-H157, NCI-H460, NCI-H1792, H1299, H520, H1975, H2009, H358, LU99, H727, H460, H2030, A549, H23, BCap37, Bads-200, Bats-72 cells *In vivo*: ADSC/ECFC xenografts in female athymic nude mice; B16-F10 xenografts in C57/BL6 mice; U-87MG, SK-OV-3x-luc#1, 786-O, A549, and MDA-MB-468 xenografts in athymic nude mice; A-2780 xenografts in CD1 nu/nu mice; OPM-2 xenografts in CB-17 SCID mice; MDA-MB-231 xenografts in NU-Foxn1^{nu} and Balb/c mice *Clinical*: adult colorectal, breast, ovarian, non-small cell lung, glioblastoma multiforme cancer patients		

RWJ 67657	Inhibition of p38α and p38β	Inhibition of TNF-α, COX-2, IL-6, IL-8, and IL-1β; downregulation of ER, SRC-1, SRC-2, and SRC-3; decreased ATF and NF-κB signaling	Antiproliferative; anti-inflammatory; inhibition of tumor growth; reversal of EMT	*In vitro*: LPS-activated human peripheral blood mononuclear cells, primary macrophages, synovial fibroblasts, MCF-7, MCF-7TN-R, MCF10A, HEK293 cells, HUVECs *In vivo*: MCF-7 and MCF-7TN-R xenografts in female immunocompromised, ovariectomized mice. *Clinical*: healthy male human subjects	No	[264–272]
SB202190 (FHPI)	Selective inhibition of p38α and p38β	P38 inhibition though competitive binding in the catalytic domain; reduction of ERK1/2 phosphorylation; increase in mutant p53 phosphorylation (Ser15); downregulation of COX-2 and leukocyte-adhesion molecule expression	Antiproliferative; induction of autophagy; inhibition of growth; inhibition/induction of migration; anti-inflammatory	*In vitro*: CHO, HK-1, MDA-MB-231, RIF-1, LY-R, SKOV3, HUVECs, ARPE-19, IPEC-1, TOV112D, A375, G361, COLO-800, MEL-JUSO, SK-MEL-30, IPC-298, HEK293, HCT116, DLD-1, PC12 cells, human embryonic lung fibroblasts, human fetal microglial cells, iPSC-derived macrophages, human melanoma and human corneal epithelial cells *In vivo*: Swiss albino mice	Yes	[240, 273–288]

(continued)

Table 2
(continued)

Name	Mechanism	Pharmacological effect	Biological effect	Tested in	Tested in PDT	References
SB203580	Selective inhibition of p38 through binding in the catalytic ATP-binding domain	P38 inhibition though competitive binding in the catalytic domain; reduction of COX-2, PGE_2, and MMP-9 protein levels; suppression of P-gp	Antiproliferative; anti-migratory; anti-invasive; induction of apoptosis	*In vitro*: A375, MCF-7, THP-1, HeLa, Cos-7, MG 63, C20A4, Caki-1, CT6, BA/F3 F7, PBMC/T, T47D, Colo 853, B16, FO-1, HK-1, NuTu-19, WiDr, M8, MDA-MB-453, MDA-MB-231, MEF, A-498, ACHN, BCG-823, SGC-7901, SH-SY5Y, HT-29, L1210/VCR, SK-Mel-19 cells	Yes	[177, 178, 187, 192, 274, 289–299]
SB239063	Inhibition of p38	Attenuation of IL-6 and blockade of TNFα release; inhibition of TGF-β and bFGF; inhibition of NF-κB activation and translocation; suppression of MMP-3 production; downregulation of VEGF protein levels; restoration of BCL-2 protein levels and inhibition of apoptosis	Anti-inflammatory; antiproliferative; anti-angiogenic; anti-migratory; inhibition of tumor growth; neuroprotective	*In vitro*: HaCaT, SH-SY5Y, HCECs, ECV304 (primed with HepG2-derived BMP2), MES 23.5, HUVECs, PANC-1, BxPC-3, LoVo human diploid fibroblasts, human primary lung fibroblasts, human peripheral blood monocytes, human lung macrophages, guinea pig eosinophils, IMR32, guinea pig primary parasympathetic nerve	No	[243, 273, 300–316]

				cells, rat bone marrow mesenchymal cells *Ex vivo*: human gestational membranes, ovine gestational membranes *In vivo*: HepG2 xenografts in female nude mice, BALB/c mice, Hartley guinea pigs, Wistar rats, CL57BL/6 J mice, Lewis rats, Sprague-Dawley rats, spontaneously hypertensive rats		
Thymoquinone	Inhibition of p38; inhibition of TNF-α-induced ASK-1 phosphorylation	Prevention of p38 and JNK activation, reduction of inflammatory signaling (IL-6 and IL-8); attenuation of mTOR activity and downstream signaling; co-downregulation of p-AKT, EP2, and MMP-9; downregulation of XIAP, BCL-2, BCL-xL, and survivin	Anti-inflammatory; induction of autophagic cell death; induction/inhibition of apoptosis; reduction of metastatic propensity; induction of oxidative stress; anti-migratory; anti-invasive	*In vitro*: primary RA synovial and osteoarthritis chondrocyte cell cultures, SW-626, HCT-116, HCT-116/p53KO, LNCaP, HEp-2, HL-60, WEHI-1, BV-2, A375, B16F10, PANC-1, BxPC-3, MG63, MNNG/HOS, primary human osteoblasts, AsPC-1, U937, Caco2, Caki-1, HCT-166, LoVo, DLD-1, HT-29, FHs74Int, T28, N28, T24, HTB-9, MCF-7, MDA-MB-231, FG/COLO357, CD18/HPAF cells *In vivo*: in mice and rats for various toxicity,	No	[203, 273, 296, 317–333]

(continued)

Table 2
(continued)

Name	Mechanism	Pharmacological effect	Biological effect	Tested in	Tested in PDT	References
				inflammatory disease, and injury studies; Msh2 (loxP/loxP) Villin-Cre intestinal tumor mouse models; PANC-1 xenografts in BALB/c nude mice; cholestatic rats with liver injury; MDA-MB-231 xenografts in female nude BALB/c OlaHsd-foxn1 mice		
VX-702	Inhibition of p38 and NLK	Inhibition of L-6, IL-1β, and TNF-α production; increase in BAX and reduction of BCL-2 levels	Anti-inflammatory; induction/inhibition of apoptosis; attenuation of fibrosis (in chronic graft-versus-host disease); suppression of immune cell infiltration	*Ex vivo*: blood assay primed with LPS *In vitro*: HC116, A549, Calu-3, HCoEpiC, BT483, MCF-7, T47D, MDA-MB-415 cells *In vivo*: Sprague-Dawley rats, T47D-TamR xenografts in nude-Foxn1^{nu} mice, B10.D2 (H-2d) and BALB/c (H-2d) mice *Clinical*: rheumatoid arthritis patients	No	[256, 334–340]

and -8 were upregulated by CMPD1. Furthermore, CMPD1 can selectively inhibit the growth of leukemia (Kasumi-1) cells at the level of MK2a, as MK2a phosphorylation is a key leukemogenic event, but not p38 and without affecting healthy cells [245]. Lastly, a study investigating the cytotoxic activity of CMPD1 in glioblastoma cells (U87, U87-EGFRvIII, A172, and U251) revealed cytotoxic effects independent of MK2a inhibition [242]. CMPD1-treated cells showed no change in phosphorylation of MK2a and heat shock protein 27 (HSP27), and knockdown of MK2a did not attenuate the cytotoxicity of CMPD1. Further analysis revealed that CMPD1 inhibits tubulin polymerization in a dose-dependent manner, disrupting mitosis and inducing cell cycle arrest and apoptosis.

Studies are needed to investigate the pharmacodynamics of CMPD1 and PDT in cancer cells.

4.2.2 PD 169316

PD 169316 is a synthetic substituted 2,4,5-triarylimidazole that acts as a cell-permeable, reversible, competitive, and selective p38 inhibitor with an IC_{50} of 89 nM. As alluded to in Subheading 3, p38 inhibition by PD 169316 augmented the extent of apoptosis in hypericin-PDT-treated human cervix carcinoma (HeLa) cells [186], providing proof of concept for the successful implementation of the combinatorial modality.

Furthermore, but in the absence of PDT, PD 169316 amplified apoptosis triggered by TNF-α by 14% in human histiocytic lymphoma (U-937) cells, an effect that was even more pronounced when PD 169316 was administered jointly with another pyridinylimidazole-based compound, PD 98059, but not with SB203580 (*see* Subheading 4.2.6). The combination yielded a 67% increase in apoptosis compared to TNF-α-primed control cells and was mediated by caspase-3 [247]. The role of TNF-α on the ASK-1 signaling axis in the context of PDT has been explained in Subheading 2 and essentially encompasses dual ASK-1 activation following PDT (ROS-induced and activation by paracrine signaling via TNFR). A plethora of synthetic pyridinyl imidazole analogs with low IC_{50} values (for p38) have been synthesized [342] that all qualify as candidate adjuvants for PDT, either as singular or as combined adjuvants.

It should be noted that numerous studies in various types of cells and cell lines have shown that blockade of p38 MAP kinase by PD 169316 deters apoptosis. For instance, Nath et al. [188] reported an increase in p38 kinase activity and consequent apoptosis following an episode of potassium deprivation in rat cerebellar granule neurons, which was attenuated by PD 169316 in a concentration-dependent manner. Similarly, Kummer et al. [248] found increased p38 activity in serum-deprived Rat-1 fibroblasts and nerve growth factor-deprived differentiated rat phaeochromocytoma (PC12) cells, which was accompanied by apoptotic cell

death. PD 169316 blocked the increase in p38 activity and reduced the degree of apoptosis by up to 80%. The manifestation of p38-mediated cell death signaling should be investigated before developing combinatorial therapeutic regimens aimed at cell death induction.

4.2.3 Ralimetinib (LY2228820)

Ralimetinib (LY2228820) is a tri-substituted imidazole derivative and a potent oral selective inhibitor of p38α/β that derives its inhibitory action from competitive binding in the ATP catalytic domain (IC_{50} = 5.3 and 3.2 nM for the α- and β-isoform, respectively) [260]. Like many other p38 inhibitors, much of the ralimetinib research has been centered on inflammation. However, unlike many other p38 inhibitors, the drug has primarily been investigated for oncological indications, which underscores the importance of inflammation in the field of oncopharmacology. Ralimetinib has undergone phase I clinical trials [258, 261–263] unveiling that the compound is orally bioavailable and potentially associated with mild adverse events (rash, fatigue, nausea, constipation, pruritus, vomiting, anorexia, tremors, and dizziness) and more severe adverse events (anemia, leukocytopenia, thrombocytopenia, confusion, and ataxia).

As a monotherapeutic, ralimetinib reduced angiogenesis in an in vitro co-culture endothelial cord formation assay, which was accompanied by endothelial p38 activation following growth factor stimulation and a corollary reduction in VEGF, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and IL-6. This in turn resulted in inhibition of endothelial as well as tumor-driven cord formation and pro-angiogenic cytokine secretion by stromal cells. The drug was further shown to decrease VEGF-A-stimulated vascularization in a mouse ear model [251]. A study by Zhang et al. [253] added that Rac family small GTPase 3 (Rac3)-induced cell invasion, migration, and epithelial-mesenchymal transition (EMT) of lung adenocarcinoma (A549) cells were subdued by ralimetinib. Ralimetinib-mediated p38 inhibition has also been demonstrated by Campbell et al. [260], which led to dose-dependent inhibition of MK2 (p38 downstream target, Subheading 2.1) in mouse melanoma (B16-F10) tumors 24 h after a single oral gavage dose of 10 mg/kg. Most importantly, ralimetinib significantly and considerably reduced tumor size in a mouse model of human lung cancer (A549), ovarian cancer (A2780), breast cancer (MD-MBA-468), glioma (U87MG), and multiple myeloma (OPM-2) subcutaneous xenografts, mouse skin melanoma (B16-F10) lung metastases, as well as human ovarian cancer (SK-OV-3) and renal cell cancer (786–0) orthotopic xenografts.

Ralimetinib has also been tested in combination with other drugs. Wiegmans et al. [252] showed in human triple-negative breast cancer (MDA-MB-231, MDA-MB-436, and PMC42-ET) cells that concurrent inhibition of p38 with ralimetinib and DNA

repair proteins RAD51 and PARP with B02 and ABT-888, respectively, synergistically improved drug cytotoxicity. These drug combinations were deemed useful inasmuch as pharmacological intervention into RAD51 signaling triggered the activation of counterproductive pathways (including p38 and PARP) that needed to be co-downmodulated to restore therapeutic efficacy of the initial singular modality. Corroboratively, the triple inhibition strategy significantly reduced cultured cell confluence and colony formation and tumor size in mice bearing MDA-MB-231 xenografts compared to each monotherapeutic regimen. In another example, ralimetinib combined with the MAPK/ERK kinase (MEK) inhibitor selumetinib synergistically impaired growth in Kirsten rat sarcoma virus (KRAS)-mutated non-small cell lung cancer (NCI-H23, NCI-H157, NCI-H460, and NCI-H1792) cells, which were sensitized to ralimetinib by increased p38 activity as a result of pharmacological MEK inhibition [259]. Ralimetinib also restored sensitivity to the inhibitor of apoptosis (IAP) antagonist birinapant in liver kinase B1 (LKB1)- and KRAS-mutated non-small cell lung cancer (H1299, H520, H1975, H2009, H358, LU99, H727, H460, H2030, A549, H23) cells [254]. Moreover, ralimetinib combined with the microtubule-targeting therapeutics paclitaxel and vinorelbine conferred strong synergistic cytotoxicity through G_2/M cell cycle arrest and apoptosis in MDR human breast cancer (Bads-200 and Bats-72) cells. The cytotoxic effects were mediated by the inactivation of HSP27 by ralimetinib but not paclitaxel. Therapeutic synergy achieved with ralimetinib and paclitaxel was confirmed in a mouse model of MDR human breast cancer (Bats-72 xenografts) [255].

These studies collectively attest to the fact that the pharmacological shutdown of one (vital) pathway in cancer cells opens the door to another pro-survival pathway and underpins the necessity of multimodal therapeutic strategies for optimally combatting malignancies. At the time this chapter was written, no studies had been performed that investigated the potentially synergistic effects of ralimetinib and PDT.

4.2.4 RWJ 67657

RWJ 67657 is an inhibitor of the α and β isoforms of p38 with a ~ten-fold greater potency compared to SB203580 (*see* Subheading 4.2.6) [267]. The compound has been primarily researched in the context of inflammation and inflammation-based diseases [269–271].

RWJ 67657 was tested in humans in a single-center double-blind study, showing a concentration-dependent inhibition of pro-inflammatory cytokines TNF-α, IL-6, IL-8, and IL-1β [264]. The compound is administered orally and a single dose abides by nonlinear pharmacokinetics with a half-life between 5.1 and 7.7 h at higher doses (0.25–30 mg/kg) and a half-life of 1.3–2.8 h at lower doses (0.25–1 mg/kg). Adverse events were

mild (dizziness, nausea, headache, and somnolence). In stimulated peripheral blood mononuclear cell isolated from patients, RWJ 67657 inhibited the production of TNF-α (IC_{50} = 0.18 μM), IL-6 (IC_{50} = 0.43 μM), and IL-8 (IC_{50} = 0.43 μM) in a concentration-dependent manner at > 85% inhibition for a 20 mg/kg dose. The sedation of a pro-inflammatory phenotype of immune cells was echoed in monocyte-derived macrophages from healthy subjects and rheumatoid arthritis patients, where RWJ 67657 (0–10 μM) reduced protein levels of TNF-α (IC_{50} = 0.03 μM in patients), IL-6, IL-8 (IC_{50} = 1.2 μM in patients), and matrix metalloproteinase-9 (MMP-9) and transcript levels of TNF-α, IL-1β, IL-6, IL-8, and COX-2 [272]. RWJ 67657 inhibited the activity of p38 but not its phosphorylation in lipopolysaccharide-stimulated monocyte-derived macrophages. A study in IL-1β- and TNF-α-stimulated human umbilical vein endothelial cells showed similar inhibitory behavior of RWJ 67657 toward IL-6, IL-8, and E-selectin but had no effect on intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1). Combination treatment with RWJ 67657 and the NF-κB inhibitor MOL-294 strongly augments the anti-inflammatory effects [268]. Given the importance of inflammatory signaling in tumor biology as well as cellular and molecular responses to PDT (*see* Subheading 2), pharmacological immunomodulation at the level of p38 after PDT is expected to confer beneficial effects on therapeutic outcome.

Unfortunately, only a few studies examined the effects of RWJ 67657 on cancer cells and none have been conducted in the framework of PDT. In human breast cancer (MCF-7 and MCF-7TN-R) cells, RWJ 67657 dose-dependently inhibited p38 and decreased ATF and NF-κB signaling, resulting in impaired clonogenic survival as well as stalled tumor growth in immunocompromised female ovariectomized mice bearing MCF-7TN-R xenografts (dosing: 60 mg/kg for 9 d) [265]. Additionally, RWJ 67657 (0–10 μM) blocked critical proteins that mediate EMT (Twist, Snail, Slug, and zinc finger E-box binding homeobox 2 (ZEB2) [343]) and favorably modulated expression levels of miRNAs that are involved in resistance to chemotherapy and endocrine therapy (miR-199, miR-200, miR-302, miR-303, and miR-328 [344–347]). An earlier study by the same group also looked into the effect of RWJ 67657-mediated p38 inhibition on estrogen receptor activity in breast cancer (MCF-7) cells, nonmalignant breast epithelial (MCF10A) cells, and human embryonic kidney (HEK 293) cells [266]. RWJ 67657 was able to reduce cell growth via p38 inhibition, which in turn induced downregulation of the estrogen receptor and its co-activators steroid receptor coactivator-1 (SRC-1), SRC-2, and SRC-3.

4.2.5 SB202190 (FHPI)

SB202190 or FHPI (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole) is a pyridinyl imidazole derivative that selectively inhibits p38α and p38β isoforms via competitive binding in the catalytic ATP-binding site [240]. The compound has been tested for antiviral and anti-inflammatory properties in neurodegenerative diseases [240, 282–284].

SB202190 has been investigated in PDT alongside other p38 inhibitors. Luna et al. [275] reported that Photofrin-PDT of mouse fibrosarcoma (RIF-1) cells resulted in transcriptional engagement of the NF-κB-, CRE-2-, C/EBP-, and AP-1 response elements (*see* Fig. 2). Photofrin-PDT further induced phosphorylation of p38, c-JUN, ERK1/2, and SAPK/JNK and promoted nuclear protein binding to the NF-κB, CRE-2 (activated by p38 and ERK1/2), c-FOS, and c-JUN response elements. COX-2 expression was subdued by inhibitors of p38 (SB202190 and SB203580), slightly reduced following MEK1 inhibition by U0126 (directly upstream of ERK1/2), and not influenced after NF-κB inhibition with SN50. Given that COX-2 promotes tumor cell proliferation, metastasis, and therapeutic recalcitrance [278] and given the steering role of the p38 pathway herein, the results reflect adjuvant potency of SB202190 in PDT.

Another study on Pc4-PDT in mouse leukemic lymphoblasts (LY-R) and Chinese hamster ovary (CHO) cells showed that SB202190 blocked PDT-induced apoptosis in mainly LY-R cells and to a lesser extent in CHO cells. While PDT strongly activated p38/HOG in CHO cells, no such activation occurred in LY-R cells despite the p38-mediated cell death. The authors contended that the high level of constitutively active p38/HOG in LY-R cells may have predisposed the cells to rapid activation of apoptosis following PDT. Constitutive p38 overexpression in itself could serve as a barometer to gauge whether cancer cells are more amenable to PDT [277], especially in light of the fact that prolonged activation of AP-1 transcription factors (that are activated by p38 upstream) poises cells for apoptosis (*see* Fig. 3).

In contrast, a study by Chan et al. [276] on hypericin-PDT demonstrated that p38 inhibition by SB202190 and SB203580 enhanced apoptosis in nasopharyngeal carcinoma (HK-1) cells, which was mediated by caspase-9 and caspase-3. P38 and JNK were rapidly activated by PDT, an effect that in turn was inhibited by $^1O_2$ scavengers. Blockade of p38 but not JNK (by SP600125) accelerated the proteolytic cleavage of caspase-9 and execution of the apoptotic program.

Besides effects on PDT-induced apoptosis, studies have shown that SB202190 inhibition of p38 has additional downstream effects that could benefit cancer therapy. SB202190 downregulates leukocyte-adhesion molecules such as ICAM-1 and various pro-inflammatory cytokines that collectively could hamper tumor sustenance [285–287, 348]. SB202190 was able to inhibit spheroid invasion in ovarian cancer (SKOV3) cells [279], although this

was contrary to another study showing that SB202190 increased cell migration through inhibition of p38 in human corneal epithelial cells as a result of the involvement of p38 in the EMT process [288]. In a combined treatment regimen with SB203580 and SB590885, SB202190 exerted an antiproliferative effect on melanoma cells carrying a BRAF V600E mutation and induced endolysosomal perturbations possibly as a result of interference in the endocytic transport machinery [280]. Furthermore, SB202190 was able to induce transcription factor EB (TFEB)/transcription factor binding to IGHM enhancer 3 (TFE3)-dependent autophagy and lysosomal biogenesis independently of p38 inhibition [281], which might be related to its aforementioned effect on lysosomal processing [280]. Autophagy can have cytoprotective functions, which should be ruled out before combining PDT with SB202190.

4.2.6 SB203580

As PD 169316, SB203580 is a synthetic 2,4,5-triarylimidazole that selectively inhibits p38 catalytic activity by binding to the ATP-binding pocket without inhibiting phosphorylation of p38 by upstream kinases [298]. Inhibition of p38 by SB203580 has generally been associated with an increase in apoptosis in human cancer cells, as was shown in melanoma (Colo 853 and FO-1) cells following p38 induction with, respectively, farnesylthiosalicylic acid [294] and adenoviral melanoma differentiation-associated gene-7/IL-24 [290]; in breast cancer (MDA-MB-453 and MDA-MB-231) cells where p38 was activated with, respectively, α-tocopheryloxybutyric acid [297] and aplidin [291]; in neuroblastoma (SH-SY5Y) cells where p38 was induced by the prion protein mimetic peptide PrP106–126 [292]; and in colon cancer (HT-29) cells where p38 was stimulated with indomethacin [293]. The activated p38-mediated apoptosis proceeded via caspase-3 in those studies that had assayed caspase-3. Ye et al. [299] further demonstrated that SB203580 can reverse p38 activation and consequent apoptosis by the phytochemical 3,3′-diindolylmethane even when the upstream ASK-1 activator TRAF2 (*see* Fig. 3) is repressed. Also, p38 has been associated with moderating P-glycoprotein (P-gp) levels in murine leukemia (L1210/VCR) cells, which imparted MDR against vincristine. SB203580 treatment considerably reversed the MDR and resensitized the cells to vincristine [295]. Finally, SB203580 inhibited human renal cancer (Caki-1) cell migration and invasion induced by butaprost by inactivating p38 and consequently downregulating MMP-9 protein levels and activity [296].

SB203580 has been investigated in the context of PDT and p38 as exemplified in Subheading 3 with the data from Song et al. [187], who demonstrated the deleterious effects of SB203580 on pro-inflammatory (survival) signaling. Wang et al. [178] reported that PDT with the PS berberine sensitized cisplatin-resistant human melanoma (A375/DDP, SKMel-19/DDP, and

M8/DDP) cells to cisplatin, an effect that coincided with an increase in protein levels of p-p38, p-JNK, p-ERK1/2, BAX, cleaved caspase-9, and cleaved caspase-3 and a reduction in Bcl-2. Neutralization of ROS with NAC and blocking of p38 with SB203580 reduced the extent of PDT-induced cell death by roughly 100% and 50%, respectively, whereby NAC treatment also downregulated p38 protein levels. Lastly, Ge et al. [179] demonstrated that ALA-PDT of human squamous carcinoma (SCL-1) increased levels of p-MEK, p-ERK1/2, p-p38, p-Elk-1, p-JNK, and p-c-Jun and that inhibition of ERK1/2 with PD98059, p38 with SB203580, and JNK with SP60125 reversed these changes and amplified apoptotic cell death.

4.2.7 SB239063

SB239063 (trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-[(2-methoxy)pyrimidin-4-yl] imidazole) is a potent and selective p38 inhibitor (IC_{50} = 44 nM [309]) that has chiefly been investigated for protection against inflammatory and neurological disorders [311–315, 349].

SB239063 has not been tested in combination with PDT. Nevertheless, the p38 inhibitor harnesses several interesting modulatory properties that make it an eligible adjuvant drug candidate for cancer chemotherapy or PDT. First, inhibition of the p38 signaling pathway by SB239063 decreased cell proliferation, migration, VEGF protein levels, and angiogenic ability in human endothelial (ECV304) cells that had been primed with human hepatocellular carcinoma (HepG2) cell-derived bone morphogenic protein (BMP2) [306]. BMP2 moderates liver cancer development [350] and activates p38 under hypoxic conditions in human articular chondrocytes [351]. These effects could be pertinent in select PDT applications since BMP2 is a driver of the abovementioned processes in cancers of non-hepatic origin as well [352–354]. Also, PDT induces hypoxia by damaging and occluding vasculature [355] that consequently may undergo remodeling, where endothelial cells and VEGF occupy a central role [356]. Second, SB239063 blocks p38-dependent release of TNF-α [302, 303], attenuates IL-6 [300], and inhibits NF-κB activation and translocation [304, 305], which could deter inflammation-driven tumor cell survival (*see* Fig. 2), including the propagation of the immediate early stress response via the TNF-α/TNFR signaling axis (*see* Fig. 3). Finally, the anticancer attributes of SB239063 emanate from its ability to (1) inhibit transforming growth factor β (TGF-β)- and bFGF-induced cell migration (in human corneal epithelial cells) [301]; (2) suppress invasion and MMP-3 production in pancreatic cancer cells [243] and invasion of nicotine-primed human colorectal cancer cells [316]; and (3) reduce tumor volume, intratumoral vascularization, and migration proneness in BMP2-overexpressing HepG2 xenografts in mice [306].

As with other p38 inhibitors, SB239063 may confer cytoprotection. Kim et al. [308] reported that SB239063 inhibited apoptosis and restored anti-apoptotic BCL-2 protein levels in human diploid fibroblasts that had been treated with hydrogen peroxide and staurosporine. Accordingly, SB239063 can protect cells from oxidative stress and apoptotic fallout. Another study demonstrated that SB239063 can attenuate radiation-induced vascular inflammation and recruitment of immune cells [307], which may have negative implications for immune control of PDT-treated tumors (*see* Subheading 1).

4.2.8 Thymoquinone

Thymoquinone is a monoterpene phytochemical extracted from the seeds of *Nigella sativa* that has been extensively researched for its antioxidant, anti-inflammatory, and anticancer properties [323, 357]. The phytochemical is toxic to cancer cells, including colon cancer cells [323, 326, 327], pancreatic cancer cells [319, 328], prostate cancer cells [329], laryngeal carcinoma cells [330], and leukemia cells [331], where it generally induces apoptosis that can proceed in a p53-dependent [332] and p53-independent manner [332, 333].

As numerous other anticancer compounds, thymoquinone generates ROS in cancer cells [320, 333], serving as a trigger for apoptotic signaling [358–363]. A study by El-Najjar et al. [317] convincingly demonstrated that thymoquinone incites oxidative stress in human colon cancer (Caco-2, HCT-116, LoVo, DLD-1, and HT-29) cells by triggering ROS generation, which was abrogated by NAC. However, ROS activated the ASK-1 downstream target JNK as well as ERK, but not p38. The null effect on p38 may be related to the p38-inhibiting properties of thymoquinone that may have countered stimulation by ROS, possibly via ASK-1 given that JNK was activated. Direct ASK-1 activation by thymoquinone has never been reported. Only one investigation [203] could be retrieved on rheumatoid arthritis synovial fibroblasts where the body of proof regarding thymoquinone activation of ASK-1 was somewhat compelling. Thymoquinone inhibited TNF-α-induced p-p38 and p-JNK expression. The p-p38 and p-JNK downregulation was mediated by ASK-1, as evidenced by the finding that TNF-α selectively induced phosphorylation of ASK-1 at the Thr845 residue that in turn was inhibited by thymoquinone in a dose-dependent manner.

Several studies have confirmed that thymoquinone blocks p38, although the data are not consistent. For example, Park et al. [296] demonstrated that thymoquinone inhibits p38 in cultured human renal carcinoma (Caki-1) cells, where it co-downregulated protein levels of p-AKT, prostaglandin E_2 receptor 2 (EP2), and MMP-9 and with it hampered cell migration and invasion. The p38-inhibiting properties of thymoquinone have been reproduced in oral cancer cells [318], but not in other studies. In human

bladder cancer (T24 and HTB-9) cells, for instance, thymoquinone induced pathways related to EMT and attenuated mTOR activity and downstream signaling, but had no effect on ERK 1/2 or p38 MAPK activity. Conversely, it was shown that thymoquinone induced apoptosis in human pancreatic cancer (FG/COLO357 and CD18/HPAF) cells by the activation rather than inhibition of JNK and p38 [319]. Similar observations were made by Woo et al. [320] in human breast cancer (MCF-7 and MDA-MB-231) cells, where thymoquinone activated p38, JNK, and ERK that culminated in apoptosis. The p38 activation and cell death were attenuated by the selective p38 inhibitor SB203580 (*see* Subheading 4.2.6) and NAC. Thymoquinone-induced p38 activation, reduction of X-linked inhibitor of apoptosis protein (XIAP), BCL-2, BCL-xL, and survivin as well as increased apoptosis and reduced proliferation were further confirmed in human breast cancer (MDA-MB-231) xenografts in mice. Finally, thymoquinone reportedly induces autophagic cell death and reduces metastatic propensity of irinotecan-resistant (CPT-11-R) LoVo colon cancer cells, both of which involve p38 activation [321, 322].

Taken altogether, the effect direction of thymoquinone on p38 activity is variable in cancer cells and difficult to predict. Inasmuch as thymoquinone has never been assayed in cells treated by PDT, studies are needed to investigate a potential synergistic or additive effect of such a combinatorial modality and rule out therapeutic antagonism.

4.2.9 VX-702

VX-702 is an orally dosed selective p38α inhibitor [364]. P38α mediates the biosynthesis of TNF-α and IL-1β at the transcriptional and translational level and with it occupies a central role in pro-inflammatory signaling (*see* Subheading 2.1). Accordingly, p38α is pharmacologically targeted for the modulation of cytokine production [365] and researched for the treatment of rheumatoid arthritis and other inflammatory diseases [334, 340, 366–368]. Although the drug does not cause serious clinical side effects, VX-702 lacked efficacy in human trials and was therefore discontinued in the framework of the aforementioned conditions [367].

In terms of anticancer properties, VX-702 is efficacious although there is paucity in the number of supporting studies. A recent investigation elucidated that VX-702 is an inhibitor of p38 as well as Nemo-like kinase (NLK), which is responsible for survival signaling in endocrine-resistant breast cancer [335]. Combined treatment of VX-702 with the mTOR inhibitor everolimus produced a significant anticancer effect in therapy-resistant cell line-derived and patient-derived xenograft models [336]. VX-702 further inhibited apoptosis induced with the histone deacetylase (HDAC) inhibitor Trichostatin A in cultured colonic epithelial (HCoEpiC) cells. Apoptotic signaling concurred with increased BAX and reduced BCL-2 levels. Trichostatin A also promoted

p38 expression and activation, all of which were reversed by VX-702 [338].

VX-702 has never been tested in the context of PDT. It is notable that VX-702 attenuates fibrosis in chronic graft-versus-host disease and suppresses infiltration of immune cells [339], which might negatively impact PDT given its reliance on antitumor immunity for long-term tumor control (*see* Subheading 1).

4.3 Other Inhibitors of the ASK-1 Pathway

In this section inhibitors are described that do not directly target ASK-1 or p38 but are considered potentially relevant inhibitors of the ASK-1 pathway (*see* Fig. 6) because they affect regulators located upstream or downstream of ASK-1 and p38 or impact nodes in other pathways that intersect with ASK-1 signaling. A summary of their mechanism of action, pharmacological and biological effects, test systems, and application in PDT is provided in Table 3.

BAY 11–7082 and BAY 11–7085 (synonym: BAY 11–7083) are inhibitors of nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor alpha (IκBα) that itself inhibits NF-κB. The compounds act by blocking TNF-α-induced phosphorylation of IκBα (IC_{50} = 10 μM for BAY 11–7085), which leads to decreased levels of NF-κB and subsequently reduced expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin in HUVECs. Both compounds also induce JNK-1 and p38 and reduce ERK-1 in TNF-α-stimulated endothelial cells [370]. BAY 11–7082 also inhibits ubiquitin-specific protease (USP)7 and USP21 (IC_{50} = 0.19 and 0.96 μM, respectively) [371] that constitute druggable targets in cancer therapy [426]. However, BAY 11–7082 could stabilize HIF-1α by blocking its proteasomal degradation [372] and possibly interfere in therapeutic modalities targeting the HIF-1 survival pathway (*see* Subheading 1, Fig. 2) or aid in tumor survival signaling. With respect to PDT, IκBα is downregulated and NF-κB is upregulated, at least after hypericin PDT [186]. These molecular targets should therefore be susceptible to BAY 11–7082 and BAY 11–7085. BAY 11–7082 completely abolished ALA-PDT-induced JNK activation, which almost completely abrogated PDT-induced apoptosis in human oral cancer (Ca9–22) cells [373]. BAY 117085 was employed to mechanistically elucidate hypericin-PDT-mediated changes in COX-2 expression in human cervix carcinoma (HeLa) wild-type cells in comparison to genetically modified HeLa cells that stably overexpress IκBα and that are devoid of NF-κB DNA-binding activity. The study revealed that PDT did not result in altered COX-2 expression levels when NF-κB was inhibited pharmacologically or genetically compared to non-illuminated controls, indicating that NF-κB was not involved in the upregulation of COX-2 by hypericin-PDT [186].

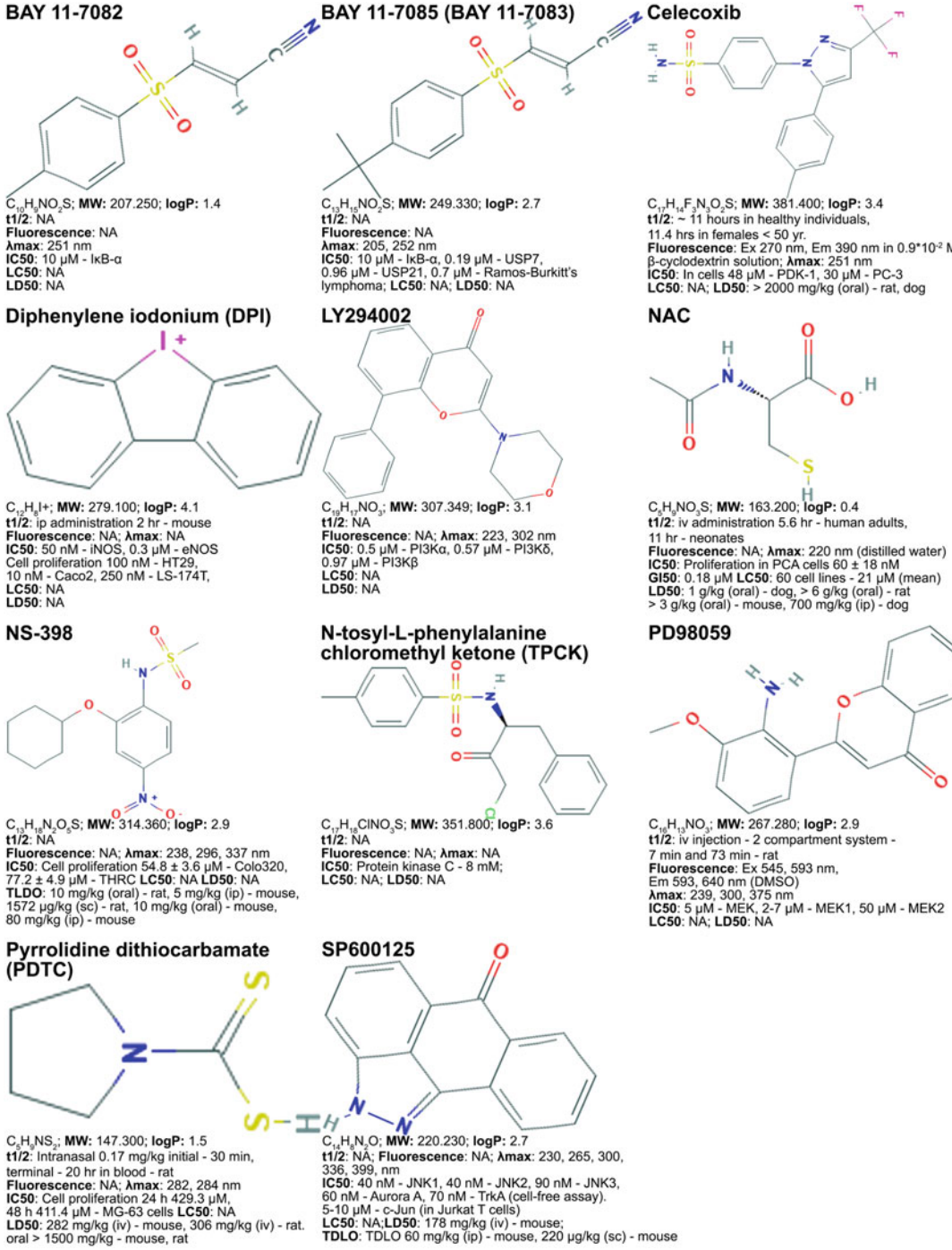

Fig. 6 Overview of inhibitors that affect the immediate early stress response upstream or downstream of ASK-1 and p38 that are eligible candidates for use as adjuvants in PDT. LogP (octanol:water partition coefficient) values were retrieved from PubChem and were predicted with XLogP2 or XlogP3 software. The half maximum inhibitory concentration (IC_{50}, enzymes), half maximum lethal concentration (LC_{50}, in vitro), half maximum lethal dose (LD_{50}, in vivo), $t_{1/2}$ (circulation half-life), and spectral properties were obtained from the material safety data sheets (retrieved from the Cayman Chemicals and Spectrum Chemical website), PubChem, LC Laboratories, Merck-Millipore, Pfizer, Selleckchem, and TargetMol. The half maximum inhibitory concentration (IC_{50}, used for proliferation and enzymes) and half maximum growth inhibitory concentrations (GI_{50}) were obtained from available literature. This also applies to LC_{50}, LD_{50}, and $t_{1/2}$ data that were missing from or inconsistent in the abovementioned databases. Abbreviations: *Em* emission, *Ex* excitation, *ip* intraperitoneal, *iv* intravenous, *MW* molecular weight, *NA* information not available, *sc* subcutaneous, *TDLO* the lowest dose causing a toxic effect, λ_{max} the wavelength at which there is an absorption maximum (may be multiple absorption bands)

Table 3
Overview of up- and downstream inhibitors of ASK-1 and their pharmacological and biological characteristics

Name	Mechanism	Pharmacological effect	Biological effect	Tested in	Tested in PDT	References
BAY 11–7082	Inhibition of TNF-α-induced IκBα phosphorylation; inhibition of USP7 and USP21	Inhibition of NF-κB; induction of JNK-1 and p38; reduction of ERK-1 (in TNF-α-stimulated endothelial cells); abrogation of JNK activation (by PDT); downregulation of BCL-2 and upregulation of BAX protein levels; inhibition of TNF-α-induced surface expression of ICAM-1, VCAM-1, and E-selectin; stabilization of HIF-1α	Tumor growth inhibition; increased apoptosis; cell cycle arrest (S-phase)	*In vitro*: HBL-1, RAW 264.7, IL-1R, Ca9–22, HGC-27, HUVECs, MGC80–3, AGS, NCI-N87, L-02 cells *In vivo*: HGC-27 xenografts in male BALB/c nude mice	Yes	[369–373]
BAY 11–7085 (BAY 11–7083)	Inhibition of IκBα phosphorylation	Inhibition of p65 NF-κB binding to κB; inhibition of AP1 binding to DNA-binding motif; inhibition of surface expression of ICAM-1, VCAM-1, and E-selectin; induction of JNK-1 and p38 and reduction of ERK-1 (in endothelial cells)	Cell death; loss of mitochondrial potential	*In vitro*: HUVECs, HeLa, T24, Nalm6, Farage, Pfeiffer, Ramos, Raji, ARH-77, TRF, NAD, HDMAR, U937, K562, HF1, 1063, SaSO2 cells	Yes	[186, 370, 374]
Celecoxib	Inhibition of COX-2 (noncompetitive) and PDK-1,	AKT dephosphorylation; inhibition of P70-S6 kinase activity; downregulation of PGE_2,	Cell death; anti-inflammatory; analgesic; antipyretic	*In vitro*: PC-3, MCF-7, AZACB, CF33, CF41. MG cells	Yes	[187, 375–382]

	cadherin-11 binding	VEGF, COX-2, TNF-α, IL-1β, and p53 protein levels (after PDT)		*In vivo*: EMT6 xenografts in female BALB/c mice		
Diphenylene iodonium (DPI)	Inhibition of NOX	Downregulation of *Nox1, HDAC4, FEN1*; upregulation of p21, DUSP4 protein levels and PDK4, PPAR-α, PPAR-γ transcript levels; downregulation of cyclin A, D1, and E protein expression	Antiproliferative; growth inhibition; cell cycle arrest (G_1/S)	*In vitro*: HT-29, LS-174 T, LS-180, Caco2, COH-BR6, MDA-MB-468, BT474, A2058, SK-MEL5, MCF-7, DU145, MCF12A cells *In vivo*: HT-29 and LS-174 T xenografts in male athymic nude mice	Yes	[383–386]
LY294002	Inhibition of PI3K and PIM1	Downregulation of HIF-1α protein levels (no effect on mRNA); inhibition of mTOR at higher doses; downregulation of EGFR, PI3K, and AKT	Anti-angiogenic; antiproliferative; synergistic reduction of growth and migration (after PDT)	*In vitro*: HepG2, 3-T3-L1, J774.2, U87MG, LN18, GL261, Caco-2, human osteoarthritic chondrocytes, PC-3, DU145, Eca-109 cells, human primary neutrophils, rabbit primary smooth muscle cells	Yes	[387–396]
NAC	Antioxidant	Reduction of COX-2 protein levels and inhibition of PGE_2 release; inhibition of JNK phosphorylation; reduction of p300HAT expression (all after PDT)	Anti-inflammatory; inhibition of autophagy (after PDT)	*In vitro*: MCF-7, A375, MG-63 cells	Yes	[187, 189, 397, 398]
NS-398	Inhibition of COX-2	Reduction of PGE_2, COX-2, and VEGF (after PDT)	Antiproliferative; inhibition of tumor growth; induction of apoptosis	*In vitro*: Colo320, BA, RIF, LLC, AA/C1, RG/C2, RR/C1, KS, JW2, HT29, HeLa, THRC cells *In vivo*: RIF cell xenografts	Yes	[186, 189, 399–401]

(continued)

Table 3
(continued)

Name	Mechanism	Pharmacological effect	Biological effect	Tested in	Tested in PDT	References
				in female C3H/HeJ mice, C26 xenografts in male BALB/cByJ mice		
N-tosyl-L-phenylalanine chloro methyl ketone (TPCK)	Inhibition of NF-κB	Inhibition of NF-κB through direct thiol modification at cys-179 of IKKβ and at cys-38 of p65/RelA; decay of subunit IκB; inhibition of α-chymotrypsin, protein kinase C, some cysteine proteases such as bromelain, ficin, and papain; downregulation of COX-2 protein and decrease in secreted PGE_2	Anti-inflammatory; antiproliferative; acceleration of cell death of glucose-deprived cells	*In vitro*: MCF-7, TNF-α-stimulated HeLa cells	Yes	[187, 402–404]
PD98059	Inhibition of MEK and AhR	Binding to inactive form of MEK, the upstream activator of ERK and AP-1; inhibition of ERK1/2 phosphorylation	Antiproliferative; accelerated cell death of glucose-deprived cells	*In vitro*: Hep3B, HepG2, PLC, SKHep, glucose-deprived HepG2, HeLa, T24, 3T3, KNRK, NRK-47F, NRK-52E, KB, PC12, MCF10A, MCF10A-Neo, MCF10A-NeoT, 143B, 143Bp0 cells, rat primary sympathetic neurons, 129/Ola mice-derived astrocytes *In vivo*: male CD mice, Sprague-Dawley rats	Yes	[186, 405–411]

Pyrrolidine dithiocarbamate (PDTC)	Inhibition of IκB	Downregulation of COX-2 protein; decrease in secreted PGE_2	Anti-inflammatory; cell cycle arrest (G_1); reversion of deoxynivalenol-induced mitochondrial dysfunction and apoptosis	*In vitro*: Sprague-Dawley rat-derived vascular smooth muscle cells, MCF-7, MG-63, GH3 cells *In vivo*: Balb/c mice, MRL/lpr mice, NMRI mice, Sprague-Dawley rats	Yes	[187, 412–418]
SP600125	Selective inhibition of JNK	Competition with ATP to inhibit c-Jun phosphorylation; downregulation of inflammatory genes (coding for COX-2, IL-2, IL-10, INF-γ, and TNF-α)	Inhibition of cell activation and differentiation; anti-inflammatory; inhibition of autophagy (after PDT)	*In vitro*: HeLa, T24, HK-1, MG-63, U2OS, human CD4+, HCE-T, RAW 264.7, MIN6, HCT116 cells *In vivo*: CL57BL/6 and JNK-KO CL57BL/6 mice, neonatal BALB/c mice	Yes	[186, 192, 276, 397, 419–425]

Celecoxib is a noncompetitive inhibitor of COX-2 that is classified as a nonsteroidal anti-inflammatory drug with analgesic, anti-inflammatory, and antipyretic properties [378, 427]. Celecoxib further inhibits 3-phosphoinositide-dependent kinase-1 (PDK-1)-mediated apoptosis (IC_{50} = 48 uM), causes AKT dephosphorylation [375], and binds to cadherin-11 (CDH11) [376] that is overexpressed in several types of cancer [428], together accounting for the compound's anticancer properties. Photofrin-PDT (i.v. injection of 5 mg/kg, light-dose interval of 16 h, cumulative radiant exposure of 135 J/cm^2) substantially increased protein levels of PGE_2, COX-2, VEGF, IL-1β, and TNF-α in syngeneic mammary carcinomas (EMT6) implanted into the dorsal scapula region of female BALB/c mice. Celecoxib (10 mg/kg, i.p. injections directly after and at 4 h and 24 h post-PDT) reduced the levels of all listed proteins to pre-PDT baseline values, suggesting a role of NF-κB signaling. In human breast cancer (MCF-7) cells, PDT upregulated p53 protein levels that were downmodulated to pre-PDT control levels by celecoxib [187].

Diphenylene iodonium (DPI) is an iodonium-class flavoprotein dehydrogenase inhibitor that blocks the activity of NADPH oxidases (NOX). The compound has been explored as an oncotherapeutic for a subset of human cancers that overexpress NOX, including colorectal cancer [383] and breast cancer [385], which require NOX for their survival and growth [429]. Doroshow et al. [383] demonstrated that DPI retarded the growth of cultured cancer cells that overexpressed NOX1 (human colon cancer cell lines Caco2, HT-29, LS-174T) at 0.01–0.25 μM concentration by decreasing steady-state ROS production (coinciding with decreased mRNA expression of NOX1 and antioxidant enzymes) and causing G_1/S cell cycle arrest, reduced proliferative signaling at the level of the transcriptome, and apoptosis in some of the cell lines. DPI decreased the expression of cyclin A, D1, and E in vitro. In vivo, DPI reduced tumor volume by ~40% compared to vehicle control in HT-29 and LS-174T xenografts in athymic nude mice. In light of the above, DPI could be used in conjunction with PDT of cancer types that rely on NOX signaling for sustenance, especially given that NOX isoforms may be activated by PDT [430, 431] and hence aid in survival. It is imperative that DPI is administered after PDT inasmuch as it acts as an antioxidant [430, 432] that could otherwise enfeeble the efficacy of PDT.

LY294002 is a morpholine-based compound that is a strong inhibitor of PI3K [391] with an IC_{50} of 0.5 μM/0.57 μM/0.97 μM for the PI3Kα/δ/β isoforms [393], but also inhibits other proteins such as the proto-oncogene serine/threonine-protein kinase (PIM1) [392] that is overexpressed in some forms of cancer [394, 395, 433–435]. The PI3K pathway regulates key biological processes such as cell growth, survival, proliferation, and angiogenesis [436]. Every key node in the PI3K pathway is

frequently mutated or amplified in many cancers [437] that not only fortifies the tumor, but also causes loss of sensitivity to some chemotherapeutics [438], which is why this pathway is widely targeted by singular or hybrid chemotherapeutic modalities [439, 440]. LY294002 is further interesting for application in PDT in that the drug can downregulate HIF-1α [389], which is a key survival regulator in PDT-subjected, sublethally afflicted cancer cells (*see* Subheading 1, Fig. 2). In a study on ALA-PDT and LY294002, Zhang et al. [390] showed that the combinatorial modality exerted a synergistic inhibitory effect on the migration of human esophageal cancer (Eca-109) cells and reduced gene and protein expression levels of epidermal growth factor receptor (EGFR), PI3K, and AKT.

NAC is commonly used as a nutritional supplement with strong antioxidant properties, acting directly as a scavenger of ROS and other types of oxidants and radicals [441]. Pretreatment of human breast cancer (MCF-7) cells with NAC (2.5 mM) before Photofrin-PDT reduced COX-2 protein levels to baseline values and strongly inhibited PGE_2 release, indicating that PDT-induced ROS generation is responsible for pro-inflammatory signaling via COX-2 and PGE_2 [187]. Equally important was the finding that pretreatment of cells with a potent antioxidant did not abrogate the photooxidative destruction of cells by PDT but inferentially improved therapeutic efficacy, as was the case for COX-2 inhibition using small interfering RNA (siRNA). Moreover, NAC pretreatment of human osteosarcoma (MG-63) cells was found to inhibit MPPa-PDT-induced autophagy and JNK phosphorylation [397]. Finally, Tsai et al. [189] showed that ALA-PDT of human melanoma (A375) cells strongly induced p300 HAT mRNA that led to elevated HAT activity and PS concentration-dependent cell death. Oxidative stress can activate p300HAT and result in increased histone acetylation and subsequent regulation of gene expression [442, 443] that could favor cell survival. In that respect, the ALA-PDT also augmented survivin protein levels in A375 and mouse colon carcinoma (C26) cells. NAC abolished the p300HAT transcriptional response induced by ALA-PDT.

NS-398 [N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide] is a nonsteroidal anti-inflammatory drug and a selective inhibitor of COX-2 with antiproliferative and pro-apoptotic attributes [399, 400]. Hypericin-PDT of cultured human cervix carcinoma (HeLa) cells pretreated with 50 μM of NS-398 completely blocked the release of PGE_2 induced by PDT and slightly increased the extent of apoptosis, although to a lesser degree than the p38 inhibitor PD 169316 (*see* Subheading 4.2.2) [186]. In PH-PDT-treated cultured radiation-induced fibrosarcoma cells, the addition of NS-398 directly after illumination entirely eliminated detectable protein levels of PGE_2 and COX, which were both strongly upregulated by PDT. PH-PDT of radiation-induced fibrosarcomas in

C3H/HeJ mice resulted in protein overexpression of COX-2, PGE_2, and VEGF. NS-398 treatment (10 mg/kg i.p.) reduced PGE_2 and VEGF protein levels by ~50% and ~75%, respectively, and ensured that ~55% and ~75% of animals remained tumor free during 90 d post-PDT follow-up when PDT was performed at a cumulative radiant exposure of 200 and 300 J/cm^2, respectively, compared to 0% and ~25% in the PDT-only treatment group [401]. Finally, Tsai et al. [189] demonstrated that the inclusion of NS-398 in liposomal chlorin e6-PDT of mouse colon carcinomas (C26) improved PDT efficacy by about 45%.

N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) is an inhibitor of serine/cysteine endopeptidases that also inhibits the expression of inflammatory mediators by blocking NF-κB through direct thiol modification at Cys-179 of inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) and at Cys-38 of p65/RelA [404]. Pretreatment of human breast cancer (MCF-7) cells with TPCK (10 μM) in Photofrin-PDT substantially attenuated PDT-induced COX-2 and PGE_2 protein levels [187].

PD98059 is a non-ATP competitive MEK inhibitor ($IC_{50} = 2$ μM) that specifically inhibits MEK-1-mediated activation of MAPK without directly inhibiting p38, JNK, and ERK1/2 [409, 410], although causing phosphorylation of ERK1/2 [405]. PD98059 is also a ligand for the aryl hydrocarbon receptor (AhR) and functions as an AhR antagonist [411]. AhR ligands are produced by the tumor microenvironment and via intracrine routes [444]. Sustained transcriptional activation of AhR promotes tumor growth and impairs antitumor immunity [445]. AhR further mediates proteasomal processing of estrogen receptor α and affects ERK kinase activity and signaling by direct cross talk [446] while stimulating cell proliferation through interactions with EGF [447]. Blockade of AhR induced cell cycle arrest in the G_1 phase in rat hepatoma (5 L) cells and G_2/M phase in murine hepatoma (1c1c7) cells [448]. AhR is targeted pharmacologically to inhibit its pro-tumorigenic properties and to re-sensitize tumor cells to therapies [445, 449]. Despite these anticancer properties of PD98059, hypericin-PDT-treated human cervix carcinoma (HeLa) cells and bladder cancer (T24) cells that had been pretreated with 20 μM PD98059 elicited no effect on COX-2 protein levels that were increased by PDT itself. PDT-induced downmodulation of COX-2 as well as other regulators of that inflammatory pathway such as PGE_2 were effectuated by other inhibitors, including PD 169316 (*see* Subheading 4.2.2) and NS-398 (this section) [186]. Similar non-responsiveness in the COX-2/PGE_2 signaling axis was observed in human breast cancer (MCF-7) cells [187]. Actual anticancer effects of PDT + PD98059 were not studied.

Pyrrolidine dithiocarbamate (PDTC) is a metal chelating compound that can induce G_1-phase cell cycle arrest in vascular smooth muscle cells [412] and inhibits NF-κB [416–418]. Accordingly,

PDTC reverted deoxynivalenol-induced mitochondrial dysfunction and apoptosis via the NF-κB/iNOS pathway [413, 450]. Deoxynivalenol is an inducer of stress responses in the ER and ribosomes and triggers mitochondrial dysfunction and intrinsic apoptosis through oxidative stress [451, 452]. In PDT an opposite (i.e., non-protective) effect is induced by PDTC. Pretreatment of human breast cancer (MCF-7) cells with PDTC (10 μM) in Photofrin-PDT substantially attenuated PDT-induced COX-2 and PGE_2 protein levels [187].

SP600125 is an anthrapyrazolone that acts as a potent, cell-permeable, selective inhibitor of JNK and dose-dependently competes with ATP to inhibit the phosphorylation of c-Jun. Through JNK inhibition, SP600125 prevents the activation of inflammatory genes such as COX-2, IL-2, interferon (IFN)-γ, and TNF-α [419]; regulates TLR-mediated inflammatory signaling [423, 424]; and downregulates Beclin-1 and reduces autophagy while increasing apoptosis [425]. Treatment of squamous nasopharynx carcinoma (HK-1) cells with Zn-BC-AM-PDT was shown to upregulate p-p38, p-JNK, and p-ATF, where SP600125 slightly reduced p-JNK and considerably attenuated p-ATF that mediates the proteotoxic stress response (*see* Subheading 1, Fig. 2) [192]. However, pretreatment of cells with SP600125 did not induce apoptotic cell death regardless of drug concentration (0–20 μM) and light dose (0–2 J/cm^2). On the other hand, SP600125 was able to reduce pyropheophorbide-α methyl ester (MPPa)-PDT-induced autophagic signaling in human osteosarcoma (MG-63) cells, which involved p-JNK and microtubule-associated protein 1A/1B-light chain 3, phosphatidylethanolamine conjugate (LC-3 II) downregulation by SP600125 following their induction by PDT [397]. Both JNK and LC-3 II are involved in autophagy [453, 454]. The effect of SP600125 on PDT-induced cell death was not studied.

5 Conclusions

When activated, the immediate early stress response protects the cell from oxidative stress and can activate other PDT-induced downstream survival pathways. However, long-term activation can induce apoptosis. Since the immediate effect of ASK-1 activation is protection against oxidative stress and since its downstream effects can have pro-inflammatory (i.e., survival) consequences, ASK-1 inhibition in combination with PDT is expected to improve treatment efficacy. However, the combination of ASK-1 inhibitors and PDT has currently not been tested in any experimental setting. In contrast, downstream targets of the ASK-1 pathway, such as p38, have been used in combination with PDT. Nevertheless, the results show that inhibition can both sensitize cancer cells to PDT and

promote cell survival. Outcomes are determined by various factors including the type of PS used, PS localization, cancer type, mutations in relevant pathways, and experimental design.

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