PHARMACOLOGICAL AND CELLULAR CHARACTERIZATION OF GRI977143, A NOVEL NONLIPID LPA_2 RECEPTOR AGONIST IDENTIFIED BY VIRTUAL SCREENING

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1. LIST OF ABBREVIATIONS

AC	adenylyl cyclase
AIF	apoptosis inducing factor
Akt	protein kinase B
APAF-1	apoptotic protease activating factor 1
ARS	acute radiation syndrome
ATM	ataxia-teleangiectasia-mutated
ATX	autotaxin
B103	rat neuroblastoma cells
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer 1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 homology 3
Bid	BH3 interacting domain death agonist
Bok	Bcl-2 related ovarian killer
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
caspase	cysteinyl aspartic acid-protease

c-FLIP	cellular FLICE like inhibitory protein
c-IAP	cellular inhibitor of apoptosis proteins
CHO	chinese hamster ovary
CHOP	C/EBP homologous protein
CHX	cycloheximide
CM	conditioned medium
Cs	Cesium
C_t	threshold cycle value
DAG	diacylglycerol
DISC	death inducing signaling complex
DKO	double knockout
DlgA	drosophila disc large tumor suppressor
DMEM	Dulbeco's modified Eagle medium
DMSO	dimethyl sulfoxide
E2F	E2F transcription factor
EDG	endothelial differentiation gene
EGFP	enhanced green fluorescent protein
EGFR	endothelial growth factor receptor
ENPP2	autotoxin

ErbB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERK1/2	extracellular signal regulated kinases 1/2
Fas	fatty acid synthase
FADD	Fas-associated protein with death domain
FBS	fetal bovine serum
FGF-2	basic fibroblast growth factor
Fura-2AM	Fura-2-acetoxymethyl esther
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
GRI	Genome Research Institute
Gy	Gray (radiation unit)
H2L	Hit2Lead
HRPO	horseradish peroxidase
HtrA2/Omi	high-temperature requirement protein A2
HUVEC	human umbilical vein endothelial cells
IACUC	Institutional Animal Care and Use Committee
ICAD	inhibitor of caspase-activated DNAse
IEC-6	intestinal epithelial cell line 6
IGF-1	insulin-like growth factor 1

IND	Investigational New Drug
IP3	inositol 1,4,5-triphosphate
IUPHAR	International Union of Basic and Clinical Pharmacology
JNK	c-Jun N-terminal kinase
KO	knockout
LIM	Lin-11, Isl-1 and Mec-3 proteins
LPA	lysophosphatidic acid;
LPAR	LPA receptor
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase/extracellular signal regulated kinase
MEF	mouse embryonic fibroblast
MM1	rat hepatoma cells
MMFF94	Merck Molecular Force Field 94
MMP	matrix metalloproteinase
MOMP	mitochondrial outer membrane pore
MPTP	mitochondrial permeability transition pore
MYC	myelocytomatosis viral onkogen
NADPH	nicotinamide adenine dinucleotide phosphate

NFκB	nuclear factor κB
NHERF2	Na^+-H^+ exchange regulatory factor 2
OTP	octadecenyl thiophosphate
PARP-1	poly (ADP-ribose) polymerase 1
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
PDZ	PSD95/Dlg/ZO-1 domain
p53	protein 53
PI3K	phosphoinositide-3-kinase
PKC	protein kinase C
PLC	phospholipase C
poly-HEMA	poly(2-hydroxyethyl methacrylate)
PPAR	peroxisome proliferator-activated receptor gamma
PSD95	postsynaptic density protein 95
QPCR	real-time quantitative polymerase chain reaction
Ras V12	Rat sarcoma V12
RH7777	McArdle rat hepatoma cell line
RIP1	receptor-interacting protein kinase 1
Rock	Rho associated kinase

RT	room temperature
Smac	second mitochondrial activator of caspases
S1P	sphingosine-1-phosphate
SRF	serum response factor
tBid	truncated Bid
TBX	T-box transcription factor
TGF-β	transforming growth factor β
TM	transmembrane
TNF-α	tumor necrosis factor α
TRADD	TNF receptor-associated protein with death domain
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRIP6	thyroid receptor interacting protein 6
UC-DDC	University of Cincinnati Drug Discovery Center
U937	human monocyte lymphoma cell line
uPA	urokinase
US FDA	United States Food and Drug Administration
Zn	Zinc
ZO-1	Zonula Occludens 1 protein

2. LIST OF FIGURES AND TABLES

3. ABSTRACT

Growing number of validated and putative lysophosphatidic acid (LPA) receptors and related downstream targets underscore the importance and diversity of the physiological and pathological roles of LPA. Selective targeting of the individual LPA receptors would provide a powerful tool to discover additional LPA regulated pathways and cellular targets and to exploit the potential therapeutic benefits of LPA. As a results of a virtual screen using single-reference similarity searching we identified GRI977143 a novel nonlipid and drug-like hit that is specific for the LPA_2 receptor subtype, which was identified as an important molecular target of LPA mediating the antiapoptotic signaling. Pharmacological, cellular and antiapoptotic properties of GRI977143 were compared to that of LPA and its metabolically stabilized mimic, octadecenyl thiophosphate (OTP) in different extrinsic and intrinsic forms of apoptosis *in vitro*. GRI977143 reduced activation of caspases 3, 7, 8, and 9 and inhibited Bax translocation, poly (ADP-ribose) polymerase 1 cleavage and DNA fragmentation induced by serum withdrawal, Adriamycin, TNF-α and γ-irradiation. These antiapoptotic cellular signaling responses were present selectively in mouse embryonic fibroblast cells derived from $LPA_{1\&2}$ double knockout mice reconstituted with the LPA_2 receptor and were absent in vector-transduced control cells. GRI977143 shows the features of a radiomitigator because it is effective in rescuing the lives of mice from deadly levels of radiation when administered 24 h after radiation exposure. GRI977143 also inhibited bystander apoptosis elicited by soluble proapoptotic mediators produced by irradiated cells. Our findings suggest that by specifically activating LPA_2 receptors GRI977143 effectively stimulated extracellular signal regulated kinases 1/2 activation and promoted the assembly of a macromolecular signaling complex consisting of LPA_2, Na^+-H^+ exchange regulatory factor 2, and thyroid receptor interacting protein 6, which has been shown previously as a required step in LPA-induced antiapoptotic signaling. Furthermore, GRI977143 promoted carcinoma cell invasion of human umbilical vein endothelial cell monolayers and fibroblast proliferation. The present findings indicate that nonlipid LPA_2-specific agonists represent an excellent starting point for development of lead compounds with radiomitigative effect or potential therapeutic utility for preventing the

programmed cell death involved in many types of degenerative and inflammatory diseases.

4. GENERAL INTRODUCTION

4.1 LYSOPHOSPHATIDIC ACID (LPA) SIGNALING

The collective name LPA (1-O-acyl-2-hydroxy-sn-glycero-3-phosphate) refers to a diverse group of lysophospholipids consisting of either a saturated (e.g., 16:0,18:0) or unsaturated (e.g., 16:1, 18:1, 18:2, 20:4) fatty acid chain esterified at the *sn*-1- or *sn*-2-position of the phospholipids (Tigyi, 2010). These LPA species, generated both intra- and extracellularly are present in serum, saliva, follicular fluid, seminal fluid, malignant effusions and facilitate cell survival, proliferation, migration, smooth muscle contraction, platelet aggregation, actin stress fiber formation, as well as cytokine and chemokine secretion (Shrivastava and Ray, 2011; Tigyi and Parill, 2003). Biological activities of LPA species are mediated through interactions with specific plasma membrane and intracellular receptors (Tigyi, 2010). The six validated plasma membrane LPA receptors (LPAR) are seven-transmembrane domain G protein-coupled receptors (GPCR), which belong either to the endothelial differentiation gene subfamily (EDG, LPAR 1-3) or to the P2Y purinergic gene subfamily of GPCRs (LPAR 4-6; Chun et al., 2010). According to the 2010 report of the International Union of Basic and Clinical Pharmacology (IUPHAR) three other members of the purinergic GPCR subfamily, GPR35, GPR87 and P2Y10 are considered putative LPA receptors, which require further experimental validation (Chun et al., 2010). Signaling pathways initiated by activation of the LPA GPCRs involve their coupling to multiple classes of heterotrimeric G proteins ($G_{i/o}$, G_s, $G_{q/11}$ and $G_{12/13}$) at the plasma membrane (Anliker and Chun, 2004). Individual G-protein targeting of LPA_{1-6} receptors, additional downstream signaling intermediates and cellular responses are illustrated in **Fig. 4.1.** Intracellular actions of LPA are elicited through activation of the nuclear hormone receptor, peroxisome proliferator-activated receptor gamma (PPARγ; Tigyi, 2010).

Expression pattern of LPA GPCRs shows overlapping tissue distribution (Fujiwara et al., 2005). Analysis of LPA_1 mRNA expression levels in human organs revealed that this LPA receptor subtype is abundant in brain, heart, colon, small intestine, placenta,

prostate, ovary, pancreas, testis and spleen, scarce in skeletal muscle, kidney, lung and thymus and completely absent in liver and peripheral blood leukocytes (An et al., 1998). LPA_2 is highly expressed in testis and leukocytes, moderately in pancreas, thymus, spleen and prostate and is not expressed in adult brain, heart, lung, liver, kidney, muscle, ovary, placenta, intestine and colon (An et al., 1998). LPA_3 shows the highest expression levels in the heart, prostate, pancreas and testis but moderate levels of LPA_3 exist in lung, ovary and brain (Bandoh et al., 1999; Im et al., 2000). High levels of LPA_4 can be detected in the brain, ovary, uterus, placenta and platelets. However, this receptor subtype is not present in the liver, spleen and testis (Ishii et al., 2009). LPA_5 transcripts are present in the heart, placenta, brain, dorsal root ganglia, small intestine, spleen and platelets, while LPA_6 transcripts in the brain, skeletal muscle and the reproductive organs (Tigyi, 2010).

LPA receptor knockout murine models revealed important physiological roles of the individual LPA receptors in intact organism. $LPA_1^{-/-}$ mice show increased (50% or higher) perinatal lethality most likely due to defective olfaction and consequential suckling impairment (Contos et al., 2000). Survivor $LPA_1^{-/-}$ pups develop reduced body size, craniofacial dysmorphism, increased apoptosis of Schwann cells in the sciatic nerve (Contos et al., 2000), resistance to neuropathic pain (Inoue et al., 2004) and attenuated renal and pulmonary fibrosis (Pradere et al., 2007; Tager et al., 2008). $LPA_2^{-/-}$, $LPA_3^{-/-}$, $LPA_4^{-/-}$, and $LPA_5^{-/-}$ mice are viable, grossly normal, however specific characteristics have been reported for each phenotype. $LPA_2^{-/-}$ mice show increased radiation sensitivity of the intestinal crypt (Deng et al., 2007), attenuated tumorigenesis in the colon (Lin et al., 2009) and decreased allergic airway inflammation (Zhao et al., 2009). $LPA_3^{-/-}$ mice have reduced litter size attributed to delayed implantation and embryo spacing abnormalities (Ye et al., 2005). Reports about the $LPA_4^{-/-}$ mice are not uniform. The three independent research groups generating this phenotype reported increased trabecular bone density (Liu et al., 2010), partial embryonic lethality with blood and lymphatic vessel malformation (Sumida et al., 2010) or found no abnormalities (Lee et al., 2008). $LPA_5^{-/-}$ mice fail to develop neuropathic pain after partial sciatic nerve ligation, suggesting a role for LPA_5 in nociceptive transmission and

pain perception (Lin et al., 2012; Oh et al., 2008). Furthermore, LPA_5 has been shown to mediate LPA induced platelet activation (Khandoga et al., 2008; Williams et al., 2009). Constitutive deletion of LPA_6 receptor in mice has not yet been reported but humans carrying a defective gene for this receptor develop a hair growth defect (Pasternack et al., 2008; Shinkuma et al., 2010).

Dysregulated LPA signaling has been suggested to play a role in the pathogenesis of various human disorders. In the cardiovascular system activation of LPA_1 and LPA_3 receptors has been shown to influence cardiomyocyte contraction (Cremers et al., 2003) and lead to cardiac hypertrophy (Chen et al, 2008; Hilal-Dandan et al., 2004). LPA promotes tumor cell invasion, metastasis and angiogenesis via stimulation of LPA_2 and LPA_3 receptors (Kato et al., 2012; Pustilnik et al., 1999), while LPA_1 might act as a tumor suppressor (Murph et al., 2008; Obo et al., 2008; Yamada et al., 2009). Different studies revealed the connection between activation of LPA_1 receptor and the development of organ fibrosis (Heusinger-Ribeiro et al., 2001; Tager et al., 2008), neuropathic pain (Inoue et al., 2004; Renback et al., 1999), osteoarthritis (Mototani et al., 2008), the absence of LPA_1 and schizophrenia (Braff and Geyer, 1990; Harrison et al., 2003), as well as the inhibitory role of LPA_2 receptor in cholera toxin-induced secretory diarrhea (Li et al., 2005). The use of LPA_3 or $LPA_{1/2/3}$ receptor knockout mouse models provided an insight into the LPA mediated regulation of embryo implantation, sperm production and germ cell survival (Ye et al., 2005; Ye et al., 2008). Very high expression levels of LPA_6 have been detected in squamous cell carcinomas, lung adenocarcinomas and transitional carcinomas of the urinary bladder (Glatt et al., 2008; Gugger et al., 2008; Zhanget al., 2009).

Discovery of individual LPA receptors followed by the effort to develop receptor-subtype selective agonists and antagonists accelerated understanding of LPA signaling and raised the possibility of pharmacotherapeutic LPAR modulation (Im, 2010; Choi et al., 2010). However, the presence of multiple LPARs on most of the cells, their overlapping tissue distribution, the differences in their G-protein coupling and ligand preferences towards the individual LPA species represent a great challenge to designing LPA-based therapeutics (Tigyi, 2010). Despite the lack of ligand-bound GPCR crystal structures,

mutagenesis studies combined with computational analysis led to the generation of numerous LPAR agonist and antagonist candidates (Blaho and Hla, 2011; Im, 2010). LPAR agonists and antagonist reported so far include phosphatase-resistant long-lasting LPA derivatives as well as non lipid compounds identified by virtual screening (Im, 2010). Most LPAR modulators were validated in vitro and differ in their potency and selectivity (Choi et al., 2010). Commercially available LPAR modulators are the LPA_2 agonist dodecylsulphate, the LPA_3/LPA_6 agonist OMPT (1-oleoyl-2-O-methyl-rac-glycerophosphothionate) and α-fluoromethylene phosphonate, the LPA_5 agonist/LPA_3 antagonist farnesyl diphosphate and several LPA_1/LPA_3 antagonists: KI16425, VPC12249, VPC32183 and diacylglycerol pyrophosphate (Choi et al., 2010; Im, 2010; Tigyi, 2010). Efforts aiming the design of LPAR specific modulators culminated in the development of the LPA_1 antagonist AM152, which is currently in clinical trials for the treatment of idiopathic pulmonary fibrosis (Swaney et al., 2011).

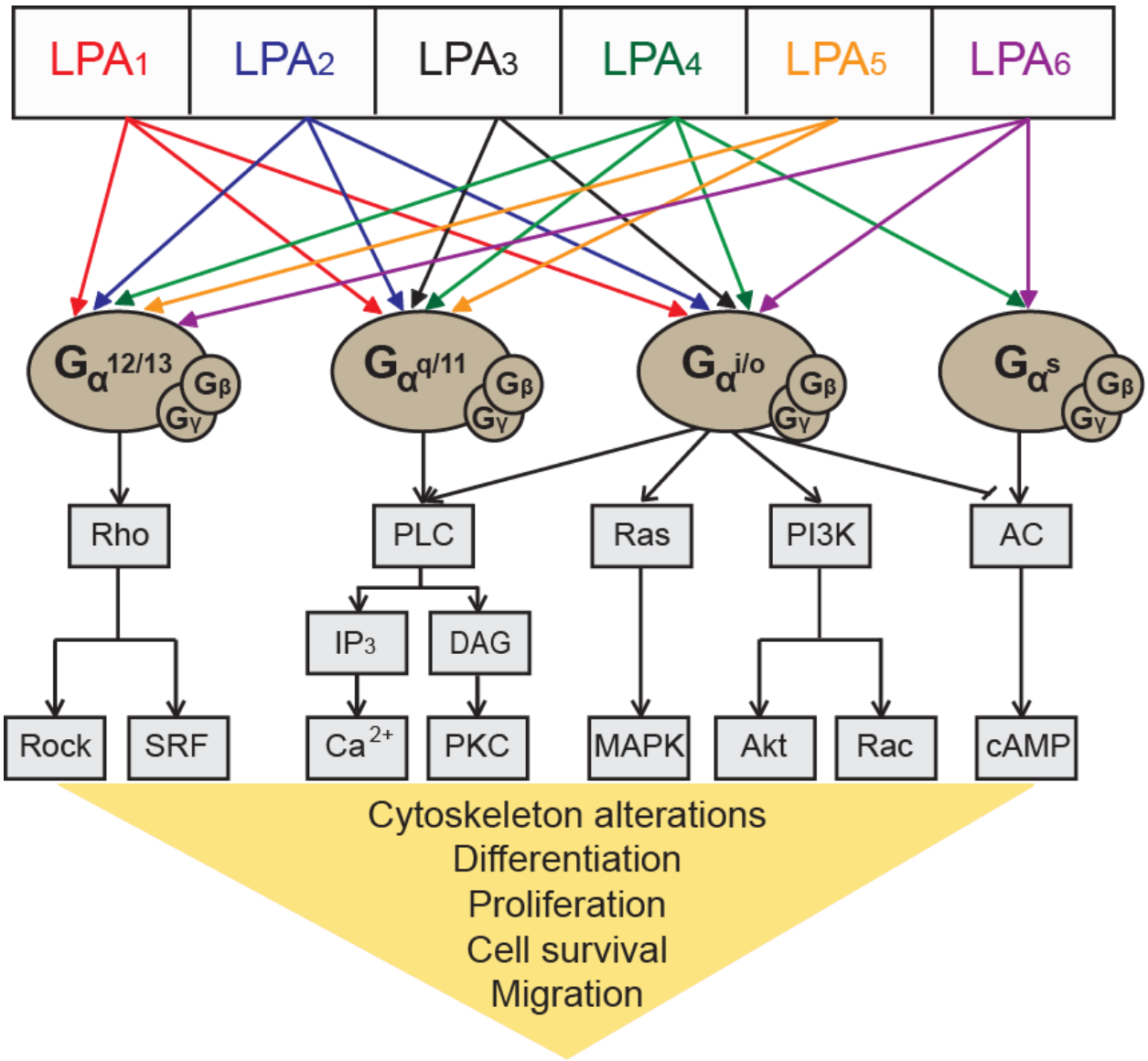


Figure 4.1 LPA receptor signaling. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; MAPK, mitogen-activated protein kinase; IP3, inositol 1,4,5-triphosphate; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; Rock, Rho associated kinase; SRF, serum response factor (Figure constructed based on figure 1., Choi and Chun, 2012)

4.2 ANTIAPOPTOTIC SIGNALING OF THE LPA_2 RECEPTOR

4.2.1 Apoptosis

Pharmacological modulation of apoptosis in physiologic and pathologic processes as a therapeutic alternative has made this form of programmed cell death extensively studied and well characterized. Apoptotic cell populations feature distinct morphologic and biochemical hallmarks of the cellular demolition mediated by the cysteinyl aspartic acid-protease (caspase) cascade (Elmore, 2007; Strasser, 2011; Ulukaya, 2011). Activation of the caspase cascade ultimately results in perinuclear chromatin condensation, internucleosomal DNA fragmentation, cell shrinkage, plasma membrane blebbing and disassembly of the cell into membrane encapsulated apoptotic bodies. Externalization of phosphatidyl serine to the outer leaflet of the plasma membrane promotes recognition and facilitates noninflammatory elimination of apoptotic bodies by the neighboring cells and macrophages. Numerous stimuli can trigger execution of apoptosis by activating the caspase cascade; however, they utilize different signaling pathways of activation. Extracellular signals initiate the extrinsic apoptotic pathway via activation of cell surface death receptors, whereas intracellular stimuli affect the permeability of the inner mitochondrial membrane leading to activation of the intrinsic apoptotic pathway (**Fig. 4.2.1**; Elmore, 2007; Strasser, 2011; Ulukaya, 2011). Extrinsic and intrinsic apoptotic pathways can influence and reinforce each other during the apoptotic process by co-activation or crosstalk at the level of several key molecular mediators (Elmore, 2007; Klener 2006).

Various cytokines of the tumor necrosis factor (TNF) protein family act as death ligands and activate the extrinsic apoptotic pathway via interaction with their cognate plasma membrane death receptors. Fatty acid synthase (Fas) ligand, TNF-related apoptosis-inducing ligand (TRAIL), TNF-α and their receptors are among the best-studied death ligand-receptor pairs (Guicciardi, 2009; Schütze, 2008). Ligand induced conformational change of activated Fas, TRAIL, and TNF receptors allows the recruitment of intracellular adaptor/docking proteins (*e.g.:* Fas-associated protein with death domain, FADD; TNF receptor-associated protein with death domain, TRADD) via death domain-

death domain interactions. Following death receptor binding, adaptor proteins associate with the initiator caspase pro-caspase 8 (or 10), resulting in the formation of the death inducing signaling complex (DISC). Unique to each death receptor, other proteins including receptor-interacting protein kinase 1 (RIP1); multiple isoforms of cellular FLICE like inhibitory protein (c-FLIP); cellular inhibitor of apoptosis proteins (c-IAP); TNF receptor associated factor (TRAF) 2 and 5 may also participate in the DISC formation, influencing activation of pro-caspase 8 (or 10) and apoptotic signaling. Execution of the receptor mediated apoptotic pathway is cell type dependent. In type I cells (*e.g.:* lymphocytes), proteolytic cleavage of pro-caspase 8 (or 10) at the DISC leads to a high amount of active caspase 8, which in turn activates caspases 3, 6, and 7, which are the effector caspases of apoptosis. In type II cells (*e.g.:* hepatocytes) the low amounts of active caspase 8 generated at the DISC require an amplification loop to activate the effector caspases, which involves cleavage of the Bcl-2 homology 3 (BH3) interacting domain death agonist (Bid) by caspase 8 and activation of the intrinsic apoptotic pathway by the mitochondria-permeabilizing fragment truncated Bid (tBid) (**Fig. 4.2.1**; Guicciardi, 2009; Schutze, 2008). Beside the TNF receptor superfamily death receptors, a heterogeneous group of receptors designated dependence receptors have also been shown to transmit apoptotic signals in the absence of their ligands, although these mechanisms are not yet clearly understood (Galluzzi, 2012).

Mitochondrial outer membrane permeability may be altered in response to various intracellular stress signals including growth factor withdrawal, oxidative stress, DNA damage, cytosolic Ca^{2+} overload, and endoplasmic reticulum stress (Bratton, 2010; Elmore, 2007). Pore formation in the outer or inner mitochondrial membrane and release of mitochondrial proapoptotic factors into the cytosol may activate the intrinsic pathway of apoptosis (Galluzzi, 2012). Members of the Bcl-2 protein family are important regulators of the mitochondrial outer membrane permeability. Proapoptotic family members can be divided into the subgroup of effectors (Bcl-2-associated X protein, Bax; Bcl-2 antagonist killer 1, Bak; Bcl-2 related ovarian killer, Bok) participating actively in the disruption of the mitochondrial outer membrane, and that of the initiators (*e.g.:* Bid; Bcl-2 antagonist of cell death, Bad) promoting apoptosis by facilitating activation of the effectors. Role of the prosurvival family members (*e.g.:* Bcl-XL, Bcl-2)

is to sequester and thereby inhibit pore forming activity of the proapoptotic effectors (Kelly, 2011; Strasser, 2011; Ulukaya, 2011). Several protein kinases (*e.g.:* Akt, ERK1/2; c-Jun N-terminal kinase, JNK) and transcription factors (*e.g.:* p53, NFκB; C/EBP homologous protein, CHOP) modulate apoptosis by altering the activity and/or ratio of the pro- and antiapoptotic Bcl-2 protein family members (Fulda, 2010; Strasser, 2011; Ulukaya, 2011). In the presence of certain sensitizers such as reactive oxygen species and inorganic phosphate, mitochondrial Ca^{2+} overload may result in the formation of the inner membrane mitochondrial permeability transition pore (MPTP) with subsequent matrix swelling, rupture of the outer membrane and release of mitochondrial proapoptotic factors. The actual protein components of the MPTP are still debated and unlike the mitochondrial outer membrane pore formed by the members of the Bcl-2 protein family, MPTP seems to play a more important role in necrosis rather than apoptosis (Kinnally, 2011; Rasola, 2011; Vaseva, 2012). Upon release into the cytosol, mitochondrial proteins activate apoptosis in both a caspase dependent and independent manner. Cytochrome c binds to apoptotic protease activating factor 1 (APAF-1) froming the apoptosome, a platform to activate intrinsic pathway initiator caspase 9, while two other mitochondrial proteins, second mitochondrial activator of caspases (Smac) and high-temperature requirement protein A2 (HtrA2/Omi) bind and inactivate members of the caspase inhibitor IAP family. Cleavage and activation of effector caspases 3, 6 and 7 by caspase 9 initiates the apoptotic execution. Independent of caspase activation, mitochondrial apoptosis inducing factor (AIF) and endonuclease G translocate to the nucleus leading to DNA fragmentation and chromatin condensation (**Fig. 4.2.1**; Elmore, 2007; Galluzzi, 2012; Ulukaya, 2011).

Activation of effector caspases 3, 6, and 7, a common endpoint of both the extrinsic and intrinsic apoptotic pathways is characterized by the cleavage of several caspase substrates including but not limited to inhibitor of caspase-activated DNAse (ICAD), poly (ADP-ribose) polymerase 1 (PARP-1), lamin A, and fodrin, leading to the unique morphology of the apoptotic cells (Elmore, 2007; Fan, 2005; Galluzzi, 2012).

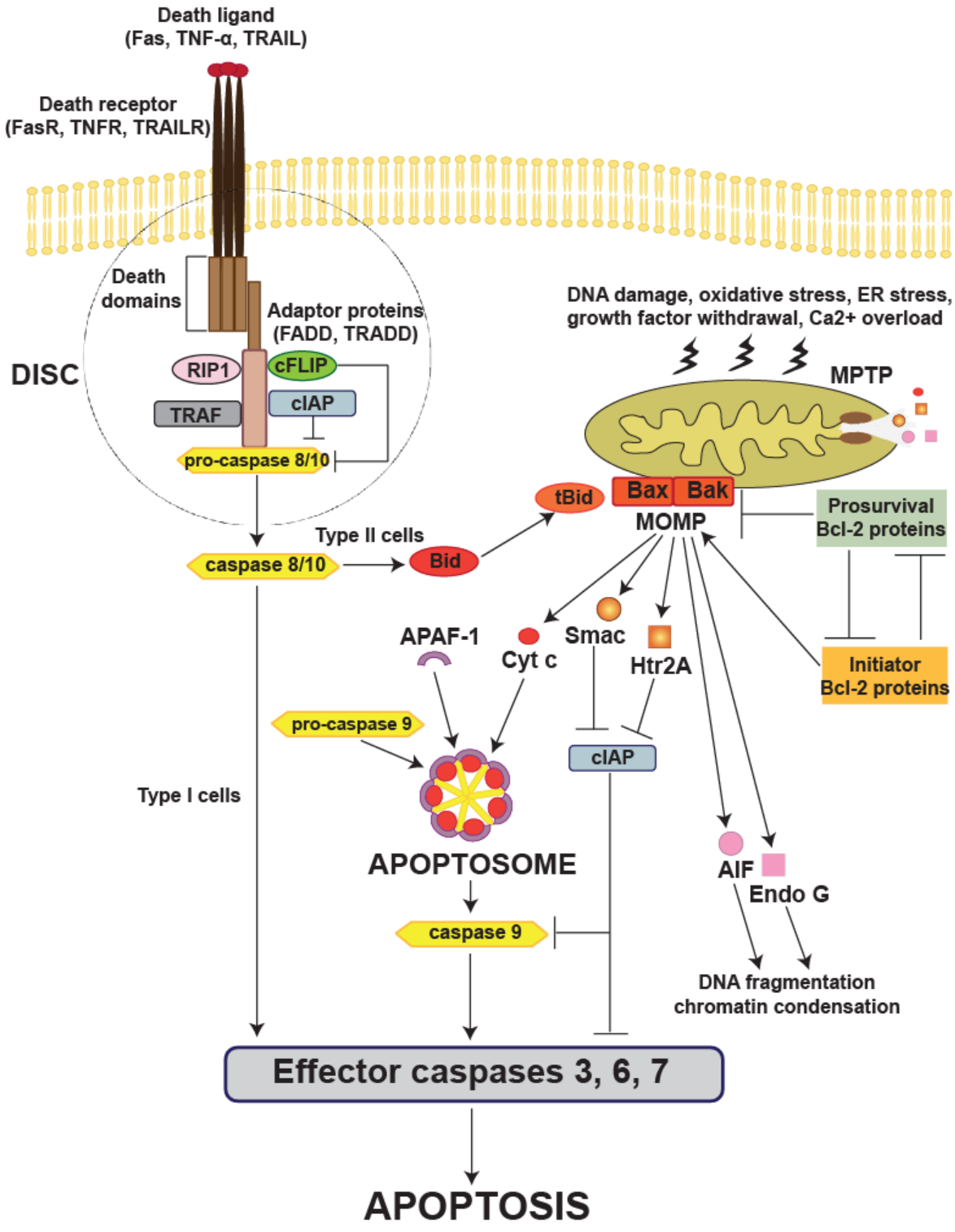


Figure 4.2.1 Extrinsic and intrinsic apoptotic pathways. Cyt c, cytochrome c, MOMP, mitochondrial outer membrane pore, MPTP, mitochondrial permeability transition pore.

4.2.2 Mechanism of LPA_2 receptor mediated cell survival

Identified as the second LPA receptor, LPA_2 shares ~60% amino acid sequence homology with LPA_1. Human LPA_2 consists of 348 predicted amino acid residues and has a molecular mass of ~39 kDa (Chun et al., 2010). Generally, LPA_2 signaling has been linked to mediation of cell survival and cell migration (Choi et al., 2010).

Our laboratory focused on developing metabolically stabilized analogs of LPA that could be used as long-acting stimulators of the prosurvival signaling mediated by LPA receptors. In parallel with the development of LPA mimics, we have begun to study the antiapoptotic mechanisms elicited by LPA. These studies led to the previously unrealized role of the LPA_2 GPCR as a center of a macromolecular signaling complex mediated through unique sequence motifs present in its C-terminal domain (E et al., 2009; Lin et al., 2007). We discovered that LPA_2 via a C_{311}xxC half zinc-finger-like motif interacts with the proapoptotic protein Siva-1 from the LIM family of proteins (Lin et al., 2007). LIM domain proteins are named after the Lin-11, Isl-1, and Mec-3 proteins, which contain Zn-finger-like domains in their sequences that are important for oligomerization and interaction with other proteins. Siva-1 is an immediate-early response gene product whose expression is triggered by the DNA damage-mediated activation of the p53 and E2F1 transcription factors. Siva-1 mediates the progression of apoptosis by making a complex with the antiapoptotic B-cell lymphoma 2 related protein, long isoform (Bcl-X_L). Binding of Siva-1 to Bcl-X_L reduces the availability of this protein to protect the mitochondrial outer membrane, thus promoting the progression of the mitochondrial apoptosis cascade. However, upon activation of LPA_2, the C-terminal domain of LPA_2 binds Siva-1 and this complex is withdrawn from GPCR recycling, undergoes polyubiquitination and is degraded in the proteasome (Lin et al., 2007). In a subsequent study, we have determined that the LPA_2 GPCR makes a ternary complex with two other proteins, the thyroid receptor interacting protein 6 (TRIP6) and the Na^+-H^+ exchange regulatory factor 2 (NHERF2). LPA_2 and TRIP6 contain motifs in their last three C-terminal amino acids that interact with PSD-95, DlgA, and ZO-1 (PDZ) binding domains of proteins. NHERF2 contains tandem PDZ-binding domains near its N-terminus. We showed that TRIP6 with its LIM domain physically binds to the C_{311}xxC

motif of LPA_2 and at the same time the PDZ motif of TRIP6 binds to the PDZ-binding domain of NHERF2. NHERF2 homodimerizes, leaving an additional PDZ-binding domain available to bind to the $S_{351}TL$ PDZ motif of LPA_2. The ternary complex consisting of LPA_2 – TRIP6 – 2x(NHERF2) is formed upon LPA stimulation of the GPCR leading to enhanced, long-lasting activation of the mitogen activated protein kinase/extracellular signal regulated kinase (MEK)-extracellular signal regulated kinases 1/2 (ERK1/2) and phosphoinositide-3-kinase (PI3K)- protein kinase B (Akt)- nuclear factor κB (NFκB) prosurvival pathways required for the LPA_2-mediated antiapoptotic effect (**Fig. 4.2.2**; E et al., 2009). The role of the ternary complex recruitment in the LPA_2-mediated antiapoptotic response is supported by the lack of LPA protection against apoptosis when cysteines 311/314 and leucine 351 in the C-terminus of LPA_2 are simultaneously mutated to alanine (E et al., 2009). Ternary complex formation upon LPA_2 receptor activation plays a unique role in chemoresistance (Tigyi et al., 2010).

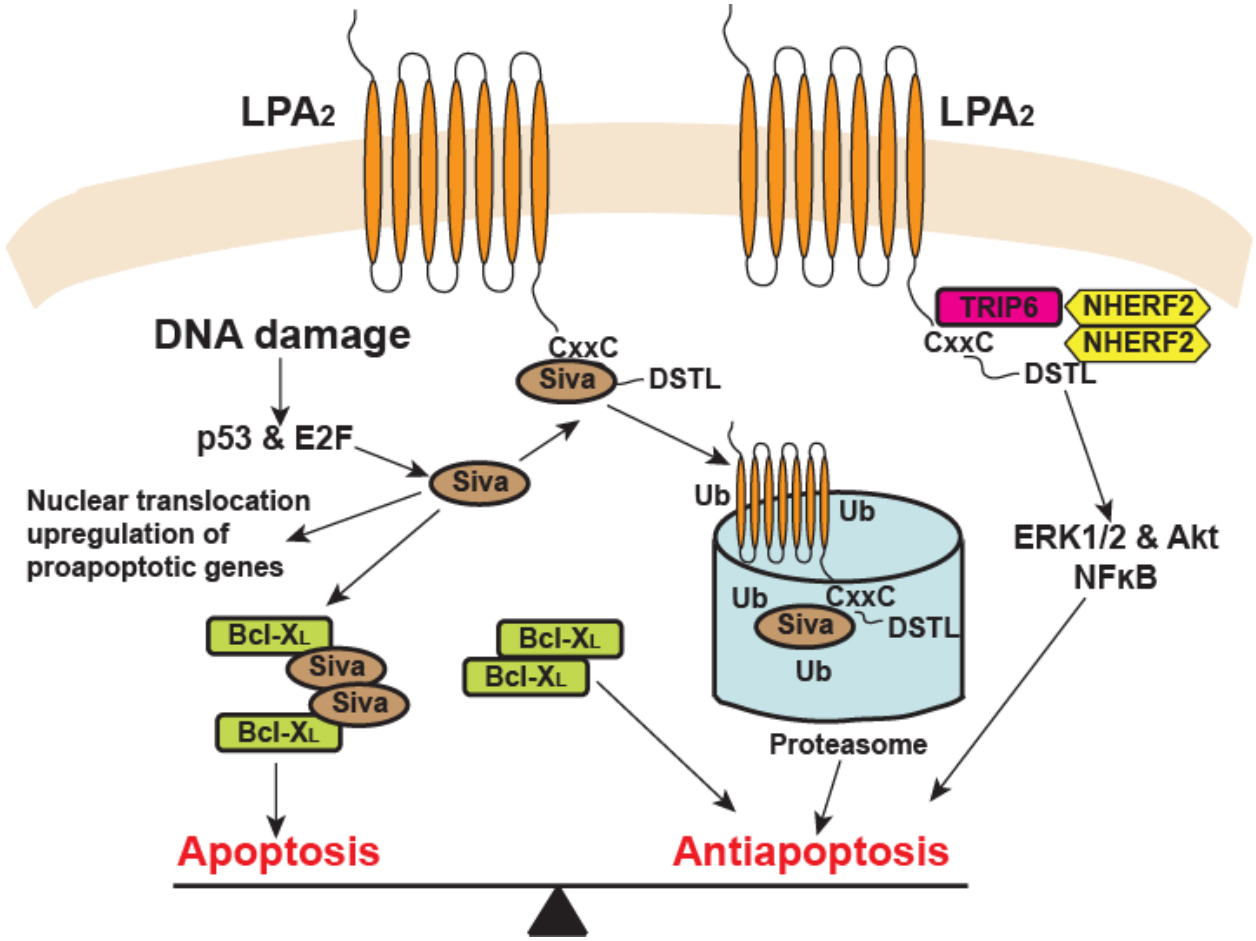


Figure 4.2.2 Antiapoptotic signaling mediated by the LPA_2 receptor. (Figure constructed based on figure 6., Tigyi 2010)

4.3 IONIZING RADIATION

Forms of photon (X-rays and γ-rays) and particle radiation (electron, positron, proton and neutron beams) are referred to as ionizing radiation because of their ability to deposit energy and induce ionization in the penetrated biological matter, leading to damage (Podgorsak, 2005; Iliakis, 1991). Ionizing radiation is commonly used in the treatment of various cancers, in diagnostic medical imaging, as well as for industrial and military purposes. Exposure to high doses of ionizing radiation may occur from nuclear power plant accidents or from nuclear weapons. The biological consequences of exposure to ionizing radiation depend on many factors that include the type, dose, and dose distribution of radiation, the type and volume of the absorbing tissue, the time of exposure, as well as genetic and environmental factors (Baskar et al., 2012; Selzer and Hebar, 2012). At the cellular level ionizing radiation can disrupt both the chemical integrity and the function of biomolecules, either by direct ionization or indirectly through the radiolysis of water and consequential free radical generation (Choi et al. 2007, Iliakis, 1991). A critical target of ionizing radiation within the cell is the DNA (Podgorsak, 2005; Selzer and Hebar, 2012). Damage to the DNA in the form of single and double strand breaks, DNA cross linking and nucleotide base damages affects cell survival and may ultimately result in mutations, malignant transformation or cell death (Huang et al., 2003; Iliakis, 1991). In addition, low dose ionizing radiation may induce a series of non-targeted effects such as bystander responses, adaptive responses and genomic instability (Prise et al., 2005; Selzer and Hebar, 2012).

Cell death in response to ionizing radiation mainly occurs via apoptosis and mitotic catastrophe (Baskar et al., 2012; Selzer and Hebar, 2012). Ionizing radiation induced apoptosis may involve activation of the DNA damage response signaling network and/or membrane associated events, which trigger the intrinsic, mitochondrial apoptotic pathway (Prise et al., 2005; Verheij and Bartelink, 2000; Watters, 1999). A central member of the DNA damage response signaling network is the tumor suppressor protein p53, although p53 independent apoptosis has also been reported (Prise et al., 2005; Verheij and Bartelink, 2000; Watters, 1999). Ionizing radiation has been shown to increase cellular p53 levels through a post-translational mechanism and also to activate

p53 via upstream DNA damage sensors PARP-1, ataxia-teleangiectasia-mutated (ATM) and DNA protein kinase. Upon activation, p53 delays the cell cycle progression at the G1-S transition to allow DNA repair or to commit the cell to apoptosis if the repair mechanisms fail. Apoptosis could be mediated by p53 dependent upregulation of Bax, a proapoptotic Bcl-2 family member, the cell death ligands or receptors or activation of ceramide synthase (Prise et al., 2005; Verheij and Bartelink, 2000; Watters, 1999). Activation of ceramide synthase leads to the generation of ceramide, a second messenger known to mediate ionizing radiation-induced apoptosis by stimulating incorporation of Bax into the outer mitochondrial membrane. Cell membrane damages may also lead to ceramide formation via activation of acid sphingomyelinase. As a result of mitochondrial membrane alterations release of cytochrome c and activation of caspases 3 and 7 may initiate the execution phase of apoptosis (Kolesnick and Fuks, 2003; Prise et al., 2005). Irradiated cells, unable to undergo mitosis due to improper distribution of chromosomes are eliminated via a cell death modality called mitotic catastrophe (Selzer and Hebar, 2012; Vakifahmetoglu et al., 2008). Exact molecular mechanisms of mitotic catastrophe are still unclear, although similarities to both apoptosis and necrosis have been reported (Kroemer et al., 2009, Vakifahmetoglu et al., 2008).

Ionizing radiation-induced cellular responses that manifest in non-irradiated naïve cells are collectively termed the 'bystander effect' or distant effects of radiation injury and include DNA and chromosome damage associated cellular responses such as proliferation, differentiation, mutations, transformation, and cell death (Chaudhry, 2006; Hamada et al., 2007; Terzoudi et al., 2007). Signals evoking the bystander effect are generated by the irradiated cells and may be transmitted to the non-irradiated cells either through gap-junction mediated intercellular communication or via secretion of low molecular weight soluble factors into the growth medium (Baskar et al., 2007; Hei et al., 2011). It is hypothesized that upon stimulation by these signals non-irradiated cells respond with generation of intracellular mediators and/or activation of signal transduction pathways ultimately leading to the bystander effect (Terzoudi et al., 2007). Despite numerous studies providing evidence to the existence and manifestation of the bystander effect, the nature of these signaling molecules and the underlying intracellular

events are still poorly understood (Asur et al., 2010; Ilnytskyy and Kovalchuk, 2011). So far members of stress-induced cytokines (e.g. TNF-α; transforming growth factor β, TGF-β), growth factors (e.g. insulin-like growth factor 1, IGF-1; basic fibroblast growth factor, FGF-2), membrane-permeable reactive species (e.g. H_2O_2, NO) and connexin43-mediated intercellular communication have been reported to participate in the transmission of damage signals to the non-irradiated cells (Hamada et al., 2007; Hei et al, 2011; Ilnytskyy and Kovalchuk, 2011). Intracellular events implicated in the bystander effect include the activation of stress-related kinases (NFκB, JNK, ERK1/2) and downstream transcription factors, activation of the plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase and generation of reactive oxygen species, as well as signaling through the p53 DNA damage response pathway (Hamada et al., 2007; Hei et al., 2008; Hei et al., 2011). The actual endpoint of the ionizing radiation-induced bystander effect will ultimately depend on the fine interplay of multiple parameters determined by the type of the target cells, the level of the secreted factors, as well as the severity of the DNA damage (Hamada et al., 2007).

Acute and late effects of ionizing radiation exposure include the acute radiation syndromes (ARS) of the hematopoetic, the gastrointestinal and the nervous system, carcinogenesis, organ fibrosis and dysfunction, sterility, and death (Podgorsak, 2005). According to a comprehensive review published in 2006 by the National Academy of Sciences' National Research Council no ionizing radiation exposure levels can be considered truly safe (National Research Council, 2006). Occupational and public ionizing radiation dose limits, protection standards, and clinical supportive care protocols have been established (International Atomic Energy Agency Safety Standards, 2011); however, the possibility of nuclear accidents and the threat of nuclear weapons necessitate the development of targeted pharmacological countermeasures suitable both for hospital and non-hospital settings (Xiao et al., 2009). Pharmacological agents currently available are drugs that block the effects of specific internalized radioisotopes (e.g. Prussian blue, potassium iodide) and the broad-spectrum cytoprotectant amifostine for alleviating radiotherapy associated xerostomia (Weiss et al., 2009). There is ongoing extensive research aimed at the development and validation of drugs that administered either before (radioprotectants) or after

(radiomitigators) the exposure to ionizing radiation prevent the initial radiation injury, repair the molecular damage caused by radiation and/or stimulate proliferation of surviving stem and progenitor cells. As a result, four drug candidates, 5-androstenediol (Neumune, 2005), genistein (BIO300, 2007), the sodium salt of 4-carboxystyryl-4-chlorobenzylsulfone (Ex-Rad®, 2008) and CBLB502 (Entolimod™, 2008) acquired Investigational New Drug (IND) status, while granulocyte colony-stimulating factor (Neupogen®) aquired Emergency Use IND status from the United States Food and Drug Administration (US FDA) for the treatment of ARS. Promising experimental results have been published about the effects of certain cellular therapies, tocols (e.g. Vitamine E), thiols, cytokines and growth factors, enzyme mimetics and inhibitors, and antibiotics able to decrease ionizing radiation associated lethality, ARS or tumor formation (Singh et al., 2012). However introduction of these agents as pharmacological radiation countermeasures requires further studies and validations.

5. INTRODUCTION

The growth factor-like lysophospholipids LPA and sphingosine-1-phosphate (S1P) regulate many fundamental cellular responses, ranging from cell survival through cell proliferation to cell motility and migration (Tigyi, 2010). To date, specific inhibitors of the LPA and S1P receptors have taken center stage in drug discovery efforts. The functional antagonist of S1P receptors fingolimod (Brinkmann et al., 2010) has been recently approved by the US FDA for the first-line treatment of multiple sclerosis; and AM152 (Swaney et al., 2011), an LPA_1-selective antagonist has been granted orphan drug status for the treatment of fibrotic diseases. A decade ago, we had already shown that LPA has profound activity in preventing apoptosis and can also rescue apoptotically condemned cells from the progression of the apoptotic cascade (Deng et al., 2002; Deng et al., 2004; Deng et al., 2007; Deng et al., 2003). We developed an LPA mimic, OTP (Durgam et al., 2006), which has superior efficacy compared to LPA in vitro and in vivo in rescuing cells and animals from radiation-induced apoptosis (Deng et al., 2007). Development of LPA-based drug candidates has been limited to the discovery of lipid-like ligands, which is understandable due to the hydrophobic environment of the S1P and LPA GPCR ligand binding pockets (Hanson et al., 2012; Valentine et al., 2008; Fujiwara et al., 2007; Li et al., 2005; Wang et al., 2001; Parrill et al., 2000). Only a few LPA receptor ligands break away from lipid-like structural features, among which Ki16425, an $LPA_{1/2/3}$ antagonist (Ohta et al., 2003), and the AM095-152 series of LPA_1-selective compounds are of importance (Swaney et al., 2011).

In order to exploit the potential therapeutic benefits of LPA, discovery and development of drug-like nonlipid compounds might be beneficial. In the present study, we applied virtual screening strategies using similarity searching that we derived from the previously validated molecular models of these receptors, and we limited our searches to chemical libraries with drug-like compounds that satisfy Lipinski's rule of five (Lipinski, 2003). We focused our virtual screen on the discovery of ligands for the LPA_2 receptor subtype because of our long-standing interest in developing compounds that can attenuate programmed cell death elicited by radiation and chemotherapy. The choice of

this receptor subtype is based on mounting evidence that LPA_2 is unique among this class of receptors in its ability to initiate signaling events that promote cell survival and prevent the progression of apoptosis (Deng et al., 2007; E et al., 2009; Lin et al., 2007). Our objective in the present study was to identify nonlipid LPA_2 agonist scaffolds that can lead to the development of new drug candidates capable of alleviating the side effects of chemo- and radiation treatment of cancer patients and potentially function as radiomitigators against lethal levels of radiation injury. A pivotal observation recognized from our early experiments was that LPA not only prevented apoptosis induced by radiation injury when applied prior to the irradiation of the cells but also rescued apoptotically condemned cells when applied two hours postirradiation (Deng et al., 2002). With the availability of the long-acting LPA mimic OTP (Durgam et al., 2006), we began to examine the utility of LPA GPCR activation in mouse models of the acute hematopoietic and gastrointestinal radiation syndromes. The hematopoietic and gastrointestinal stem cells are among the most radiation sensitive cells in the body, thus these murine models appeared to be a logical extension of our *in vitro* studies. Our research focused on further characterization of OTP and the preclinical development of this compound as a radiomitigator in murine and nonhuman primate models of the acute gastrointestinal radiation syndrome. Radiomitigators are agents that attenuate radiation injury when applied after radiation exposure. We reported in 2007 (Deng et al., 2007) that LPA or OTP administration to mice exposed to lethal levels of radiation was effective in reducing lethality from the hematopoietic radiation syndrome and reduced radiation-induced injury to the gastrointestinal stem cells by attenuating their apoptosis and enhancing crypt regeneration. In this study, we showed that OTP and LPA were completely ineffective in protecting mice lacking the LPA_2 receptor subtype. We also obtained *in vitro* evidence that LPA or OTP failed to protect RH7777 cells, which do not express $LPA_{1/2/3}$ GPCR, unless LPA_2 was expressed by heterologous transfection of this receptor subtype. Although highly effective in protecting animals from radiation injury, OTP activates multiple LPA GPCRs including LPA_1, which has been linked to apoptosis through anoikis (Funke et al., 2012; Furui et al., 1999) and might attenuate the protective effect of LPA_2 stimulation in cells that coexpress both GPCR subtypes.

Here we report on the identification of four nonlipid compounds that are specific agonists of LPA_2 and do not activate other LPA GPCRs including $LPA_{1/3/4/5}$, GPR87, or P2Y10. We selected one of these hits GRI977143 from the Genome Research Institute (GRI) chemical library and characterized its cellular, pharmacological and signaling responses in several assay systems. Our results show that the compound GRI977143 is a specific agonist of LPA_2 and does not activate any other known or putative LPA GPCR. We also show that GRI977143 is similarly effective compared to LPA and OTP in preventing programmed cell death, although in Ca^{2+}-mobilization and caspase 3 and 7 inhibition assays it has higher EC_{50} values than the other two ligands. GRI977143 inhibited activation of caspases 3, 7, 8, and 9, Bax translocation, and PARP-1 cleavage, leading to reduced DNA fragmentation following activation of the extrinsic or intrinsic apoptotic signaling cascades. Our results show that GRI977143 is highly efficacious against radiation-induced apoptosis *in vitro* and protects mice from the acute hematopoietic radiation syndrome when administered 24 hours after the radiation exposure. We also provide evidence that GRI977143 robustly activates the ERK1/2 survival pathway and leads to the assembly of a macromolecular signalosome consisting of LPA_2, TRIP6, and NHERF2, which has been shown to be required for the prosurvival signaling elicited via this receptor subtype. GRI977143 [2-((3-(1,3-dioxo-1*H*-benzo[de]isoquinolin-2(3*H*)-yl)propyl)thio)benzoic acid] and the three other nonlipid hits NSC12404 [2-((9-oxo-9*H*-fluoren-2-yl)carbamoyl)benzoic acid], H2L5547924 [4,5-dichloro-2-((9-oxo-9*H*-fluoren-2-yl)carbamoyl)benzoic acid], and H2L5828102 [2-((9,10-dioxo-9,10-dihydroanthracen-2-yl)carbamoyl)benzoic acid] described in this paper represent a good starting point for lead development and optimization, which may yield novel LPA-based drug candidates for therapeutic applications.

6. MATERIALS AND METHODS

6.1 Materials

LPA (18:1) was purchased from Avanti Polar Lipids (Alabaster, AL). OTP was synthesized and provided by RxBio, Inc. (Johnson City, TN) as described (Durgam et al., 2006). The test compounds used in the present study were obtained from the following vendors: Genome Research Institute (GRI) GRI977143 from the University of Cincinnati Drug Discovery Center (UC-DDC; Cincinnati, OH); Hit2Lead (www.hit2lead.com) H2L5547924, and H2L5828102, from ChemBridge (San Diego, CA); and NSC12404 from the National Cancer Institute Developmental Therapeutics Program Open Chemical Repository. Ten mM stock solutions of GRI977143, H2L5547924, H2L5828102, and NSC12404 were prepared in dimethyl sulfoxide (DMSO). For the animal experiments 1 mg/kg GRI was dissolved in a vehicle consisting of PBS, 1% ethanol and 2% propanediol. One millimolar stocks of LPA and OTP as an equimolar complex of charcoal-stripped, fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrichl St. Louis, MO) were prepared in phosphate-buffered saline (PBS). A stock solution of 3.45 mM Adriamycin was prepared in distilled water.

6.2 Computational docking

Compounds were flexibly docked into the activated LPA_2 receptor homology model reported by Sardar et al. (Sardar et al., 2002) using Autodock Vina (Trott and Olson, 2010). The compounds and receptor homology model were both energy optimized with the Merck Molecular Force Field 94 (MMFF94) in the Molecular Operating Environment software (MOE, 2002) prior to docking. Docking simulations were performed using a docking box with dimensions of 65 x 63 X 50 Å and a search space of 20 binding modes, and an exhaustive search parameter was set at 5. The best docking pose was chosen based on the lowest energy conformation. Finally, the best pose was further refined using the MMFF94 in MOE.

6.3 Ligand-based similarity search

Similarity searching of NSC12404 was performed using the UC-DDC library database (drugdiscovery.uc.edu). The Tanimoto similarity indices for the reference compounds were calculated using ECFC6, FCFP4, and FCFP6 fingerprints in Pipeline Pilot software (Accelerys, Inc.; San Diego, CA). The UC-DCC library was screened using Pipeline Pilot fingerprints to identify additional LPA_2 ligands. A similarity threshold was set at 80%. Among the 225 returned hits, compounds with similarity > 80% were selected by visual inspection, carefully considering the similarity and how closely the structures reflected the reference compound. A total of 27 compounds was selected for evaluation using LPA receptor-activated Ca^{2+}-mobilization assays.

6.4 Residue nomenclature

Amino acids in the transmembrane (TM) domains were assigned index positions to facilitate comparison between GPCRs with different numbers of amino acids, as described by Ballesteros and Weinstein (Ballesteros, 1995). An index position is in the format *X.YY*., where *X* denotes the TM domain in which the residue appears, and *YY* indicates the position of that residue relative to the most highly conserved residue in that TM domain, which is arbitrarily assigned position 50.

6.5 LPA receptor-mediated Ca^{2+} mobilization assay

Stable cell lines expressing the individual LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5 established receptor subtypes (Tigyi, 2010), as well as putative LPA receptors GPR87 (Tabata et al., 2007) and P2Y10 (Murakami et al., 2008), or appropriate empty vector-transfected controls have been previously generated and described (Murakami et al., 2008; Tabata et al., 2007; Williams et al., 2009). Assays for ligand-activated mobilization of intracellular Ca^{2+} were performed using a Flex Station 2 robotic fluorescent plate reader (Molecular Devices; Sunnyvale, CA) as previously described (Durgam et al., 2006). The appropriate concentrations of the test compounds were either used alone (for agonist testing) or mixed with the respective ~EC_{75} concentration of LPA 18:1 for the LPA receptor being tested (antagonist screen). The cells were

loaded with Fura -2-acetoxymethyl esther (Fura-2/AM) in Krebs buffer containing 0.01% pluronic acid for 30 min and rinsed with Krebs buffer before measuring Ca^{2+} mobilization. The ratio of peak emissions at 510 nm after 2 min of ligand addition was determined for excitation wavelengths of 340 nm/380 nm. All samples were run in quadruplicate. The inhibition elicited by 10 μM test compound on the EC_{75} concentration of LPA 18:1 for a given receptor ($I_{10\mu M}$) was interpolated from the dose-response curves. The half maximally effective concentration (EC_{50}), and inhibitory constant (K_i) values were calculated by fitting a sigmoid function to dose-response data points using the Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

6.6 Cell culture

Mouse embryonic fibroblast (MEF) cells were isolated from E13.5 $LPA_{1\&2}$ double knockout (DKO) embryos (Lai et al., 2007). MEFs were transduced with empty vector or LPA_2-containing lentiviruses and selected with 1.5 μg/ml puromycin. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (V/V) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/ mL streptomycin. Serum-free medium contained 0.1% (W/V) BSA in DMEM. The rat intestinal epithelial cell line 6 (IEC-6) was obtained from the American Type Culture Collection (Rockville, MD) at passage 13; passages 16–21 were used in all experiments. lEC-6 cells were maintained in a humidified 37 °C incubator in an atmosphere of 90% air and 10% CO_2. Growth medium consisted of DMEM supplemented with 5% heat inactivated FBS, 10 μg/mL insulin, and 50 μg/mL gentamycin. The composition of the serum-starvation medium was the same as that of the full growth medium except that it contained no FBS. The McArdle rat hepatoma cell line (RH7777) stably expressing LPA_2 receptors was a gift from Dr. Fumikazu Okajima (Gunma University, Maebashi, Japan). RH7777 cells stably expressing LPA_1 or LPA_3 receptors were generated in-house and characterized earlier (Fischer et al., 2001). Wild type and LPA receptor stably transfected RH7777 cells were grown in DMEM supplemented with 10% FBS and 2 mM L-glutamine in the presence of 250 μg/ml G418. Chinese hamster ovary (CHO) cells stably expressing either vector or LPA_4 receptor were a kind gift from Dr. Takao Shimizu (Tokyo University; Tokyo, Japan). Cells were

cultured in Ham's F12 medium containing 10% FBS, 2 mM L-glutamine, and 350 µg/ml G418. Rat neuroblastoma cells (B103) were transduced with the lentivirus harboring wild type of FLAG-LPA_5 and selected with puromycin to establish the stable cell lines. The stable cells were maintained in DMEM supplemented with 10% FBS and 0.4 µg/ml puromycin. GPR87- and P2Y10-expressing CHO cells and vector-transfected control cells were a gift from Dr. Norihisha Fujita (Ritsumeikan University; Shiga, Japan). The highly invasive MM1 rat hepatoma cells (gift from Dr. Michiko Mukai, Osaka University, Japan) were grown in suspension in DMEM supplemented with 10% (V/V) FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Human umbilical vein endothelial cells (HUVEC) were purchased from VEC Technologies Inc. (Rensselaer, NY, USA) and cultured in MCDB-131 complete medium supplemented with 10% (V/V) FBS, 90 µg/mL heparin, 10 ng/mL EGF, 1 µg/mL hydrocortisone, 0.2 mg/mL EndoGrowth (VEC Technologies Inc.) supplement, 100 U/mL penicillin G, 100 µg/mL streptomycin, and 25 µg/mL amphotericin B. The U937 human monocyte lymphoma cell line was a kind gift of Dr. Ryan Yates (Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN). Growth medium consisted of RPMI-1640 supplemented with 10% (V/V) FBS and 50 µg/mL gentamicin while serum starvation medium contained 0% FBS.

6.7 Cell proliferation assay

For determination of the effect of the LPA receptor ligands on cell growth, vector- and LPA_2-transduced MEF cells (2×10^4) were plated in each well of a 24-well plate in full growth medium. Cells were counted the next day and the medium was replaced with medium containing 1.5% (V/V) FBS supplemented with or without 1 µM LPA, 1 µM OTP, or 10 µM GRI977143. Media containing LPA, OTP, and GRI977143 were refreshed every 24 h. The growth rate was measured by counting the number of cells in triplicate using the Z1 Coulter Particle Counter (Beckman Coulter; Hileah, FL) as a function of time.

6.8 MM1 hepatoma cell invasion of endothelial monolayer

HUVEC (1.3 x 10^5 cells at passages 5 to 7) were seeded into each well of a 12-well plate pre-coated with 0.2% gelatin (Sigma-Aldrich). Cells were grown for two days until a confluent monolayer was formed. MM1 cells were pre-labeled with 2 μg/mL calcein AM (Life Technologies; Grand Island, NY) for 2 h and rinsed twice, and 5 x 10^4 cells per well were seeded over the HUVEC monolayer. Tumor-monolayer cell invasion was carried out for 20 h in MCDB-131 complete media containing 1% FBS with or without the addition of 1 μM LPA or 1-10 μM GRI977143. Non-invaded tumor cells were removed by repeatedly rinsing the monolayer with PBS (containing Ca^{2+} and Mg^{2+}), followed by fixation with 10% buffered formalin. Tumor cells that penetrated the monolayer were photographed using a NIKON TiU inverted microscope with phase-contrast and fluorescence illumination. The fluorescent and phase-contrast images were overlaid using Elements BR software (Nikon, version 3.1x). A total of five non-overlapping fields was imaged per well, and the number of invaded MM1 cells (displaying a flattened morphology underneath the monolayer) was counted.

6.9 Induction of apoptosis by Adriamycin or serum withdrawal

Experiments were performed on vector- and LPA_2-transduced MEF cells. To measure caspase 3, 7, 8, or 9 activity and DNA fragmentation, cells were plated in 48-well plates (2 x 10^4 cells/well). To detect PARP-1 cleavage and Bax translocation, 1.5 x 10^6 cells were plated in 10-cm dishes and cultured overnight in full growth medium. The next morning, the growth medium was replaced by serum-starvation medium and cells were pretreated for 1 h with LPA (1-10 μM), OTP (1-10 μM), GRI977143 (1-10 μM), or vehicle. Caspase activity, DNA fragmentation, PARP-1 cleavage, and Bax translocation were measured 5 h after incubation with 1.7 μM Adriamycin or 24 h after serum withdrawal.

6.10 Induction of apoptosis by TNF-α in IEC-6 cells

Confluent serum-starved IEC-6 cells were treated with or without TNF-α (10 ng/ml)/cycloheximide (CHX) (20 µg/ml) (Deng et al., 2002) in the presence of OTP (10 µM), GRI977143 (10 µM), or LPA (1 µM) for 3 h. Cells were washed twice with PBS and the quantitative DNA fragmentation assay was carried out as described previously (Valentine et al., 2010).

6.11 Induction of apoptosis by direct γ-irradiation

Cell cultures were irradiated on a rotating platform, using a Cs^{137} γ-source (J.L. Shepherd & Assoc. Mark I, Model 25, San Fernando, CA). MEF cells were plated the day before the irradiation in 48 well plates (for caspase activation or DNA fragmentation assays) or in 10 cm dishes (for PARP-1 cleavage or Bax translocation assays) at a density of 2×10^4 cell/well or 1.5×10^6 cell/dish, respectively. One hour before the irradiation full growth medium was changed to serum starvation medium and the cell cultures were exposed to a dose of 15 Gy γ-irradiation, at a dose rate of 3.2 Gy/min. One hour post irradiation cells were treated with either vehicle, LPA (1-10 µM), OTP (1-10 µM), or GRI (1-10 µM). Caspase activation, DNA fragmentation, PARP-1 cleavage, and Bax translocation were measured 5 h after the irradiation. U937 cells were plated on the day of the irradiation at a density of 2.5×10^5 cell/ml in serum starvation medium and exposed to increasing doses of γ-irradiation (7–55 Gy), at a dose rate of 7.74 Gy/min. Caspase 3 and 7 activation was determined in the irradiated U937 cells 24 h later.

6.12 Induction of apoptosis in unirradiated cells using conditioned medium (CM) from γ-irradiated U937 cell cultures

For the generation of irradiated cell CM, U937 cell cultures (5×10^5 cell/ml) were irradiated with a dose of 35 Gy γ-irradiation, using a Cs^{137} γ-source and a dose rate of 7.74 Gy/min. Twenty four hours later the cells were pelleted by centrifugation at 1000 x g for 5 min and the CM was collected. This CM was supplemented with either vehicle, 10 µM LPA, or 10 µM GRI and applied to 24-h serum-starved IEC-6 cultures (1.7×10^5

cell/well in 48-well plates). Activation of caspase 3 and 7 in the IEC-6 cells treated with the different CM-drug mixtures was measured 24 h later.

6.13 Caspase activity assay

Caspase-Glow^{R} 3/7, Caspase-Glow^{R} 8 and Caspase-Glow^{R} 9 reagents were purchased from Promega (Madison, WI) and used according to the manufacturer's instructions. Briefly, cells were lysed by adding 50 µl of lysis reagent per well, followed by shaking for 30 min at room temperature. Two hundred µl lysate were transferred to a 96-well white-wall plate, and luminescence was measured using a BioTek (Winooski, VT) plate reader.

6.14 DNA fragmentation ELISA

Apoptotically-challenged cells were washed twice with PBS, and a quantitative DNA fragmentation assay was carried out using a Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Penzberg, Germany) and normalized to protein concentration using the BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.; Rockford, IL) as described previously (Valentine et al., 2010). Aliquots of nuclei-free cell lysate were placed in streptavidin-coated wells and incubated with anti-histone-biotin antibody and anti-DNA peroxidase-conjugated antibody for 2 h at room temperature. After the incubation, the sample was removed, and the wells were washed and incubated with 100 µl 2,2'-azino-di[3-ethylbenzthiazolin-sulfonate substrate at room temperature before the absorbance was read at 405 nm. Results were expressed as absorbance at 405 nm/min/mg protein as detailed in our previous report (Ray et al., 2011).

6.15 Gene expression profiling with real-time quantitative PCR (QPCR)

Relative transcript level of the following gene products was quantified using QPCR: $LPA_{1/2/3/4/5/6}$, GPR87, P2Y10, GPR35 and autotaxin (ENPP2). A list of receptor-specific primers used in the QPCR experiments is included in **Table 7.9** RNA isolation was performed using the TRIzol® reagent (Life Technologies, Grand Island, NY). cDNA synthesis was performed using the ThermoScript^{TM} RT-PCR System (Life

Technologies), and quantitative PCR was done with the RT^2 RealTime™ SYBR Green/ROX PCR Master Mix kit (Qiagen Inc., Valencia, CA). Amplification was performed for 40 cycles consisting of 15 sec at 95 °C and 60 sec at 60 °C using an ABI Model 7300 Real-Time PCR machine (Life Technologies Corporation, Carlsbad, CA). To compare relative expression levels each sample was normalized on the basis of its GAPDH gene content, which was used as an internal control. Quantitative values were obtained from the threshold cycle value (C_t) using the Comparative C_t Method ($\Delta\Delta C_t$) as described previously (Guo et al., 2008).

6.16 Radiomitigation of the acute hematopoietic radiation syndrome in mice

Experimental procedures were reviewed and approved by the IACUC of the University of Tennessee Health Science Center. Ten-week old C57BL6 female mice (Charles River Laboratories International Inc., Wilmington, MA) were acclimated for one week in the vivarium. The mice were divided into two groups (15 animals/group) and exposed to 6.6 Gy γ-irradiation from the Cs^{137} γ-source at a dose-rate of ~320 cGy/min. Twenty four hours after the irradiation the animals were treated with either vehicle or 1 mg/kg GRI via intraperitoneal injection. The mice received no additional supportive care in this study. Animal survival was recorded twice daily up to 30 days, which was the endpoint of this experiment.

6.17 Determination of colony growth of irradiated MEF cells in soft agar cultures

Poly-HEMA-coated (Sigma-Aldrich) 6 well plates containing a 0.6% bottom agar layer and a 0.35% top agar layer were used. For these layers, Noble agar (BD, Franklin Lake, NJ) and full growth MEF medium without penicillin-streptomycin was used. Unirradiated and 15 Gy-γ-irradiated LPA_2 MEF cells were mixed with the top agar layer (10^5 cell/ml) 2 h after the irradiation and 1 h after receiving vehicle or 10 μM GRI. The cells were fed every 5 days with fresh growth medium and colonies were counted 2 weeks later under a light microscope at 100 x magnification. A positive score was given to colonies with a diameter ≥ 500 μm. Results were expressed as mean number of colonies per well ± SD. MEFs overexpressing c-Myc and Tbx2 are immortal but not transformed (Taghavi et al., 2008). Such MEF cells, a kind gift from Prof. Martejn van

Lohuizen (Netherlands Cancer Institute, Amsterdam, NL), transduced with the Tbx2 transcriptional repressor with or without the oncogenic RasV12 mutant served as immortalized non-transformed controls and transformed controls, respectively in these experiments.

6.18 Immunoblot analysis

To detect ligand-induced ERK1/2 activation, vector- and LPA_2-transfected MEF cells were serum starved 3 h before exposure to 1 μM LPA, 1 μM OTP, 10 μM GRI977143, or vehicle for 10 min. For ERK1/2 activation and PARP-1 cleavage measurements, cells were harvested in 1X Laemmli sample buffer and separated using 12% Laemmli SDS-polyacrylamide gels. To assess Bax translocation, cell lysates were separated into cytosolic, mitochondrial, and nuclear fractions using the Cell Fractionation Kit-Standard (MitoSciences; Eugene, OR). Cytosolic fractions were then concentrated by precipitation with 75% trichloroacetic acid, and the pellets were dissolved in 50 mM non-neutralized Tris pH 10 buffer and 6X Laemmli buffer. Samples were boiled for 5 min and loaded onto 12% SDS-polyacrylamide gels. Western blotting was carried out as previously described (Valentine et al., 2010). Primary antibodies against pERK1/2, PARP-1, Bax (Cell Signaling Technology; Beverly, MA), actin (Sigma-Aldrich), and anti-rabbit-horseradish peroxidase secondary antibodies (Promega) were used according to the instructions of the manufacturer.

6.19 Detection of ligand-induced macromolecular complex formation with LPA_2

LPA_2 forms a ternary complex with TRIP6 and NHERF2 (E et al., 2009; Lin et al., 2007; Xu et al., 2004). This complex is assembled via multiple protein-protein interactions that include: binding of NHERF2 to the C-terminal PDZ-binding motif of LPA_2, the binding of TRIP6 to the Zinc-finger-like CxxC motif of LPA_2, and binding of NHERF2 to the PDZ-binding motif of TRIP6 (E et al., 2009). To examine ligand-induced macromolecular complex formation, HEK293T cells were transfected with FLAG-LPA_2 and enhanced green fluorescent protein (EGFP)-NHERF2, and the cells were exposed to 10 μM GRI977143 for 10 min as described in detail in our previous publication (E et al., 2009). The complex was pulled down using anti-FLAG M2 monoclonal antibody-conjugated

agarose beads (Sigma-Aldrich) and processed for western blotting using anti-EGFP (gift from Dr. A.P. Naren; UTHSC, TN), anti-FLAG (Sigma-Aldrich), and anti-TRIP6 (Bethyl Laboratories; Montgomery, TX) antibodies.

6.20 Statistical analysis

Data are expressed as mean ± SD or SEM for samples run in triplicates. Each experiment was repeated at least two times. Student's *t*-test was used for comparison between the control and treatment groups. A p value ≤0.05 was considered significant.

7. RESULTS

7.1 Rational discovery of LPA_2 agonists

In a virtual screen using a structure-based pharmacophore of LPA_1 (Perygin, 2010), we serendipitously identified compound NSC12404, which was a weak agonist of LPA_2 (**Table 7.1.1** and **Fig. 7.1.1**). Although this hit was not the intended target of that study, here we returned to this scaffold for the initiation of a virtual homology screen for other nonlipid ligands of LPA_2. With the use of this hit, we undertook a database search in the UC-DCC chemical library. The similarity search included the requirement for a fused tricyclic or bicyclic ring system and the presence of an acid moiety linked with a hydrocarbon chain. The similarity fingerprint metrics included: 1) extended connectivity fingerprint counts over 6 atoms, 2) functional class connectivity fingerprint counts over 4 atoms, and 3) functional class connectivity fingerprint counts over 6 atoms.

Similarity searches were performed separately using each similarity fingerprint to quantitate similarity. Hits meeting the 80% similarity threshold from each search were ranked based on the Tanimoto coefficient measure of similarity to the target molecule NSC12404 and the top 75 unique hits from each fingerprint search were selected for further analysis. The 225 compounds selected for further analysis were clustered based on Tanimoto coefficients calculated using Molecular ACCess System-key fingerprints (MACCS keys) and evaluated using the diversity subset function implemented in MOE. This selected a diverse subset of 27 compounds for biological evaluation by choosing the middle compounds in each cluster. These 27 compounds were tested in Ca^{2+} mobilization assays at a concentration of 10 μM using stable cell lines individually expressing LPA_2 and also in vector-transfected control cells (**Fig. 7.1.1** and **Table 7.1.1**). Hits activating LPA_2 were further tested using cells expressing the other established and putative LPA GPCRs. Experimental testing of the selected compounds identified three new selective LPA_2 agonists: GRI977143, H2L5547924, and H2L5828102 (**Table 7.1.1**). NSC12404, H2L5547924, H2L5828102, and GRI977143 only activated LPA_2 and failed to activate any of the other established and

putative LPA GPCRs when applied up to 10 μM. A 10 μM concentration of these compounds have also been tested for the inhibition of the Ca^{2+} response elicited by the ~EC_{75} concentration of LPA 18:1 at those receptors that the compound failed to activate when applied at 10 μM. We found that at this high concentration NSC12404 and GRI977143 inhibited LPA_3 but none of the other receptors we tested were either activated or inhibited by these two compounds. H2L5547924 activated LPA_2 but partially inhibited LPA_1, LPA_3, LPA_4, GPR87, and P2Y10. H2L5828102 although was a specific agonist of LPA_2 but fully inhibited LPA_3 and partially inhibited LPA_1, GPR87 and P2Y10 (**Table 7.1.1**). Based on its lower EC_{50} concentration to activate the LPA_2 receptor compared to NSC12404 and because it only inhibited the LPA_3 receptor compared to the H2L compounds we selected GRI977143 for further characterization in cell-based assays.

The LPA_2 computational model docked with LPA 18:1 suggests 13 residues that comprise the ligand binding pocket (**Fig. 7.1.2 panels B-D** and **Table 7.1.2**). Computational docking of the four hits listed in **Table 7.1.1** indicates that these LPA_2 ligands interact with some additional residues unique to a specific agonist in addition to the 13 common residues (**Table 7.1.2** and **Fig. 7.1.2 panels B-D**). The model of GRI977143 docked to the LPA_2 structure is shown in **figure 7.1.2**. The docked structure shows that GRI977143 docks in the vicinity of the key residues R3.28, Q3.29, K7.36, and W4.64, which, we have previously shown are required for ligand activation of LPA_2 (Valentine et al., 2008). In addition, the model predicted an interaction with W5.40 that was unique to this ligand.

A structure-based pharmacophore was developed using the docking function of the MOE software (MOE, 2002). Compound NSC12404 and LPA were docked into a homology model of LPA_2 (Sardar et al., 2002; Valentine et al., 2008). In the pharmacophore model, we identified three feature sites based on the interactions between the agonists and the protein. We defined the key residues as those within 4.5 Å of our LPA_2 agonists. The pharmacophore features and the corresponding amino acid residues involved in ligand interactions are shown in **figure 7.1.2 A**. This pharmacophore model has three features: a hydrophobic feature (green), a hydrogen

bond acceptor (blue), and an anionic (red) feature. The four volume spheres in the pharmacophore with radii in the 2.8–4.2 Å range delineate the regions ideal for different types of chemical interactions with the ligand in the binding pocket. The distances between chemical features along with the radii of the four volume spheres are shown in **Fig. 7.1.2 A**.

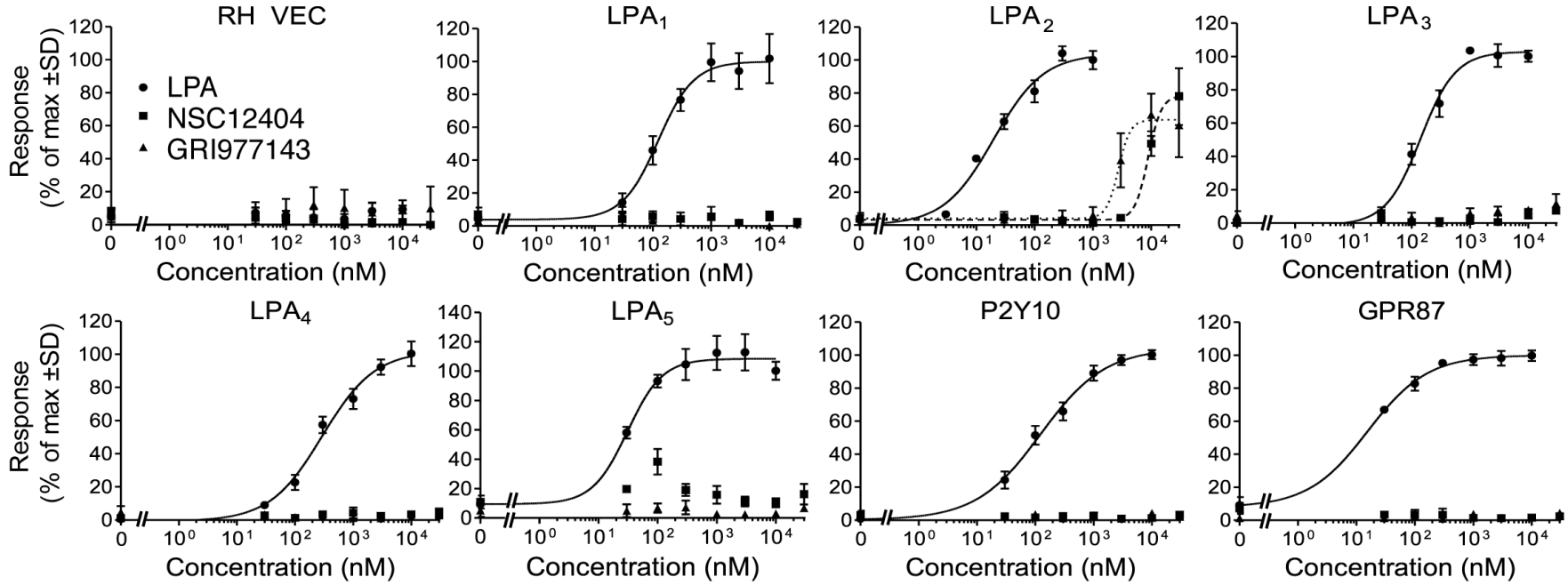
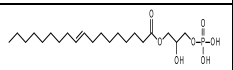
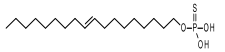
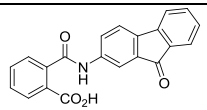
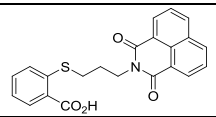
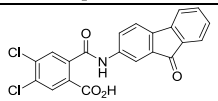
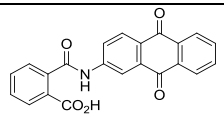


Figure 7.1.1 Receptor specificity of the prototype hit compound NSC12404 and in silico hit GRI977143 indicated by LPA GPCR-activated Ca^{2+}-transients in cell lines expressing the individual LPA GPCR subtypes. The curves shown in this figure are representative of at least two experiments.

Table 7.1.1 LPA receptor activated Ca^{2+} mobilization profiles for hit compounds.

Compound	Structure	Log P	LPA_1		LPA_2		LPA_3		LPA_4		LPA_5		GPR87		P2Y10	
LPA 18:1		6.12	E_{max}100	EC_{50} 0.13	E_{max}100	EC_{50} 0.03	E_{max}100	EC_{50} 0.08	E_{max}100	EC_{50} 0.25	E_{max}100	EC_{50} 0.015	E_{max}100	EC_{50} 0.049	E_{max}100	EC_{50} 0.03
OTP		7.72	E_{max} 50	EC_{50} 0.65	E_{max} 80	EC_{50} 0.47	E_{max} 50	EC_{50} 0.30	E_{max} 70	EC_{50} 2.0	E_{max}100	EC_{50} 0.003	E_{max} 40	EC_{50} 3.0	NE	NE
NSC 12404		3.25	NE	NE	E_{max} 82	EC_{50} 9.5	I_{max} 61	IC_{50} 8.5	NE	NE	NE	NE	NE	NE	NE	NE
GRI 977143		3.88	NE	NE	E_{max} 75	EC_{50} 3.3	I_{max} 100	IC_{50} 6.6	NE	NE	NE	NE	NE	NE	NE	NE
H2L 5547924		4.36	I_{max} 21	IC_{50} 1.90	E_{max} 34	EC_{50} 2.8	I_{max} 66	IC_{50} 3.5	I_{max} 51	IC_{50} 1.3	NE	NE	I_{max} 31	IC_{50} 0.88	I_{max} 34	IC_{50} 1.4
H2L 5828102		2.78	I_{max} 29	IC_{50} 0.20	E_{max} 37	EC_{50} 3.3	I_{max}100	IC_{50} 1.9	NE	NE	NE	NE	I_{max} 21	IC_{50} 3.9	I_{max} 39	IC_{50} 1.1

Log P = Log partition coefficient, NE = no effect up to 10 μM of the ligand, the maximal concentration tested in the present experiments. I_{max}= % inhibition of the ~E_{max75} LPA 18:1 response for a given receptor subtype using 10 μM of the antagonist. EC_{50} and IC_{50} concentrations are given in μM for dose-response curves covering the 30 nM - 10 μM range (see figure 1). For determination of IC_{50} values, dose-response curves were generated using ~ E_{max75} concentration of LPA 18:1 for any given LPA receptor subtype and the ligand was coapplied in concentrations ranging from 30 nM to 10 μM.

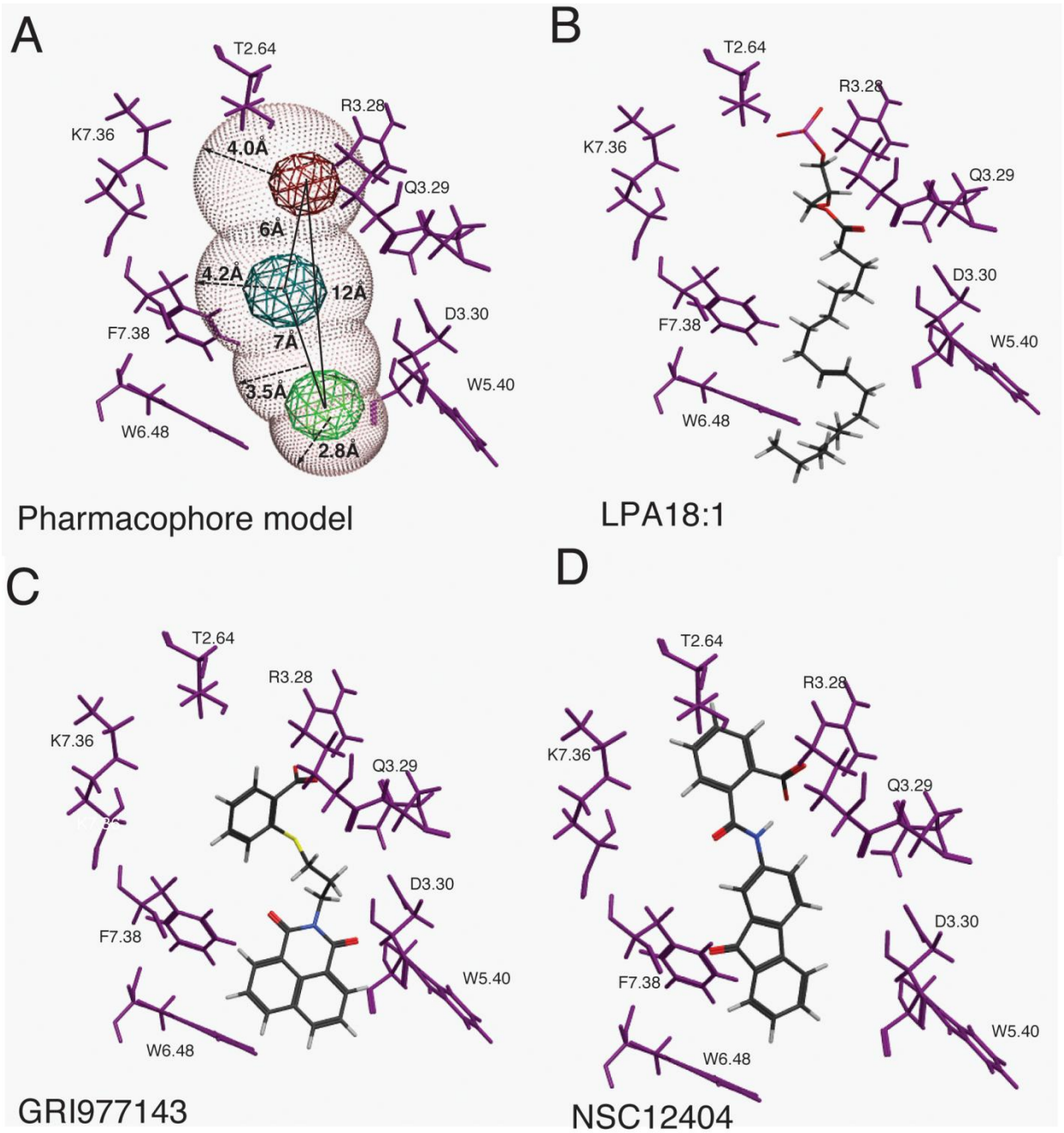


Figure 7.1.2 Pharmacophore development for the LPA_2 GPCR. The three-dimensional pharmacophore generated (panel A) was based on the common structural features of docked LPA (panel B), GRI977143 (panel C), and NSC12404 (panel D). The pharmacophore properties are shown in red (anionic), blue (hydrogen bond acceptor), and green (hydrophobic interaction) in panel 1A. The volume of the binding site is shown in white spheres. The three agonists (ball and stick) used for pharmacophore

development are shown with interactions with key amino acid residues (purple) within 4.5 Å of the previously validated ligand binding pocket.

Table 7.1.2

	EC_{50} (μM)	**Common Residues to all agonists predicted to be within 4.5 Å of the docked ligand**
LPA 18:1	0.014	R3.28, Q3.29, G3.30, L3.32, D3.33, L3.34, W4.64, L5.37, R5.38, W6.48, K7.36, F7.38, L7.39
		Unique residues to a specific ligand predicted to be within 4.5 Å of the docked ligand
OTP	0.09	W5.40, L6.55
NSC 12404	9.5	T2.64
GRI977143	3.3	W5.40
H2L 5547924	2.9	Interacts with all common residues but some interactions are less favorable than with LPA or OTP
H2L 5828102	16.7	L7.32, T2.64, S270

7.2 Effect of GRI977143 on cell growth

LPA can function as a mitogen or an antimitogen, depending on the cell type and the receptors it expresses (Tigyi et al., 1994). We tested GRI977143 for its effect on cell proliferation of vector- (**Fig. 7.2 A**) and LPA_2-transduced MEF cells (**Fig. 7.2 B**). These cells were grown in low 1.5% serum medium and supplemented with either 1 μM LPA, 1 μM OTP, or 10 μM GRI977143 complexed with equimolar BSA. LPA had no significant effect on the proliferation of empty vector-transduced MEF cells. Likewise, GRI977143 did not cause a significant increase in vector cell proliferation except at 72 hours ($p<0.05$). In contrast, OTP significantly ($p<0.001$) increased the growth of empty vector-transduced MEF cells from 24 hours onwards. The effects of LPA, OTP and GRI977143 on the growth of LPA_2-transduced MEF were all significant from 24 hours onwards.

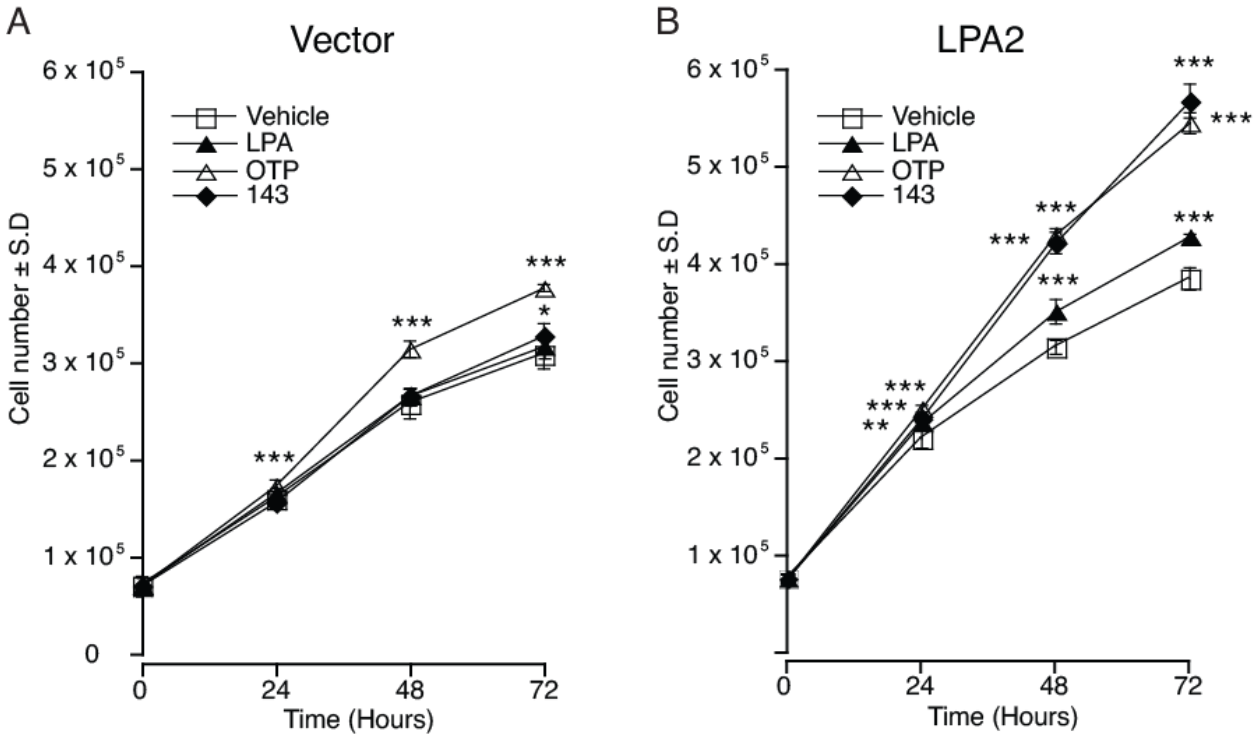


Figure 7.2 Effects of LPA (1 μM), OTP (1 μM) and GRI977143 (10 μM) on fibroblast growth. Panel A shows the growth curves of the vector- and panel B of the LPA_2-transduced MEF cells. Values are means ± S.D and representative of two independent experiments ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

7.3 Effect of GRI977143 on MM1 hepatoma cell invasion

LPA is a potent agent that increases cell motility, tumor cell invasion, and metastasis (Tigyi, 2010). The highly invasive rat hepatoma MM1 cells invade mesothelial cell monolayers in an LPA-dependent manner (Mukai and Akedo, 1999; Mukai et al., 2003; Uchiyama et al., 2007). MM1 cells express autotaxin (ATX), the enzyme that generates LPA, whereas endothelial cells express very low levels of ATX (Gupte et al., 2011). Because GRI977143 selectively activates the LPA_2 receptor subtype, we posed the question of whether activation of this receptor that is abundantly expressed in MM1 cells (Gupte et al., 2011) could stimulate the invasion of HUVEC monolayers. Fifty thousand calcein-AM prelabeled MM1 cells were plated on confluent monolayers of HUVEC in the presence of 1 μM LPA or increasing concentrations of GRI977143 in the range of 1-10 μM and allowed to invade for 20 h. The co-cultures were subsequently rinsed and fixed and the number of cells below the monolayer was counted. Our results showed that whereas 1 μM LPA already caused a significant increase in MM1 cell invasion, a higher 10 μM concentration of GI977143 was required to elicit the same significant increase in invasion (**Fig. 7.3**). Nonetheless, these findings support the hypothesis that LPA_2 promotes the invasion of this cell type in vitro.

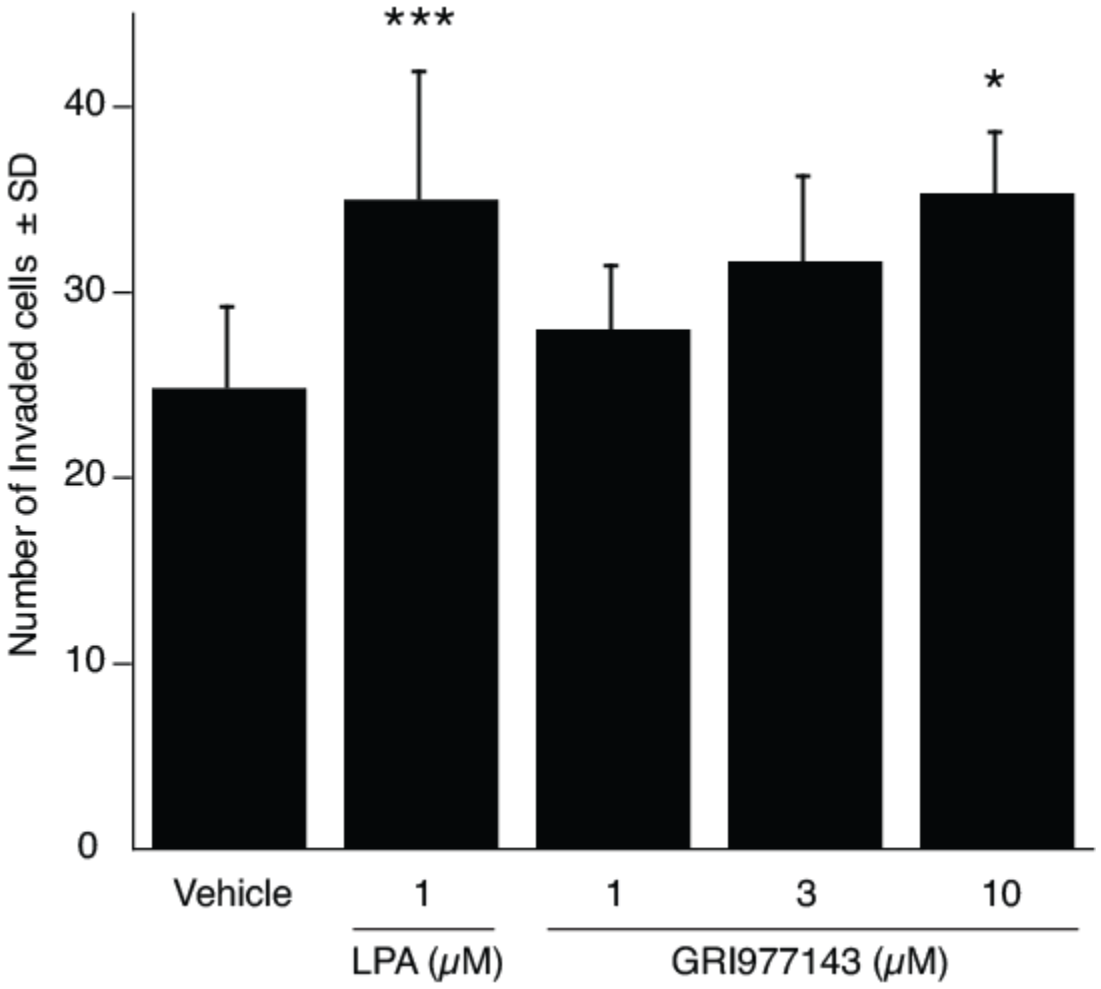


Figure 7.3 Effect of GRI977143 on the invasion of HUVEC monolayers by MM1 hepatocarcinoma cells. Data are the means of 5 non-overlapping fields and representative of two independent experiments ($^{*}p \leq 0.05$, $^{***}p \leq 0.001$).

7.4 Effect of GRI977143 on LPA_2-mediated protection against Adriamycin-induced apoptosis

LPA and its mimics like OTP, have been shown to prevent apoptosis in a variety of cell lines through LPA_2 (E et al., 2009) but also to cause cell death through LPA_1-mediated anoikis (Funke et al., 2012). The DNA-intercalating agent Adriamycin inhibits topoisomerase II resulting in DNA damage (Hortobagyi, 1997) and activation of the intrinsic apoptotic cascade (Fulda and Debatin, 2006). Because LPA_2 receptor-mediated supramolecular complex formation plays an important role in the protection against Adriamycin-induced apoptosis (E et al., 2009) we investigated the antiapoptotic properties of GRI977143 in the Adriamycin-induced apoptosis model by comparing it with LPA and OTP. Activation of caspases 3, 7, 8, 9 and DNA fragmentation was measured in serum-starved vector- and LPA_2-transduced MEF cells pretreated 1 h with 1-10 µM GRI977143, LPA or OTP followed by a 5 h treatment with 1.7 µM Adriamycin.

Apoptosome formation during intrinsic apoptosis results in caspase-9 activation which in turn activates effector caspases 3 and 7 (Riedl and Salvesen, 2007; Taylor et al., 2008). We hypothesized that GRI977143 reduces caspase 3 and 7 activation via upstream inhibition of caspase 9. GRI977143 (10 µM) decreased caspase 9 activation in LPA_2-transduced MEF cells by 46 ± 4%; this decrease was similar in its magnitude to that of 1 µM LPA, whereas 1 µM OTP resulted in a slightly smaller 38 ± 1% decrease (**Fig. 7.4.1 A**). GRI977143 did not affect caspase 9 activation in the vector-transduced cells, whereas LPA and OTP even in a 1 µM concentration reduced caspase 9 activation by 20-24% (**Fig. 7.4.1 A**). These results suggest that the LPA_2 receptor-mediated antiapoptotic effect of GRI977143 involves caspase 9 inhibition, which could attenuate caspase 3 and 7 activation downstream.

To guide our dosing considerations in the apoptosis assays, we also tested the dose-response relationship of our test compounds on Adriamycin-induced caspase 3 and 7 activation in vector- and LPA_2-transduced MEF cells. In the LPA_2-transduced MEF cells GRI977143 elicited a dose-dependent and significant protection above 3 µM ($p < 0.01$, **Fig. 7.4.2**). LPA and OTP dose-dependently protected LPA_2-transduced MEF

cells starting from a concentration as low as 30 nM; however, at the highest 10 μM concentration tested, LPA reduced caspase 3 and 7 activation in the vector-transduced cells by 26 ± 1% (**Fig. 7.4.2 panel A** and **B**). In contrast, when applied at 10 μM, GRI977143 and OTP did not attenuate caspase 3 or caspase 7 in the vector-transduced cells (**Fig. 7.4.2 A**). At 10 μM concentration, GRI977143 decreased caspase 3 and 7 activation on LPA_2-transduced MEF cells by 51 ± 3% and was approximately as potent as 3 μM LPA or OTP (**Fig. 7.4.1 B** and **Fig. 7.4.2**). These results indicate that GRI977143 effectively protects against Adriamycin-induced caspase 3 and 7 activation via the LPA_2 receptor, although with a lesser potency than LPA or OTP.

The caspase-activated deoxyribonuclease primarily responsible for apoptotic internucleosomal DNA fragmentation is activated upon caspase 3 and 7 cleavage (Georas, 2009). Because GRI977143 decreased Adriamycin-induced caspase 3 and 7 activation on LPA_2-transduced MEF cells, we next investigated its effect on DNA fragmentation. In LPA_2-transduced MEF cells GRI977143 reduced DNA fragmentation by 41 ± 2% ($p < 0.001$) compared to a modest 7 ± 1% protection in the vector-transduced cells ($p < 0.05$). LPA (3 μM) and OTP (3 μM) also protected LPA_2-transduced MEF cells by decreasing DNA fragmentation by 35 ± 4% and 32 ± 1%, respectively (**Fig. 7.4.1 C**). These findings suggest that activation of LPA_2 receptor signaling by GRI977143 protects against Adriamycin-induced apoptosis by reducing DNA fragmentation downstream of caspase 3, 7, and 9.

Caspase-8 is considered an initiator caspase of the death receptor-mediated apoptotic pathway (Kaufmann et al., 2012; Ola et al., 2011), however, it can also become activated during mitochondrial apoptosis (Ferrari et al., 1998; Lin et al., 2004; Vit et al., 2001). We also examined whether GRI977143 treatment attenuates caspase 8 activation upstream of caspase 3 and 7 in the Adriamycin apoptosis model. Administration of 10 μM GRI977143 resulted in a 41 ± 5% decrease in caspase 8 activation in LPA_2- transduced MEF cells. Treatments with 1 μM LPA or 1 μM OTP decreased caspase 8 activation by 36 ± 1% and 33 ± 2%, respectively. A similar but lesser effect of LPA and OTP was noted in the vector-transduced cells, amounting to 12 ± 2% and 15 ± 5% decreases, respectively (**Fig. 7.4.1 D**). These findings together

establish that selective activation of LPA_2 receptor signaling by GRI977143 protects against Adriamycin-induced apoptosis by inhibiting caspase 3, 7, 8 and 9, and reducing DNA fragmentation.

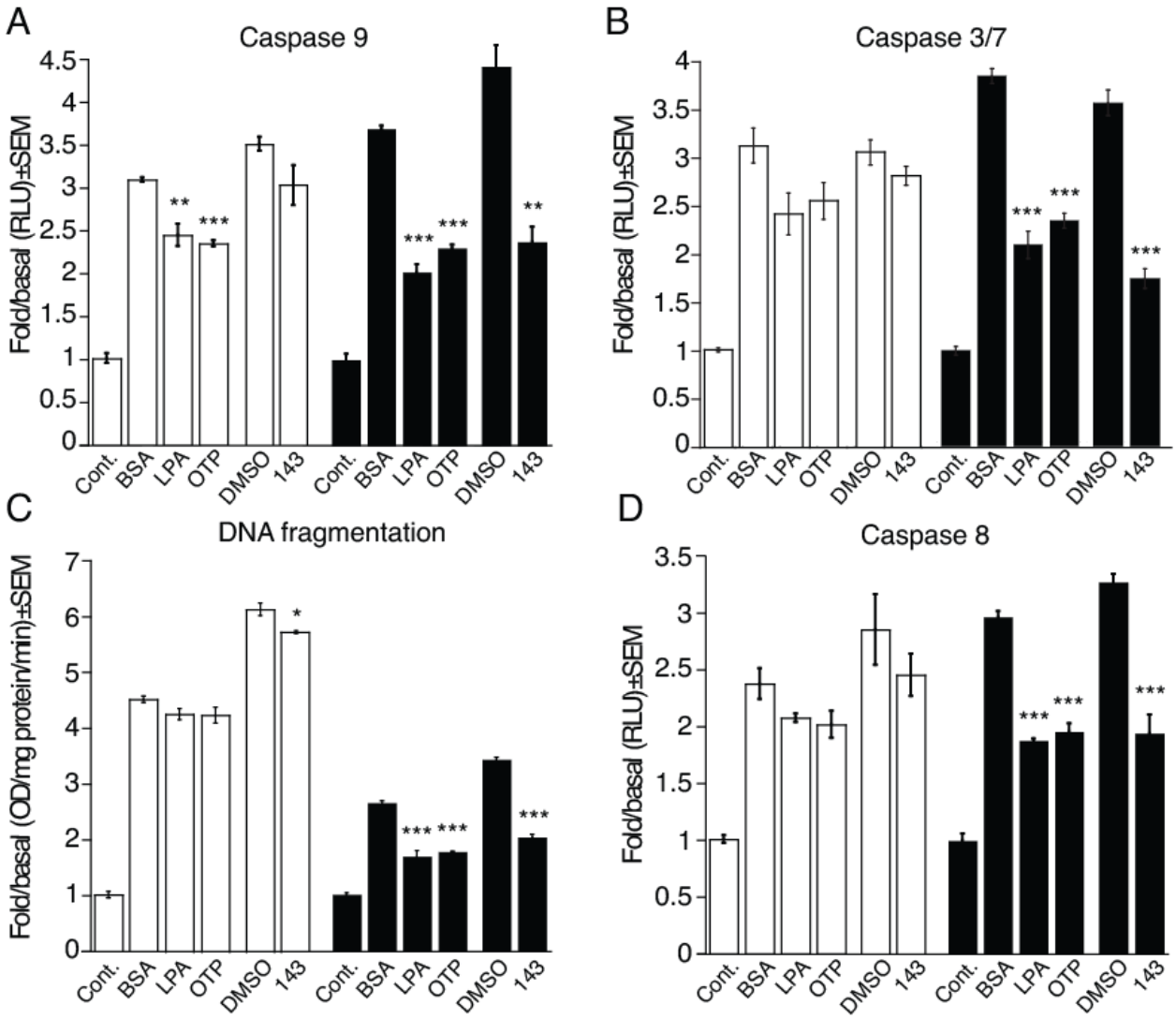


Figure 7.4.1 Effects of LPA and OTP (panels A, D: 1 μM; panels B, C: 3 μM), and GRI977143 (10 μM) on Adriamycin-induced apoptotic signaling in vector- (open bars) or LPA_2-transduced (filled bars) MEF cells. Bars represent the mean of triplicate wells and the data are representative of three independent experiments. ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

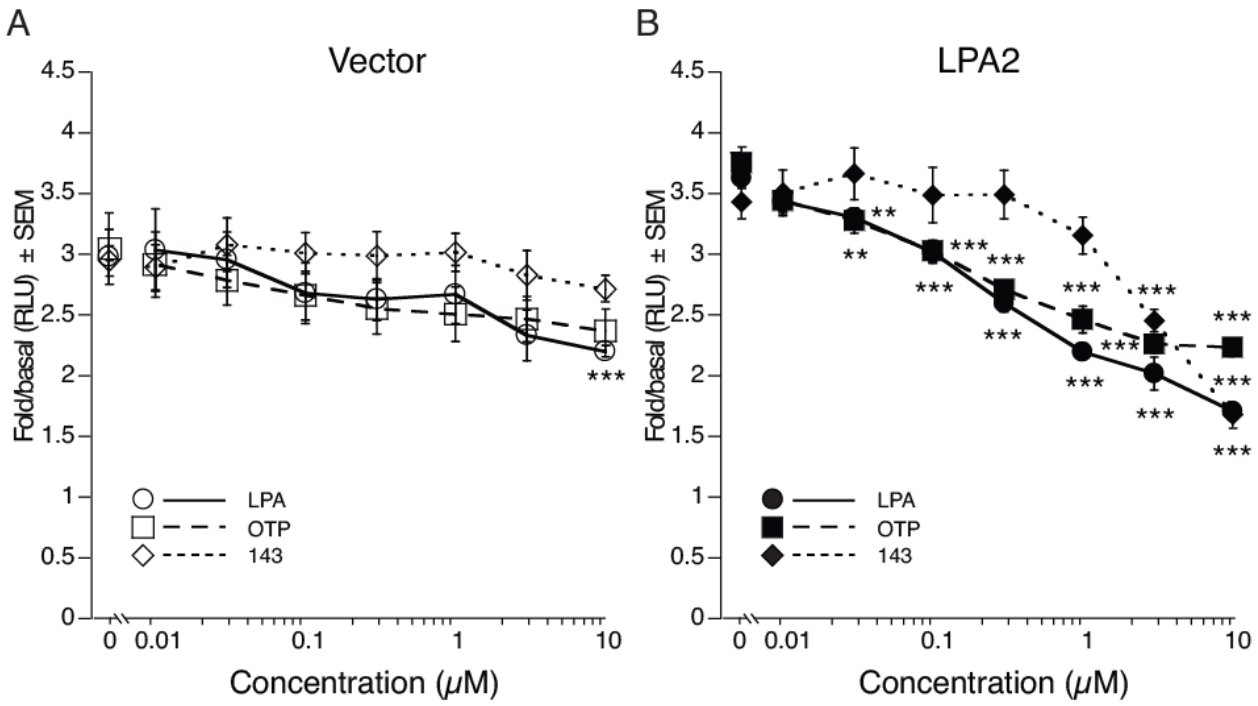


Figure 7.4.2 Effects of LPA (circles), OTP (squares), and GRI977143 (diamonds) on Adriamycin-induced caspase 3 and 7 activation in vector- (panel A) or LPA_2-transduced (panel B) MEF cells. Data points represent the mean of triplicate wells and the data are representative of three independent experiments. ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

7.5 Effect of GRI977143 on LPA_2-mediated protection against serum withdrawal-induced apoptosis

Long-term serum withdrawal and consequential trophic factor deficiency has been shown to induce intrinsic apoptosis on a variety of different cell types (al-Rubeai and Singh, 1998; Laken and Leonard, 2001). We examined whether GRI977143 could provide the necessary trophic support to serum starved MEF cells expressing or lacking LPA_2 receptors. LPA_2 or control MEFs were transferred from 10% FBS-containing medium to DMEM without serum but supplemented with either 3 μM or 10 μM LPA, 3 μM OTP or 10 μM GRI977143. Activation of caspases 3, 7, 8, 9 and DNA fragmentation was determined 24 h later.

GRI977143 (10 μM) significantly reduced activation of caspases 8 and 9 in LPA_2-transduced MEF cells by 59% and 49%, respectively. This protection was similar to the effect of 3 μM OTP but surpassed the effect of 10 μM LPA. While GRI977143 and LPA did not affect serum withdrawal-induced caspase 8 and 9 activation in the vector-transduced cells, we detected a significant 16% decrease in caspase-9 activation in the presence of OTP (**Fig.7.5 panel A** and **D**). GRI977143 also reduced serum withdrawal-induced activation of caspases 3 and 7 and DNA fragmentation in the LPA_2-transduced MEF cells by ~70%. Administration of 3 μM OTP resulted in a similar ~70% decrease in both parameters, while the effect of LPA was weaker, leading to a ~40% decrease. Unlike GRI977143, LPA and OTP reduced DNA fragmentation in the vector-transduced cells too, by 12% and 33% respectively (**Fig.7.5 panel B** and **C**). These results mirrored our findings in the Adriamycin-induced apoptosis paradigm, extending the role of LPA_2 activation to the prevention of serum withdrawal-induced apoptosis.

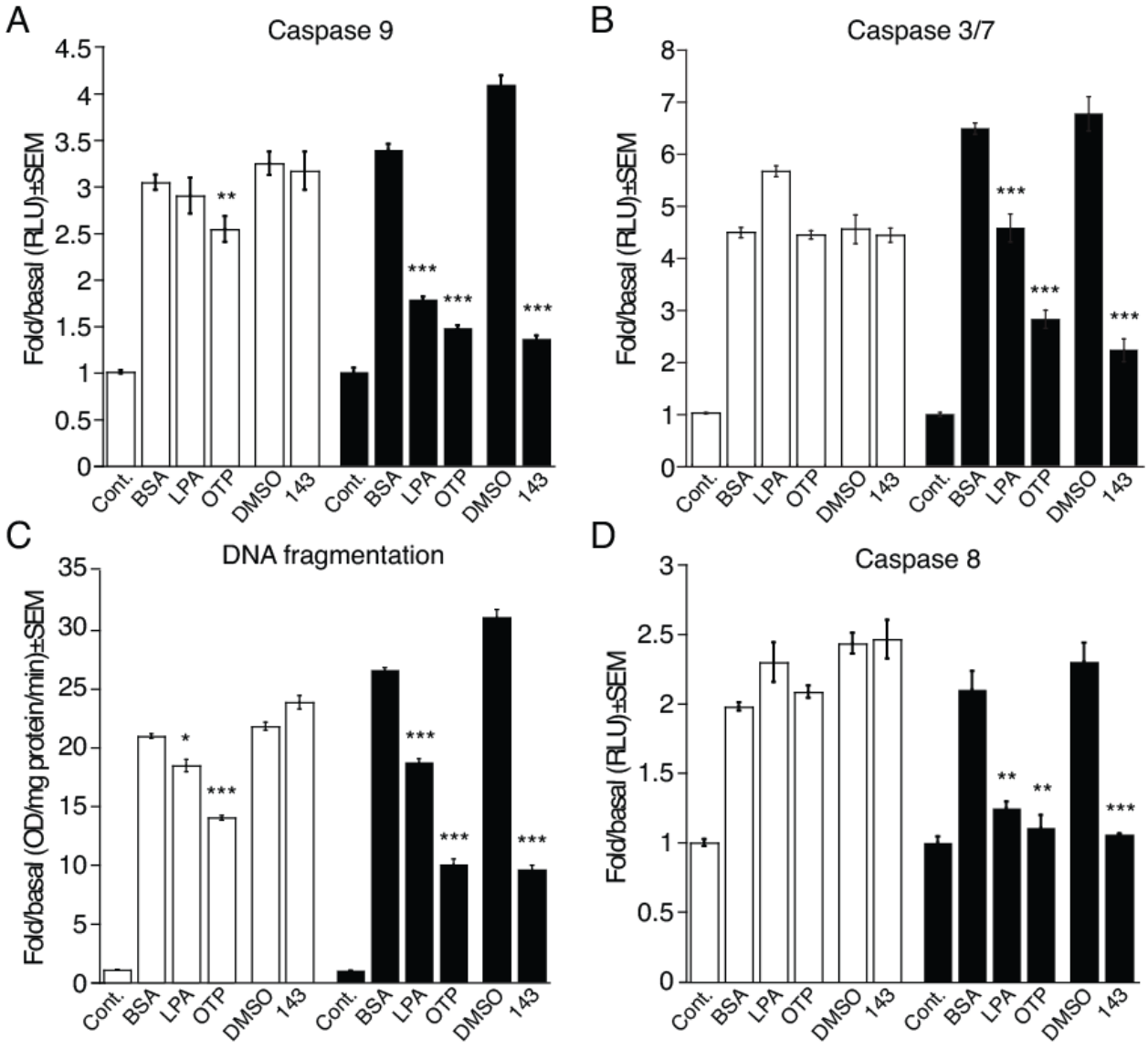


Figure 7.5 Effects of LPA (panels A, B: 3 μM; panels C, D: 10 μM), OTP (3 μM) and GRI977143 (10 μM) on serum withdrawal-induced apoptotic signaling in vector- (open bars) or LPA_2-transduced (filled bars) MEF cells. Bars represent the mean of triplicate wells and the data are representative of three independent experiments. ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

7.6 Effect of GRI977143 on TNF-α-induced apoptosis in IEC-6 intestinal epithelial cells

We showed earlier that LPA and OTP protects and rescues non-transformed IEC-6 crypt-like intestinal epithelial cells from TNF-α-induced apoptosis (Deng et al., 2002; Deng et al., 2004; Deng et al., 2007; Deng et al., 2003). IEC-6 cells endogenously express $LPA_{1/2/3/4}$ GPCRs, GPR87 and P2Y5 (Valentine et al., 2010). Based on our data concerning the agonist action of GRI977143 at the LPA_2 receptor we examined whether it can prevent the progression of TNF-α-induced extrinsic apoptosis in this model cell line. Confluent cultures of IEC-6 cells were exposed to 10 ng/ml TNF-α plus 20 μg/ml CHX in the absence of serum for 3 h with or without 1 μM LPA, 10 μM OTP or 10 μM GRI977143 and DNA fragmentation was measured. Treatment with TNF-α/CHX increased DNA fragmentation over 20-fold; the fragmentation was completely blocked by 10 μM OTP and significantly reduced by 1 μM LPA or 10 μM GRI977143 treatment. Neither LPAR agonist caused any detectable change in DNA fragmentation when added to the cultures in the absence of TNF-α/CHX (**Fig. 7.6**). These results extend our previous observations obtained in the Adriamycin-induced intrinsic apoptosis model to the TNF-α-induced model mediated via the extrinsic apoptosis pathway.

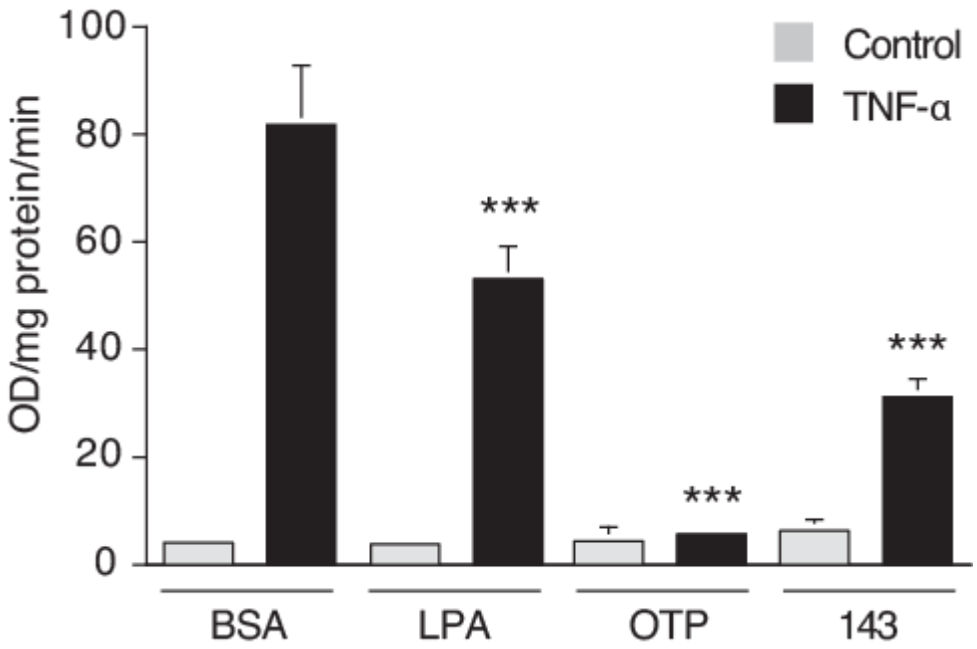


Figure 7.6 Effects of LPA (1 μM), OTP (10 μM), and GRI977143 (10 μM) on DNA-fragmentation elicited via extrinsic apoptosis induced by TNF-α and CHX treatment in IEC-6 cells. Bars represent the mean of triplicate wells, and the data are representative of two experiments. ($^{***}p \leq 0.01$).

7.7 Effect of GRI977143 on LPA_2-mediated protection against γ-irradiation induced apoptosis

Previously, our group showed, that LPA protects the intestinal epithelium against γ-irradiation-induced apoptosis both *in vitro and in vivo* (Deng et al., 2002). Rational design of OTP, a metabolically stabilized LPA mimic revealed the role of LPA_2 receptor in the protection against γ-irradiation induced apoptosis by downstream activation of the MEK-ERK1/2 and PI3K-Akt pro-survival pathways (Deng et al., 2007). Thus we extended our studies to examine the antiapoptotic properties of GRI977143 in a γ-irradiation-induced apoptosis model *in vitro*. Serum-starved vector-transduced and LPA_2 MEF cells irradiated with 15 Gy γ-irradiation were treated with 10 μM GRI, 3 μM LPA or 3 μM OTP one h after the irradiation. We chose the one h delay for the drug treatment to model radiomitigation as opposed to radioprotection. In this latter case the drug is applied as pretreatment to the cells. Activation of caspases 3, 7, 8 and 9 and DNA fragmentation was determined 5 h after drug treatment.

Γ-irradiation has been shown to induce apoptosis by activating the intrinsic or mitochondrial apoptotic pathway (Price et al., 2005). Thus we first examined the effect of GRI977143 on γ-irradiation induced activation of caspase 9, the initiator caspase of the mitochondrial apoptotic pathway. GRI977143 selectively protected LPA_2-transduced MEF cells and reduced caspase 9 activation by 37% ± 1% but was without any effect in the vector-transduced MEFs. In contrast, LPA and OTP mitigated the effects of radiation-induced caspase 9 activation in both the LPA_2- and the vector-transduced cells. LPA decreased caspase 9 activation in LPA_2-transduced MEF cells by 53% ± 2% compared to 22% ± 2% in vector-transduced cells. Administration of OTP resulted in a 40% ± 2% decrease in caspase 9 activation in LPA_2-transduced MEFs and a 19% ± 2% decrease in vector-transduced cells (**Fig. 7.7 A**).

As shown in **figure 7.7 panel B**, all three compounds mitigated activation of executor caspases 3 and 7 in the LPA_2-transduced MEF cells; GRI977143 by 40% ± 2%, while LPA and OTP by ~50%. Effect of GRI977143 was selective to the LPA_2-transduced

MEF cells. In contrast, administration of LPA resulted in 20% ± 2%, while that of OTP in 14%± 1% decrease in caspase 3 and 7 activation in the vector-transduced cells.

GRI977143 also reduced γ-irradiation-induced DNA fragmentation by 25% ± 2% selectively in LPA_2-transduced MEF cells. LPA decreased DNA fragmentation in LPA_2-transduced MEF cells by 37% ± 1%, whereas OTP decreased DNA fragmentation by 41% ± 1%. Both LPA and OTP had protective action in the vector-transduced cells reducing DNA fragmentation by 11% ± 2% and 22% ± 1%, respectively (**Fig. 7.7 C**). These data indicate that the LPA_2 receptor-mediated antiapoptotic effect of GRI in the γ-irradiation-induced apoptosis model involves inhibition of caspases 3, 7, 8, 9 and DNA fragmentation.

We also tested whether GRI977143-mediated inhibition of caspase 8 could play a role in the radiomitigation against γ-irradiation-induced apoptosis. In LPA_2-transduced MEF cells irradiation-induced caspase 8 activation was mitigated by 33% ± 5% with GRI977143, by 34% ± 1% with OTP, and by 45% ± 4% in the presence of LPA. GRI977143 did not alter caspase 8 activation in the vector-transduced cells. However, administration of LPA and OTP resulted in 23% ± 4% and 18% ± 4% mitigation of caspase 8 in the vector-transduced MEF cells, respectively (**Fig. 7.7 D**).

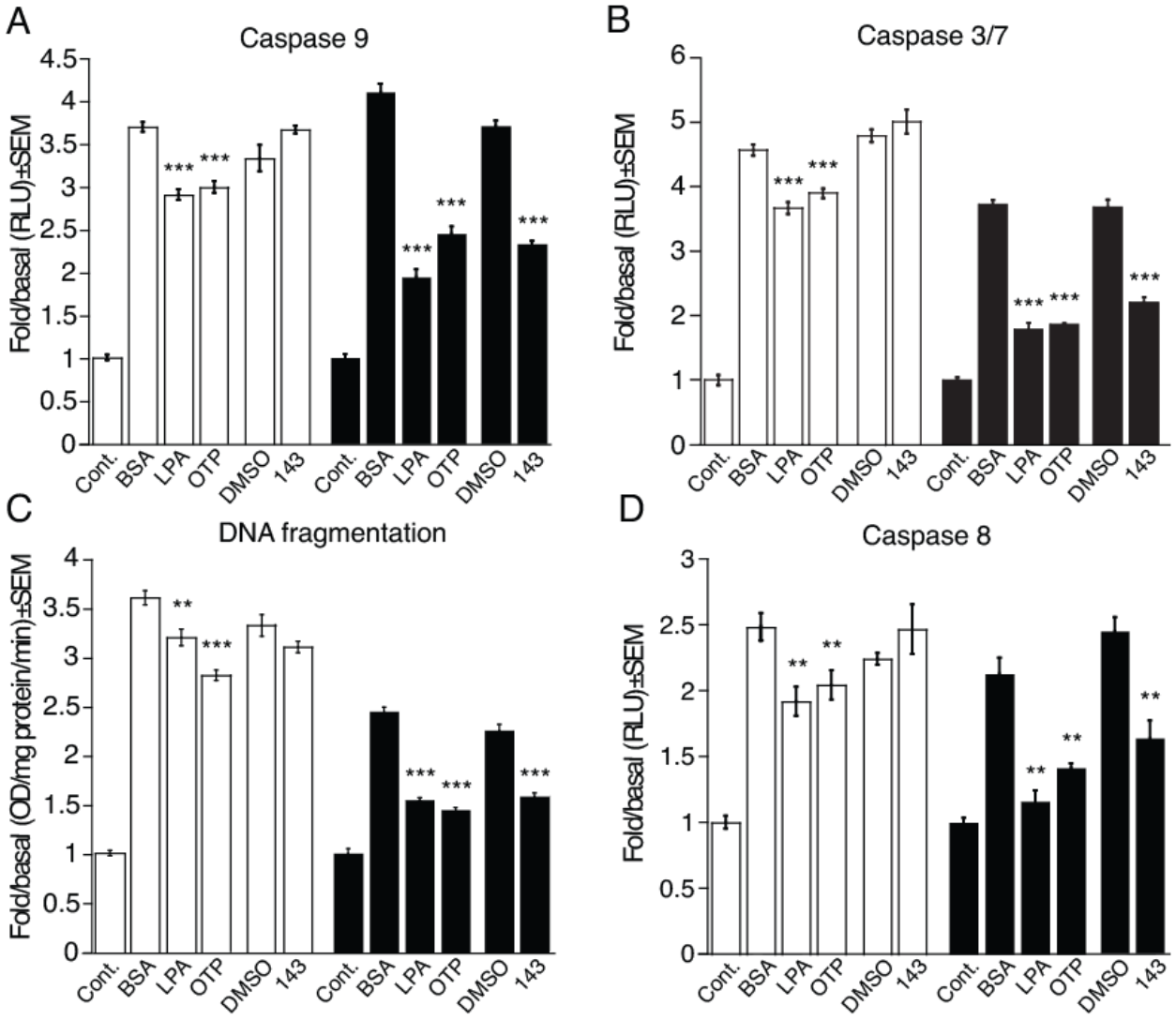


Figure 7.7 Effects of LPA (panels A, B: 3 μM; panels C, D: 10 μM), OTP (3 μM) and GRI977143 (10 μM) on γ-irradiation-induced apoptotic signaling in vector- (open bars) or LPA_2-transduced (filled bars) MEF cells. Bars represent the mean of triplicate wells and the data are representative of three independent experiments. ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

7.8 Effect of GRI977143 on Bax translocation and PARP-1 cleavage induced by Adriamycin, serum withdrawal or γ-irradiation

Bax is a proapoptotic member of the Bcl-2 protein family known to translocate to the mitochondrion and initiate the release of proapoptotic factors in response to stimuli of the intrinsic apoptotic cascade (Ghibelli and Diederich, 2010; Walensky and Gavathiotis, 2011). Because GRI977143 reduced activation of caspases 3, 7, 8, and 9, we tested the effect of 10 μM GRI977143 on Bax translocation to the mitochondria induced by Adriamycin, serum withdrawal or γ-irradiation. Serum-starved vector-transduced and LPA_2 MEF cells received 10 μM GRI977143 one hour prior being exposed to either 1.7 μM Adriamycin or prolonged serum withdrawal, or 1 h after being irradiated with 15 Gy γ-irradiation. Bax translocation was measured 5 h after exposure to Adriamycin or γ-irradition, or after 24 h serum withdrawal. As shown in **figure 7.8 panel A** and **C**, 10 μM GRI977143 treatment maintained a high level of Bax in the cytoplasm of LPA_2-transduced MEF cells after Adriamycin treatment or γ-irradiation, consequently reducing its translocation to the mitochondria. GRI977143 failed to reduce Bax translocation in the vector-transduced MEFs. In the serum withdrawal model of apoptosis we did not detect any change in the cytosolic Bax level (**Fig. 7.8 B**). These observations suggest that GRI977143 inhibits the translocation of Bax during the early stages of Adriamycin- and γ-irradiation-induced apoptosis. The reduction in mitochondrion-associated Bax is consistent with the reduced activation of caspase-9 and -3 we detected in GRI977143-treated LPA_2 expressing MEF cells.

During late stage apoptosis active caspase-3 and -7 cleave PARP-1 thus preventing PARP-1-mediated DNA repair (Bouchard et al., 2003; Woodhouse and Dianov, 2008). Because GRI977143 reduced caspase-3 and -7 activation induced by Adriamycin, serum withdrawal or γ-irradiation in LPA_2 MEF cells, we next examined the effect of GRI977143 on PARP-1 cleavage. Serum-starved vector-transduced and LPA_2 MEF cells received 10 μM GRI977143 one hour prior being exposed to either 1.7 μM Adriamycin or prolonged serum withdrawal, or 1 h after being irradiated with 15 Gy γ-irradiation. PARP-1 cleavage was determined by western blotting 5 h after exposure to

Adriamycin or γ-irradiation, or after 24 h serum withdrawal. GRI977143 treatment (10 μM) also reduced PARP-1 cleavage after all three apoptosis-inducing treatments (**Fig. 7.8 panel D, E** and **F).** This effect was not observed in the vector-transduced cells. These experiments are consistent with the hypothesis that GRI977143 attenuates the activation of the mitochondrial apoptosis pathway through a mechanism that requires the LPA_2 receptor. These experiments are consistent with the hypothesis that GRI977143 attenuates the activation of the mitochondrial apoptosis pathway through a mechanism, which requires the LPA_2 receptor.

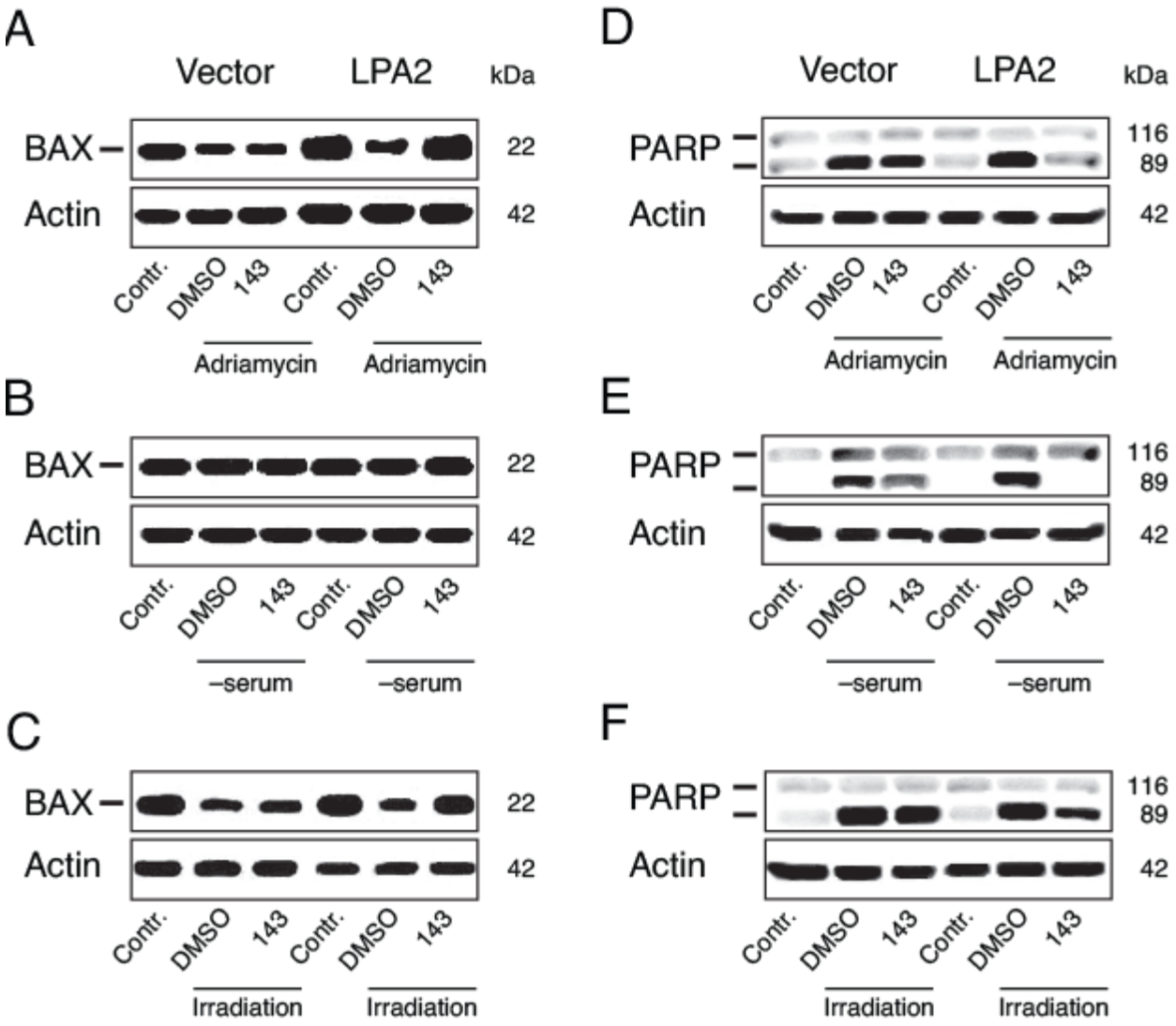


Figure 7.8 Effects of GRI977143 on cytoplasmic Bax levels and PARP-1 cleavage in vector- or LPA_2-transduced MEF cells following Adriamycin-, serum withdrawal-, or γ-irradiation-induced apoptosis. The western blots shown are representative of three experiments.

7.9 Antiapoptotic effect of LPA and OTP is mediated mainly by the LPA_2 receptor

By using GRI977143, an LPA_2 selective agonist, and MEF cells derived from $LPA_{1\&2}$ DKO mice and reconstituted with LPA_2 receptor or empty vector, we demonstrated the unique role of LPA_2 receptor in the protection against different forms of apoptosis. However, the small but significant protection detected in the vector-transduced cells in the presence of LPA and its non receptor selective mimic OTP suggested the existence of additional LPA receptors on the MEF cells. To test this hypothesis we performed quantitative real-time PCR using primers specific to $LPA_{1/2/3/4/5/6}$ receptors, GPR87, P2Y10, GPR35 and autotaxin. As shown in **figure 7.9**, both vector- and LPA_2-transduced MEF cells express appreciable amounts of $LPA_{4/5/6}$ and P2Y10, which can explain the antiapoptotic effect of LPA and OTP detected in the vector-transduced MEF cells. However the antiapoptotic efficacy mediated by one or more of these LPA receptors is much less than the protection mediated by the LPA_2 receptor.

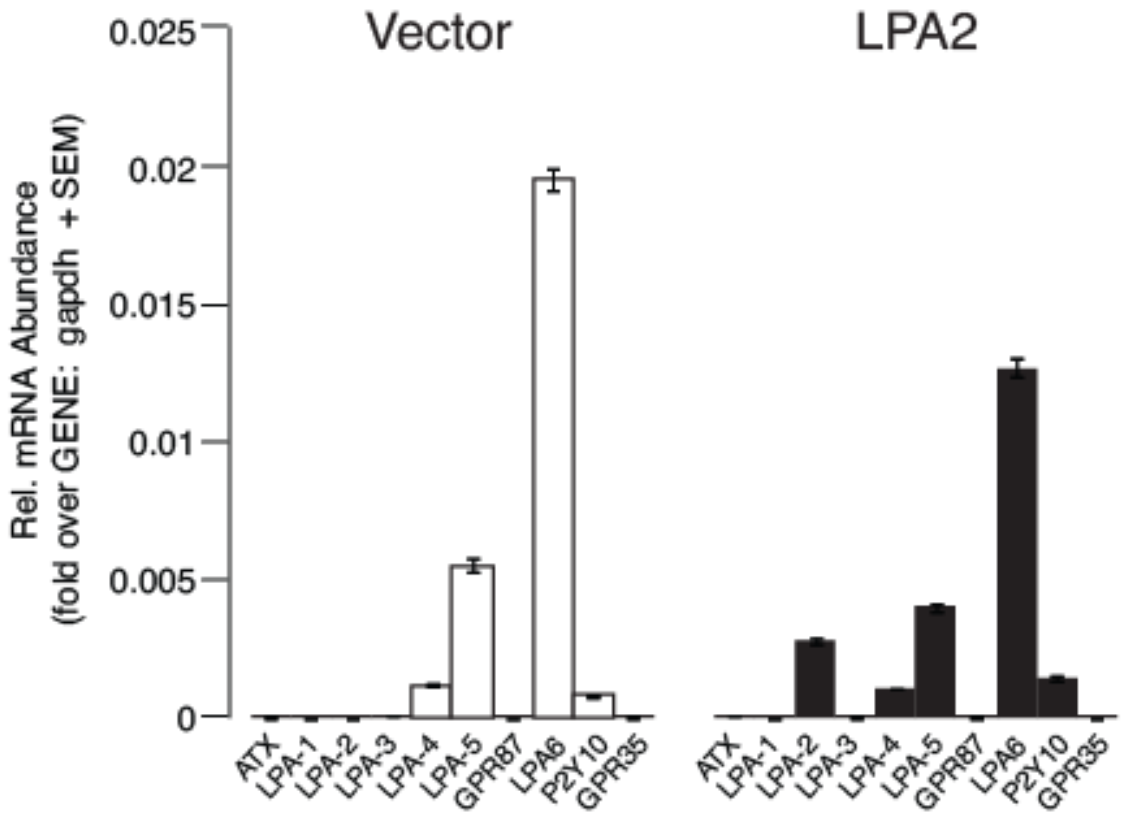


Figure 7.9 Expression profiling of LPA receptor transcripts and autotaxin (ATX) by quantitative RT-PCR in MEF cells transduced with empty vector (open bars) or the human LPA_2 receptor (filled bars). LPA GPCR expression was normalized to GAPDH gene content of the cells.

Table 7.9 List of primers used for expression profiling of LPA receptor transcripts and autotaxin (ATX) by quantitative RT-PCR in MEF cells transduced with empty vector or the human LPA_2 receptor.

Gene	Forward primer	Reverse primer
GAPDH	CTG CAC CAC CAA CTG CTT AG	GGG CCA TCC ACA GTC TTC T
EDG2	GTC TTC TGG GCC ATT TTC AA	TGA TAG TCC TCT GGC GAA CA
EDG4	ACA CTT CTG GCA CTG CCT CT	AGG CTG AGT GTG GTC TCT CG
EDG7	GAA TTG CCT CTG CAA CAT CTC	ATG AAG AAG GCC AGG AGG TT
GPR23	TCT GGA TCC TAG TCC TCA GTG G	CCA GAC ACG TTT GGA GAA GC
GPR92	CGC CAT CTT CCA GAT GAA C	TAG CGC TCC ACG TTG ATG
GPR87	ACA AAT CCA GCA GGC AAT TC	TAC GGG AGG AAG CAG GTA AA
P2Y5	TCT GGC AAT TGT CTA CCC ATT	TCA AAG CAG GCT TCT GAG G
P2Y10	TCA ACA TGT ATG CCA GCA TTT	GAA ATG GCA AAC AGG CAG TC
GPR35	ATT GCT GTG GAC CGC TAT GT	CCA GGG AGG TGA CCA CTA TC

7.10 Protective effect of GRI977143 against radiation-induced bystander apoptosis

It has been shown that irradiated cells either via soluble factors or by communication through gap junctions can induce changes in the neighboring intact cells, known as the "bystander radiation effect" or distant effects of radiation exposure, which could in turn lead to the apoptosis of these cells (Baskar et al., 2010; Rzeszowska-Wolny et al., 2009). Because GRI977143 protected γ-irradiated LPA_2 MEF cells against direct radiation-induced apoptosis, we extended our experiments to a model of γ-irradiation-induced bystander apoptosis. In this model (Kim et al., 2008) we used CM from irradiated U937 cells to elicit apoptosis in unirradiated IEC-6 cells that endogenously express multiple LPA GPCRs including LPA_2 (Deng et al., 2007).

First, we determined the radiosensitivity of U937 cells by measuring γ-radiation-induced apoptosis through caspase 3 and 7 activation 24 h after irradiation with doses in the 7-55 Gy range (**Fig. 7.10 A**). Based on the radiation-dose response of U937 cells we chose the 35 Gy dose that caused a near maximal level of caspase 3 and 7 activation. Second, we tested the effect of CM collected from U937 cells irradiated with 7-35 Gy in IEC-6 cells by measuring caspase 3 and 7 activation 24 h after the exposure to CM (**Fig. 7.10 B**). CM was highly effective in eliciting apoptosis in nonirradiated IEC-6 cell cultures indicated by the activation of the main executional caspases 3 and 7. Third, we determined the effect of GRI977143 on the bystander apoptosis using CM harvested from 35 Gy-irradiated U937 cells. The CM was supplemented with either 10 μM GRI977143, 10 μM LPA, or vehicle and transferred to nonirradiated IEC-6 cell cultures that were already serum-starved for 24 h. Bystander apoptosis of IEC-6 cells was determined 24 h later by measuring caspase 3 and 7 activation. The CM induced robust caspase 3 and 7 activation in the IEC-6 cells (**Fig. 7.10 C**) GRI977143 decreased caspase 3 and 7 activation in the IEC-6 cells by 24% ± 3%, whereas LPA caused a 42% ± 5% inhibition (**Fig. 7.10 C**). These data highlight that stimulation of LPA_2 receptor by GRI977143 protects not only against direct radiation damage but also against the bystander apoptosis induced by proapoptotic mediators present in the CM.

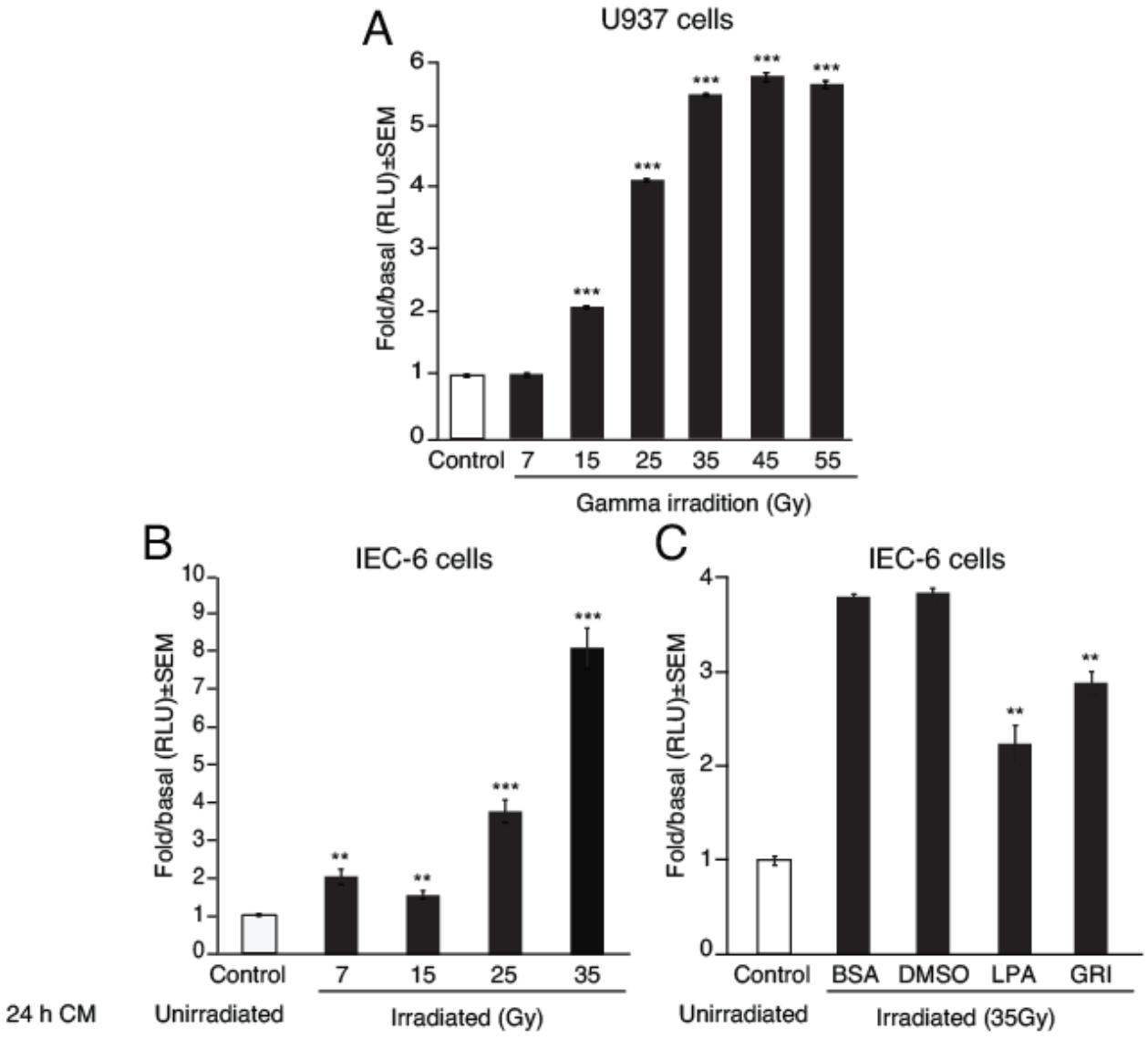


Figure 7.10 Effects of LPA and GRI977143 on the bystander apoptosis elicited by the conditioned medium of irradiated U937 cells. Radiation dose-dependence of caspase 3 and 7 activation in U937 cells (Panel A). Dose-response relationship of bystander apoptosis measured by caspase 3 and 7 activation in IEC-6 cells elicited by CM from γ-irradiated (7-35 Gy) U937 cells (Panel B). Effect of GRI977143 (10 μM) and LPA (10 μM) on the bystander apoptosis of IEC-6 cells induced by 24-h CM from U937 cells irradiated with 35 Gy (Panel C). Bars are representative of three experiments. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

7.11 Effect of GRI977143 on malignant transformation of γ-irradiated MEF cells

Ionizing radiation can lead to malignant transformation and tumorigenesis due to mutagenesis, mitotic stimulation, and alterations in the tissue microenvironment (Barcellos-Hoff et al., 2005). To examine whether the radiomitigative activity of GRI977143 promotes malignant transformation in MEF cells rescued from γ-irradiation induced apoptosis, soft-agar assays were carried out. Unirradiated or 15 Gy γ-irradiated LPA_2 MEF cells were treated with 10 μM GRI or vehicle and plated in soft-agar plates. Cells received fresh growth medium every 5 days and colonies were counted 2 weeks after plating. As shown in **Fig. 7.11** GRI977143 did not promote colony formation in irradiated LPA_2-transduced MEF cells. In contrast, oncogenic V12Ras-transformed MEF cells showed a robust colony growth under these assay conditions.

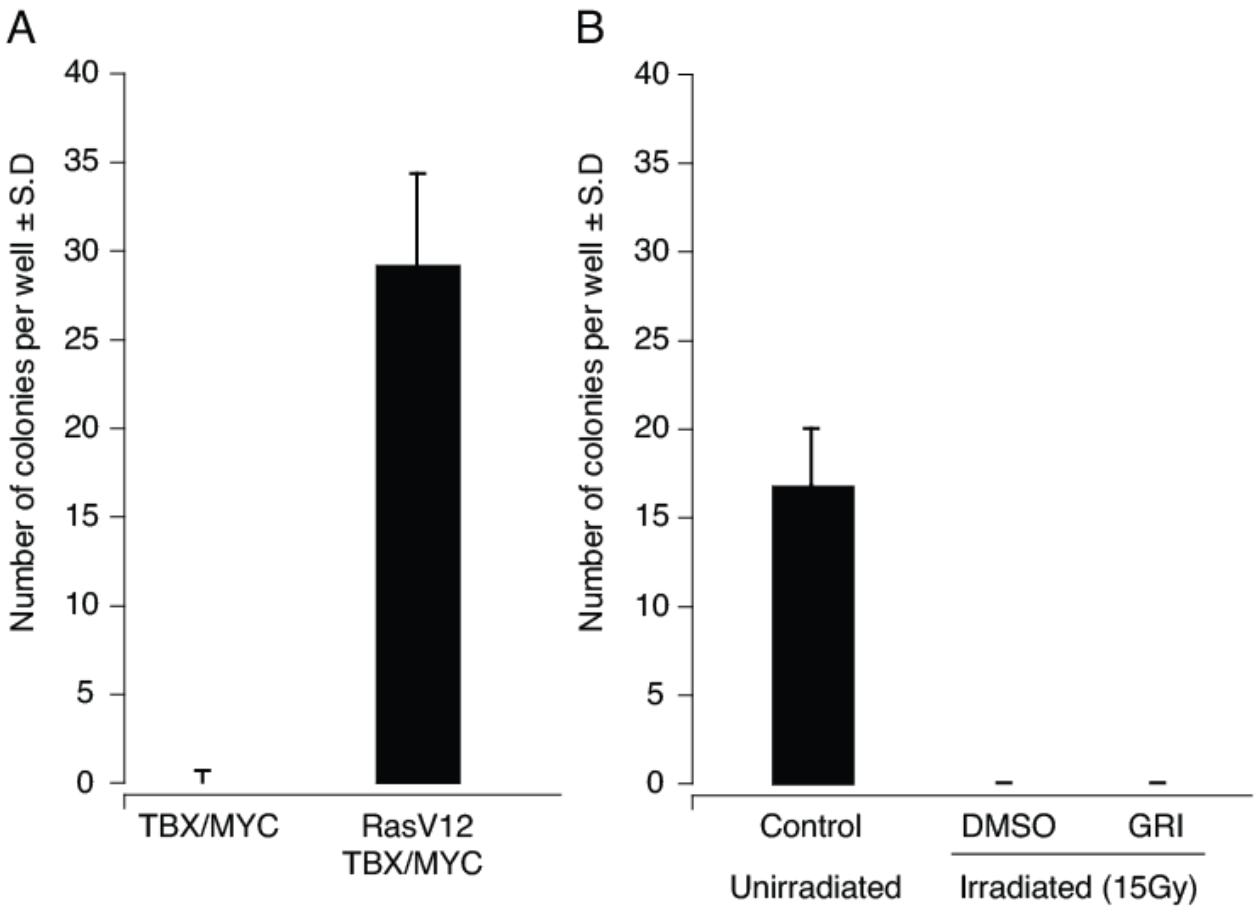


Figure 7.11 Effect of GRI977143 (10 μM) on γ-irradiation-induced colony formation in soft-agar cultures of LPA_2-transduced MEF cells. Colony growth of Tbx2 and Tbx2+V12 Ras transformed MEFs in soft agar (panel A). Colony formation of unirradiated control LPA_2-transduced MEF cells or 15 Gy γ-irradiated LPA_2-transduced MEF cells treated with vehicle (DMSO) or 10 μM GRI977143 in the soft agar (panel B). TBX, T-box transcription factor; MYC, myelocytomatosis viral onkogen; Ras V12, Rat sarcoma V12.

7.12 Effect of GRI977143 on the hematopoietic acute radiation syndrome

Targeting the LPA_2 receptor, OTP has been shown to act as a radiomitigator and rescue irradiated mice against lethal doses of γ-irradiation (Deng et al., 2007). Because the actions of GRI977143 mimicked those of OTP in the γ-irradiation apoptosis model *in vitro*, we extended our study to a murine model of the acute hematopoietic radiation syndrome. Ten-week old C57BL6 mice were exposed to 6.6 Gy total body γ-irradiation (~$LD_{100/20}$) and were treated with vehicle or a single 1 mg/kg intraperitoneal dose of GRI 24 h postirradiation. Survival of the animals was recorded for a 30-day period. In the GRI treated group 50% of the animals survived until the 20th day and 20% of the animals survived until the 30th day post irradiation, which was the endpoint of the study. On the other hand, 50% of the untreated animals died by 11 days and 100% died by 19 days after irradiation (**Fig. 7.12**).

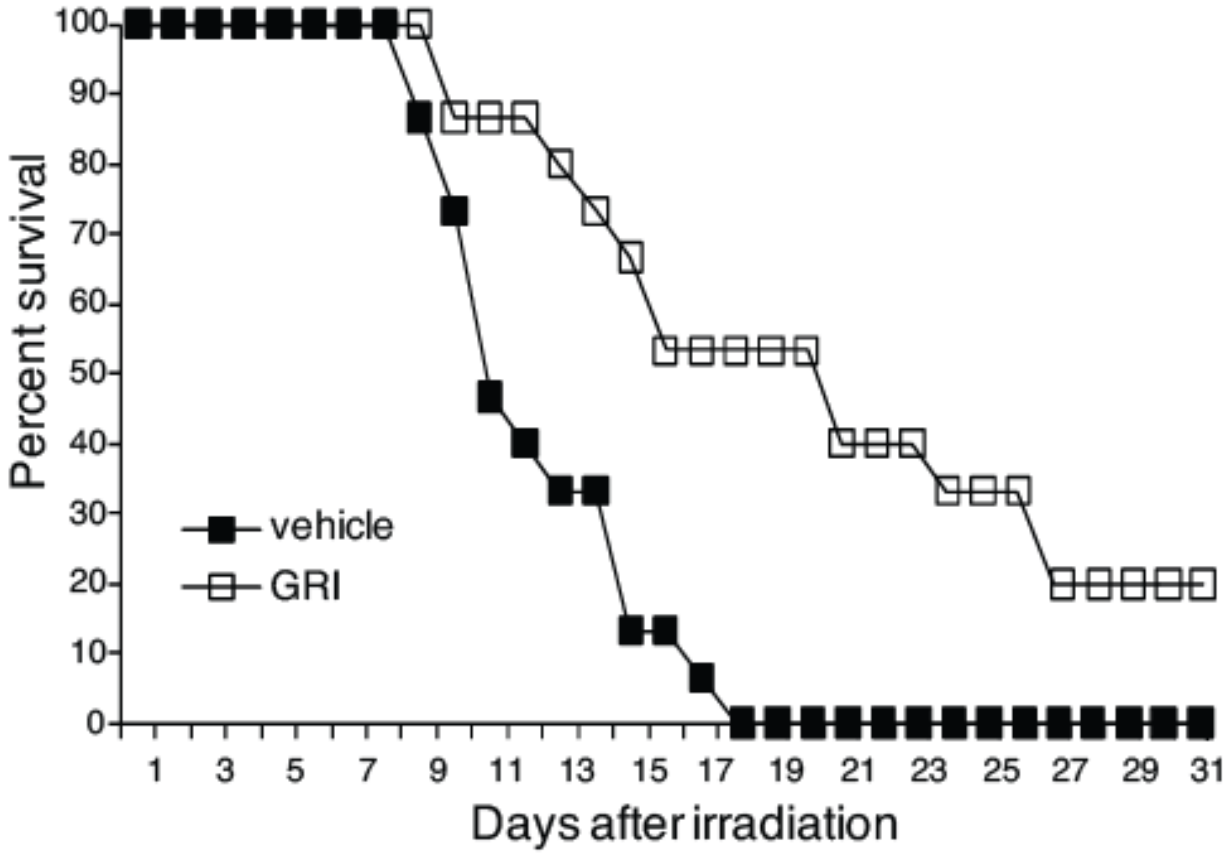


Figure 7.12 Effect of GRI977143 (1 mg/kg administered intraperitoneally at +24 h postirradiation) on mortality from the acute hematopoietic radiation syndrome in C57BL6 mice exposed to ~6.6 Gy γ-radiation at a dose rate of ~320 cGy/min.

7.13 Effect of GRI977143 on ERK1/2 activation

Activation of the extracellular signal regulated kinases 1/2 have been shown to play a required step in LPA_2 receptor-mediated antiapoptotic signaling (Deng et al., 2002; Deng et al., 2003; E et al., 2009). We investigated whether administration of GRI977143 activates $ERK_{1/2}$ kinase on LPA_2 MEF cells and vector-transduced control MEFs. Serum-starved vector-transduced and LPA_2 MEF cells were treated with 10 μM GRI977143 for 10 min and western blot analysis was done to assess $ERK_{1/2}$ activation. One μM LPA and 1 μM OTP were used as positive controls. Treatment with 10 μM GRI977143 for 10 min increased ERK1/2 activation 9.6-fold in LPA_2-transduced MEF cells but did not alter the basal activity of these kinases in the vector-transduced cells (**Fig. 7.13 panel A and B**). This result supports the hypothesis that the prosurvival effect of GRI977143 detected in different intrinsic and extrinsic apoptosis models is mediated by the LPA_2 receptor and involves ERK1/2 activation.

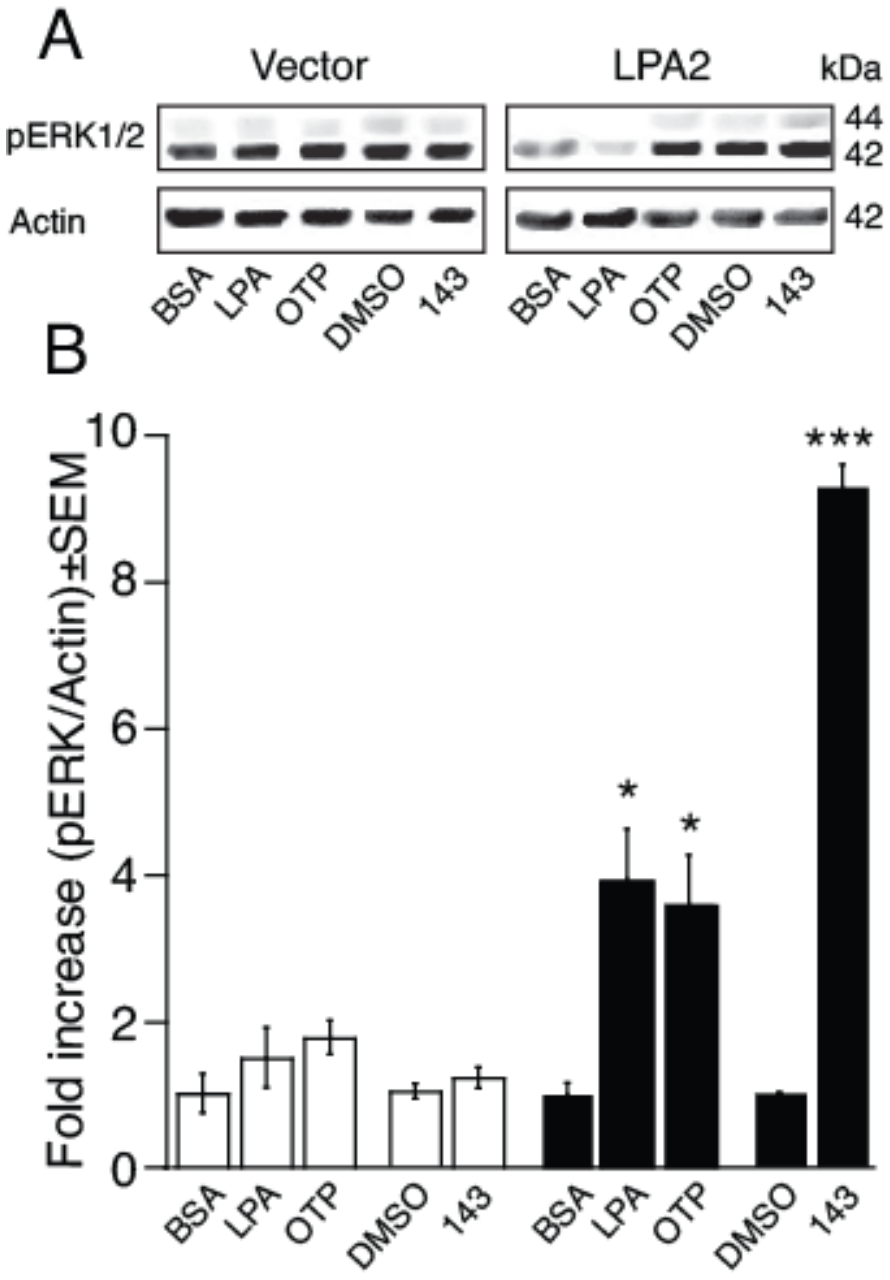


Figure 7.13 Effect of LPA (1 μM), OTP (1 μM), or GRI977143 (10 μM) on ERK1/2 activation. Representative western blots (panel A) and densitometry (panel B) of the mean ERK1/2 activation in vector- (open bars) and LPA_2-transduced (filled bars) MEF cells after GRI977143 treatment. Data were normalized for equal loading based on actin and are representative of three independent experiments ($^{*}p \leq 0.05$, $^{***}p \leq 0.001$).

7.14 Effect of GRI977143 on the assembly of a macromolecular complex between LPA_2 TRIP6 and NHERF2

LPA_2 receptor-mediated supramolecular complex formation is required for the protection against Adriamycin-induced apoptosis (E et al., 2009). To further elucidate molecular mechanisms activated by GRI977143, we investigated its effect on agonist-induced signalosome assembly between TRIP6, NHERF2 and the C-terminus of LPA_2. This macromolecular complex plays an important role in the antiapoptotic effect via stimulation of the ERK1/2 and Akt-NFκB survival pathways. HEK293 cells were transfected with EGFP-NHERF2 and FLAG-LPA_2 and exposed to 10 μM GRI977143 or vehicle for 10 min. The cells were lysed and the signalosome was pulled down using anti-FLAG beads. The complex was loaded to SDS-PAGE and western blots were performed using antibodies to TRIP6, EGFP and FLAG to monitor the presence of the different proteins constituting the LPA_2 signalosome. GRI977143 elicited the assembly of the macromolecular complex indicated by the recruitment of TRIP6 and EGFP-NHERF2 to the LPA_2 receptor (**Fig. 7.14**). Only trace amounts of the ternary complex were detected in the vehicle-treated cell lysates, indicating that activation of LPA_2 by GRI977143 is required for the assembly of the signaling complex.

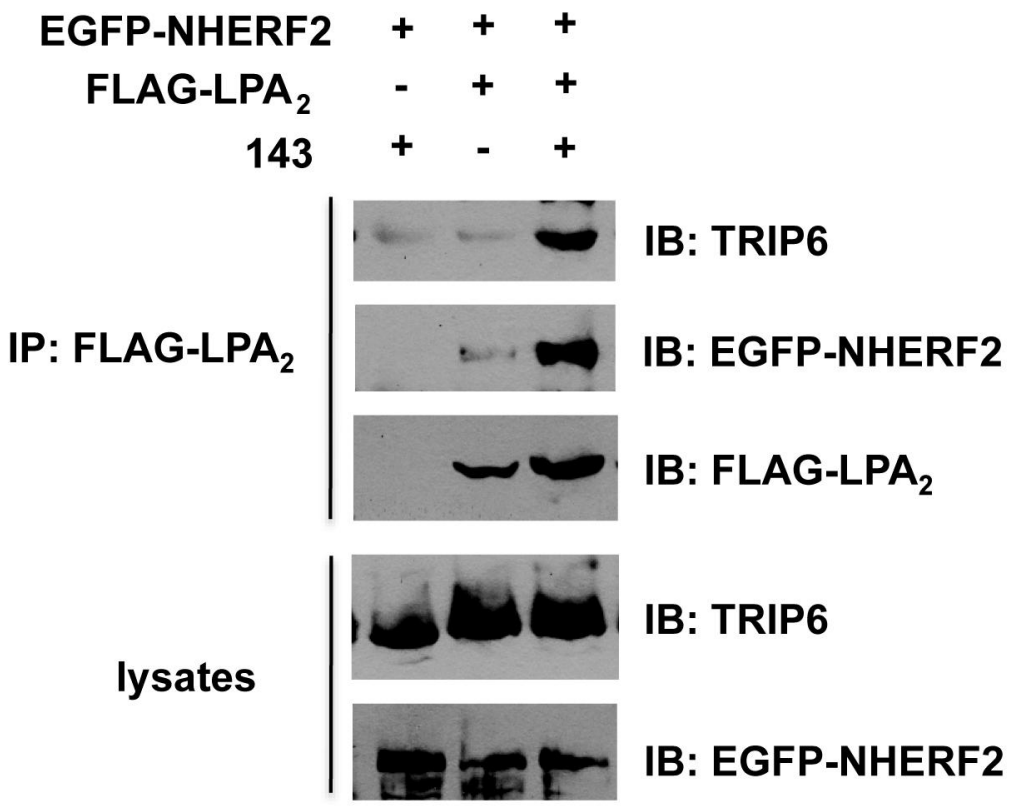


Figure 7.14 Effect of GRI977143 (10 μM) on the macromolecular complex assembly between FLAG-LPA_2, EGFP-NHERF2, and endogenous TRIP6. The blot shown is representative of two co-transfection experiments.

8. DISCUSSION

LPA's growth factor-like actions and its simple chemical structure make it an ideal candidate for drug discovery. A major obstacle in developing LPA analogs is their high degree of hydrophobicity that makes these agents nonideal drug candidates. Another complicating factor is the multiplicity of LPA GPCRs, which represents a significant challenge to the development of compounds specific to a single target such as LPA_2. Our group has been developing and validating computational models of the putative ligand binding pockets of LPA GPCRs (Fujiwara et al., 2007; Fujiwara et al., 2005; Parrill et al., 2000; Valentine et al., 2008; Wang et al., 2001). Our previous work aimed at the virtual discovery of LPA_1-specific compounds has serendipitously identified NSC12404, which is a weak but specific agonist of LPA_2 (Perygin, 2010). In the present study, we used this hit for virtual screening of the GRI and H2L chemical libraries. This approach identified three new selective nonlipid LPA_2 agonists: GRI977143, H2L5547924, and H2L5828102 (**Table 7.1.1**).

We selected GRI977143 for initial characterization in cell-based assays and compared its pharmacological and signaling properties with those of LPA and the previously identified LPA mimic OTP. The other compounds, NSC12404, H2L5547924, and H2L5828102 might be worthy of detailed characterization and synthetic improvements in the future. GRI977143 was a specific agonist of only LPA_2 when tested for agonist or antagonist activity at up to 10 μM concentration at five established and two putative LPA GPCRs. It is noteworthy that this compound above 10 μM also showed a modest partial inhibition of LPA_3. One of our strategies utilized MEF cells derived from $LPA_{1\&2}$ DKO mice (Lin et al., 2007). The parental MEF cells do not express functional $LPA_{1/2/3}$ receptors but express $LPA_{4/5/6}$ transcripts. Thus, these MEF cells can be considered an LPA receptor null host cell line for $LPA_{1/2/3}$, which belong to the EDG family LPA receptors. Knock in of LPA_2 rendered these MEF cells responsive to LPA with pharmacological properties similar to those of LPA_2 established in other cell types endogenously expressing this receptor subtype (E et al., 2009; Lin et al., 2007). GRI977143 had no effect in the vector-transduced MEF cells with the exception of a

minimal reduction in DNA fragmentation in the Adriamycin model of apoptosis (**Fig. 7.4.1 C**). There was no such detectable effect of GRI977143 in the serum withdrawal- or γ-irradiation-induced apoptosis models (**Figs. 7.5 C** and **7.7 C**). We do not know the reason for the effect of GRI977143 on DNA fragmentation in the control MEF cells in the Adriamycin model only, but it might be due to some yet unknown off-target effect of the compound. Certainly, the lack of effect of GRI977143 in vector-transduced MEF cells on Ca^{2+} mobilization (data not shown), on caspases 3, 7, 8, and 9, on DNA fragmentation, and on ERK1/2 activation are all consistent with the hypothesis that specific activation of LPA_2 is responsible for these same responses that we consistently detected in the MEF cells expressing the LPA_2 receptor. In contrast to GRI977143, we have also noticed that LPA and OTP had a slight attenuating effect in vector-transfected MEF cells. Quantitative RT-PCR analysis showed that the MEF cells derived from LPA_1 and LPA_2 double KO mouse embryos express appreciable amounts of $LPA_{4/5/6}$ and P2Y10, which can explain the radiomitigating effect of LPA and OTP (**Fig. 7.9**). Even though GRI is less efficacious than LPA in activating Ca^{2+}-mobilization via LPA_2 (EC_{50}= 3.3 μM *vs.* 0.03 μM, respectively), GRI977143 was highly effective in protecting LPA_2-transduced MEF cells compared to the protection exerted by LPA in vector-transduced cells. Both, the LPA_2-specific GRI977143 compound and the pan agonist LPA showed the highest level of protection in LPA_2 expressing MEFs. This comparison suggests that activation of LPA_2 provides effective radiomitigation compared to the activation of the other LPA receptor subtypes expressed in these MEF cells and activated by LPA. Nonetheless, this observation requires further studies with MEF cells expressing each individual LPA GPCR subtype.

We also showed that specific stimulation of the LPA_2 receptor subtype promotes cell growth (**Fig. 7.2**). This is the first pharmacological evidence that this receptor subtype mediates mitogenesis. Surprisingly, the LPA receptor panagonist OTP and GRI977143 had equally robust activity on cell proliferation. We note that OTP and GRI977143 after 3 days also promoted the growth of vector-transduced MEF cells, which might be due to off-target or indirect effects. We cannot exclude the possibility that OTP and GRI977143 somehow potentiated the effect of the 1.5% serum present in the medium.

There might be differences in the pharmacokinetic properties of these ligands, which could explain the differences we noted. Future experiments will have to address the differences on cell growth observed between these ligands.

LPA has been shown to promote cancer cell invasion and metastasis (Kato et al., 2012; Pustilnik et al., 1999). We tested the effect of GRI977143 in an in vitro invasion model that has been considered a realistic model of metastasis (Mukai et al., 2005; Mukai et al., 2000; Uchiyama et al., 2007). Stimulation of MM1 hepatocarcinoma cells with GRI977143 elicited a dose-dependent increase in the number of cells that penetrated the HUVEC monolayer (**Fig. 7.3**). However, this effect, although significant at a 10 μM concentration of GRI977143, was modest compared to that of LPA. The MM1 cells express LPA_2 >> LPA_1 > LPA_6 > LPA_5 > LPA_4 transcripts, whereas HUVECs express LPA_5 >> LPA_4 > GPR87 ~ LPA_1 > LPA_2 transcripts determined by quantitative RT-PCR (Lee & Tigyi – unpublished). The increase in GRI977143-induced invasion of MM1 cells is likely to represent the effect of selective stimulation of LPA_2 in the invading MM1 cells rather than in HUVEC due to the very low expression of this receptor subtype in the cells of the monolayer (Gupte et al., 2011). LPA_2 mediates the invasion of ovarian cancer in at least two ways. LPA_2 promotes production of vascular endothelial growth factor, urokinase (uPA), and matrix metalloproteinases (MMP) (So et al., 2005). LPA increases vascular endothelial growth factor production and in turn upregulates ATX production, which increases LPA levels that might represent a potential feed-forward loop that also promotes angiogenesis (Ptaszynska et al., 2008). Members of the MMP family play an important role in remodeling of the extracellular matrix during tumor invasion. We showed previously that MM1 cells express membrane type 1 MMP and MMP tissue inhibitor 2, which are required for activation of MMP-2 (Uchiyama et al., 2010a). We also showed that activation of Rho/ROCK, Rac/WAVE2, and Cdc42/N-WASP pathways (Uchiyama et al., 2010b), all of which are downstream elements of LPA GPCR signaling, plays an important role in the invasive properties of this carcinoma. LPA-induced uPA production promotes invasiveness of ovarian cancer cell lines (Pustilnik et al., 1999). Clinically, high uPA levels indicate poor prognosis in

ovarian cancer (Murthi et al., 2004). Thus, our present results provide new pharmacological evidence that activation of LPA_2 promotes invasion and metastasis.

Studies have already established the role of the LPA_2 receptor in protecting cells from programmed cell death (Deng et al., 2002; E et al., 2009; Lin et al., 2007; Sun et al., 2010; Taghavi et al., 2008; Yu et al., 2008). The LPA_2-specific agonist properties of GRI977143 allowed us to test this hypothesis in the LPA_2 knock-in MEF cells and in IEC-6 cells, the latter of which endogenously expresses multiple LPA GPCRs (Deng et al., 2002; Deng et al., 2004; Deng et al., 2007; Deng et al., 2003). Our experiments showed that by activating the LPA_2 receptor GRI977143 effectively reduces cytosolic Bax translocation, activation of initiator and effector caspases, DNA fragmentation and PARP-1 cleavage associated with Adriamycin-, serum withdrawal-, or γ-irradiation induced intrinsic apoptosis (**Figs. 7.4.1, 7.5, 7.7**). Both LPA and OTP activate these same mechanisms but also activate multiple LPA GPCRs (Deng et al., 2002; Deng et al., 2004; Deng et al., 2007; Deng et al., 2003). It is also important to recognize that GRI977143 protected IEC-6 cells from apoptosis, which endogenously express multiple LPA GPCR subtypes (**Fig. 7.6**). This result is the first evidence that we know of in the literature that specific activation of LPA_2 is sufficient to evoke an antiapoptotic effect and this effect is not limited to the LPA_2 knock-in MEF cells. Thus, we propose that specific activation of LPA_2 is sufficient to protect cells from apoptosis. The specific agonist properties of GRI977143 might represent an advantage over LPA and other receptor-nonselective LPA mimics that also stimulate LPA_1 receptor subtype activation, which has been shown to promote cell death via anoikis in tumor cells (Furui et al., 1999), in cardiac myocytes (Chen et al., 2006), and in pulmonary epithelial cells (Funke et al., 2012).

We also examined the effect of LPA and GRI977143 in a model of radiation-induced bystander apoptosis *in vitro*. This model has relevance to the radiomitigative action of LPA analogs because in the animal experiments the LPA analogs were not present during the first 24 h postirradiation when the initial wave of radiation-elicited apoptosis takes place. Nevertheless, administration of OTP or GRI977143 at +24 h postirradiation is effective in protecting the lives of the animals (Deng et al., 2007; **Fig. 7.12**). We

hypothesize that GRI977143-mediated activation of LPA_2 receptors in the tissues exerts some of its protective action by attenuating bystander effects of radiation injury that occur 24 – 48 h post injury and are possibly mediated by agents similar to those present in the CM of irradiated U937 cells in our *in vitro* model (Kim et al., 2008). This observation prompts further research toward the identification of these soluble mediators that could passively transfer apoptosis from U937 cells to unirradiated IEC-6 cells. Whether these factors are also present in the biological fluids of irradiated animals becomes a very intriguing question. Taken together, our results obtained with GRI in the different γ-irradiation injury models consistently suggest that this compound exerts a radiomitigative action and is capable of rescuing apoptotically condemned cells *in vitro* and *in vivo*. In this context we were surprised to find that malignant transformation of the irradiated and rescued MEF cells did not show enhancement after GRI treatment (**Fig. 7.11**). This observation will need to be followed up *in vivo* but already hints that GRI977143-treated cells have been able to repair DNA damage that otherwise could have led to a high-rate of transformation revealed by growth in soft agar.

LPA_2-mediated activation of the ERK1/2 prosurvival kinases is a required event in antiapoptotic signaling (Deng et al., 2004; E et al., 2009; Lin et al., 2007). Consistent with our previous results obtained with LPA and OTP (Deng et al., 2007; E et al., 2009; Lin et al., 2007), GRI977143 treatment resulted in a robust ERK1/2 activation. We have previously shown that in addition to the Gi protein-mediated signals demonstrated by the partial pertussis toxin-sensitivity of the effect (Deng et al., 2002; Deng et al., 2004), the LPA_2-mediated antiapoptotic effect requires additional ligand-induced assembly of a C-terminal macromolecular complex consisting of LPA_2, TRIP6, and a homodimer of NHERF2 (E et al., 2009; Lai et al., 2005; Lin et al., 2007). Activation of LPA_2 regulates the c-Src-mediated phosphorylation of TRIP6 at the Tyr-55 and Pro-58 residue, which in turn promotes LPA-induced ERK1/2 activation (Lai et al., 2005). We found that GRI977143 elicited the assembly of this signalosome (**Fig. 7.14**), which can explain the concomitant robust ERK1/2 activation (**Fig. 7.13 panel A** and **B**).

Taken altogether, the present findings indicate that nonlipid LPA_2-specific agonists, such as those described here, represent an excellent starting point for the development of lead compounds with radiomitigative effect and potential therapeutic utility for the prevention of programmed cell death involved in many types of degenerative and inflammatory diseases.

9. CONCLUSIONS

These observations with the initial characterization of GRI977143 should be taken as a prelude to a more in depth analysis of the action of LPA_2-specific pharmacons in different forms of apoptosis. We remain enthusiastic about these initial results as they provide the first line of evidence that selective pharmacological stimulation of the LPA_2 receptor subtype is sufficient to protect against the acute hematopoietic radiation syndrome *in vivo*. We also recognize that GRI is a weak, although specific LPA_2 agonist, which must undergo lead optimization to yield antiapoptotic and radiomitigative drug candidates suitable for human use.

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11. PUBLICATIONS OF THE AUTHOR

11.1 PUBLICATIONS

2012

Kiss GN, Fells JI, Gupte R, Lee SC, Liu J, Nusser N, Lim KG, Ray RM, Lin FT, Parrill AL, Sumegi B, Miller DD, Tigyi GJ (2012) Virtual Screening for LPA2-Specific Agonists Identifies a Nonlipid Compound with Antiapoptotic Actions. *Mol Pharmacol* **82**(6):1162-1173.

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2010

Valentine WJ and Kiss GN, Liu J, E S, Gotoh M, Murakami-Murofushi K, Pham TC, Baker DL, Parrill AL, Lu X, Sun C, Bittman R, Pyne NJ, Tigyi G (2010) (S)-FTY720-vinylphosphonate, an analogue of the immunosuppressive agent FTY720, is a pan-antagonist of sphingosine 1-phosphate GPCR signaling and inhibits autotaxin activity. *Cell Signal* **22**(10):1543-1553.

2009

Kovacs K, Hanto K, Bognar Z, Tapodi A, Bognar E, Kiss GN, Szabo A, Rappai G, Kiss T, Sumegi B, Gallyas F Jr (2009) Prevalent role of Akt and ERK activation in cardioprotective effect of Ca(2+) channel- and beta-adrenergic receptor blockers. *Mol Cell Biochem* **321**(1-2):155-164.

2008

Bartha E, Kiss GN, Kalman E, Kulcsár G, Kálai T, Hideg K, Habon T, Sumegi B, Toth K, Halmosi R (2008) Effect of L-2286, a poly(ADP-ribose)polymerase inhibitor and

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11.2 PRESENTATIONS, POSTERS

2011

Tigyi G, Kiss GN, Fells J, Liu D, Yue J, Thompson KE, Yates R, E S, Lai YJ, Lin FT, Parrill AL, Deng W, Gupte R, Miller DD (2011) Development of Radiomitigators Targeting the Lysophosphatidic Acid Receptors. *14th International Congress of Radiation Research, Warsaw, Poland*, **poster**

Tigyi G, Kiss GN, Valentine W, E S, Fells J, Liu J, Yue J, Thompson KE, Yates R, Parrill A, Lai YJ, Lin FT, Deng W (2011) Computational Design of a Non-lipid LPA2 Receptor Agonist with Antiapoptotic and Radioprotective Action. *Experimental Biology Meeting, Washington, D.C.*, **poster**

2010

Tigyi G, Kiss GN, Valentine W, E S, Fells J, Liu J, Yue J, Thompson KE, Yates R, Parrill A, Lai YJ, Lin FT, Deng W (2010) Radiomitigative signaling by lysophospholipid receptors. (2010) *56th Annual Meeting of the Radiation Research Society, Maui, Hawaii*, **poster**

Valentine WJ, Kiss G, Liu J, E S, Lu X, Sun C, Bittman R, Tigyi G (2010) (S)-ene Phosphonate Analog of the Immunosuppressive Agent FTY720 is a Pan-antagonist of the S1P GPCR. *Keystone Symposium on Sphingolipid Signaling, Kyoto*, **poster**

Valentine WJ, Kiss GN, Liu J, Lu X, Sun C, Bittman R, Tigyi G (2010) FTY720 S-ene-phosphonate is a novel pan-antagonist of the S1P receptors that inhibits lymphocyte egress. *Experimental Biology Annual Meeting, San Diego Ca*, **poster**

2008

Bognar E, Kiss GN, Sarszegi Zs, Bartha E, Solti I, Sumegi B, Berente Z (2008) Poly(ADP-ribose) Polymerase (PARP) Inhibitor HO3089 Enhanced Post Ischemic Myocardial Glucose Uptake Mostly By Activation Of AMP-activated Protein Kinase (AMPK). *World Congress of Cardiology*, **poster**

2007

Bartha E, Halmosi R, Kiss GN, Solti I, Bognar E, Kalman E, Kalai T, Hideg K, Sumegi B, Toth K (2007) Effect of PARP and ACE inhibitors on rat chronic heart failure model. *XXXVII. Membrane Transport Conference*, **poster**

Bartha E, Halmosi R, Kulcsar G, Kiss GN, Kalman E, Sumegi B, Kalai T, Hideg K, Toth K (2007) Effect of PARP-inhibitors and ACE-inhibitors on the progression of the isoproterenol-induced heart failure. *European Heart Journal* vol. 28, Abstract Suppl.: *48-49*, **poster**

Bartha E, Palfi A, Mark L, Kiss GN, Halmosi R, Szabados E, Sumegi B (2007) Effect of an alcohol free red wine extract on the myocardial remodeling induced by isoproterenol. *Hungarian Society of Cardiology Scientific Conference*, **presentation**

Berente Z, Kiss GN, Bognar E, Sumegi B (2007) Relationship between the cardioprotective effect of certain poly(ADP-ribose)polimerase inhibitors and the glucose metabolism of the postischemic heart. *Hungarian Biochemical Society Annual Meeting*, **presentation**

Berente Z, Kiss GN, Radnai B, Sumegi B (2007) In situ and in vivo applications of nuclear magnetic resonance for biomarker search. *Symposium on Medicinal Chemistry and Technology*, **presentation**

Kovacs K, Szabo A, Bognar E, Kiss GN, Kiss T, Sarszegi Z, Sumegi B, Gallyas F (2007) Identification of novel drug targets preventing ischemic heart diseases. *Bridges in Life Sciences Annual Scientific Review, Regional Cooperation for Health, Science and Technology*, vol.1, page 42., **presentation**

Szabo A, Kovacs K, Bognar E, Kiss GN, Sumegi B, Gallyas F (2007) Role of Akt and ERK activation in the cardioprotective effect of poly(ADP-ribose)polymerase inhibitors, Ca2+ channel- and β-blockers. *Hungarian Biochemical Society Annual Meeting*, **presentation**

2006

Bartha E, Palfi A, Mark L, Kiss G, Halmosi R, Szabados E, Kalman E, Pajor L, Toth K, Sumegi B (2006) Effect of Alcohol-free red wine extract on isoproterenol-induced cardiac remodeling in rats. *Exp Clin Cardiol* **11**(3):246, **poster**

Bognar E, Kiss GN, Solti I, Hanto K, Kovacs K, Sumegi B, Gallyas F **(**2006) Comparison of 4-hydroxy-quinazoline, Verapamil and Metoprolol in heart protection during ischemia- reperfusion. *Exp Clin Cardiol* **11**(3):247, **poster**

Bognar E, Solti I, Nemeth V, Bartha E, Tucsek Zs, Veto S, Hocsak E, Kiss GN, Sumegi B, Berente Z (2006) Study of glucose uptake and related cell signaling pathways in isolated rat heart model. *Hungarian Biochemical Society Annual Meeting*, **poster**

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Kiss GN, Bognar E, Solti I, Hanto K, Kovacs K, Gallyas F, Sumegi B (2006) Molecular mechanisms of cardioprotection afforded by poly(ADP-ribose) polymerase inhibitors, Ca2+ channel- and ß-blockers in ischemia-reperfusion: a comparative ex vivo study. *Semmelweis Symposium Nitric Oxide and Nitrosative Stress in the Cardiovascular system*, **poster**

Solti I, Bognar Z, Nemeth V, Veto S, Hocsak E, Kiss GN, Szanto A, Varbiro G, Sumegi B (2006) Effect of Taxol on the mitochondrion and the ROS production. *Hungarian Biochemical Society Annual Meeting*, **poster**

2005

Hanto K, Tapodi A, Kiss G, Varbiro G, Gallyas F, Hideg K, Sumegi B, Toth K (2005) Enhanced Akt activation induced by PARP inhibition in H9c2 cells during oxidative stress. *Hungarian Society of Cardiology Scientific Conference*, **presentation**

Hanto K, Tapodi A, Kiss G, Varbiro G, Gallyas F, Hideg K, Sumegi B, Toth K (2005) PARP inhibition induced Akt activation is dominantly responsible for the cytoprotection against oxidative stress in H9c2 cells. *VI. Hungarian Genetic Congress; XIII. Cell and Developmental Biology Conference*, **poster**

Kiss G (2005) Effect of poly(ADP-ribose) polymerase (PARP) inhibitors on the glucose uptake of isolated perfused rat hearts. *Student Science Work Conference*, *University of Pecs, Medical School*, **presentation**

Resko A, Kiss GN (2005) Cardioprotective effects of poly(ADP-ribose) polymerase inhibitors on chronic rat heart failure. *Student Science Work Conference, University of Pecs, Medical School*, **presentation**

2004

Kiss G, Pozsgay E, Resko A (2004) Study of glucose uptake and related cell signaling pathways on isolated rat heart model during ischemia-reperfusion. *Student Science Work Conference, University of Pecs, Medical School*, **presentation**

Pozsgay E, Kiss G, Resko A (2004) Experimental antioxidant compounds decrease acute cardiotoxicity induced by Doxorubicin. *Student Science Work Conference, University of Pecs, Medical School*, **presentation**

Deres P, Kiss G, Pozsgay E, Toth A, Halmosi R, Kovacs K, Palfi A, Hideg K, Sumegi B, Toth (2004) Experimental antioxidant compounds protect against acute cardiotoxicity induced by Doxorubicin. *Hungarian Society of Cardiology Scientific Conference*, **poster**

Deres P, Hanto K, Kovacs K, Halmosi R, Palfi A, Kiss G, Resko A, Pozsgay E, Sumegi B, Toth (2004) Protective effect of poly(ADP-ribose) polymerase

inhibitors on Doxorubicin induced acute cardiotoxicity. *XXXIV. Membrane Transport Conference*, **poster**

2003

Kiss G, Hanto K (2003) Effect of experimental antioxidant compounds on the activation of cardioprotective cell signaling pathways during rat heart ischemia-reperfusion. *Student Science Work Conference, University of Pecs, Medical School*, **presentation**