8-氮鸟嘌呤通过Akt/mTORC1/ULK1诱导细胞自噬增强其在肝癌细胞中的耐药性

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摘要: 细胞自噬是真核生物中进化保守的对细胞内容物进行降解的生理过程, 其利用溶酶体将细胞内物质降解再利用, 在应激条件下可以促进癌细胞的存活。8-氮鸟嘌呤 (8-azaguanine, 8-AG) 是一种嘌呤核苷酸生物合成的抑制剂, 对多种肿瘤细胞具有抗肿瘤活性。然而, 耐药性限制了8-AG作为抗癌药物的应用, 其耐药性机制尚不清楚。本研究发现8-AG通过诱导细胞自噬减弱其细胞毒性而产生耐药性。利用HepG2和SMMC-7721肝癌细胞系进行药物处理, 结果显示8-AG抑制肿瘤细胞活力, 并且通过上调促凋亡蛋白BCL-2样蛋白11 (BCL-2-like protein 11, Bim) 中的BimS亚型水平来诱导内源性凋亡。此外, Western blot实验检测结果表明8-AG通过抑制Akt (protein kinase B)/mTORC1 (mammalian target of rapamycin complex 1) 信号通路激活ULK1 (Unc-51-like autophagy activating kinase 1) 蛋白, 从而诱导自噬发生。敲低自噬相关基因7 (autophagy-related gene 7, *ATG7*) 显著增加BimS的蛋白水平, 促进8-AG引起的细胞死亡; 联合使用自噬抑制剂氯喹 (chloroquine, CQ) 或巴弗洛霉素A1 (bafilomycin A1, Baf A1) 促进8-AG诱导的肝癌细胞凋亡。以上结果表明, 8-AG诱导自噬导致肿瘤细胞产生耐药性, 抑制自噬可增加癌细胞对其的敏感性。

关键词: 8-氮鸟嘌呤; 耐药性; 细胞自噬; 蛋白激酶B/哺乳动物雷帕霉素靶蛋白复合物1; 细胞凋亡

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8-Azaguanine-induced autophagy contributes to its chemoresistance in hepatic cancer cells

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Abstract: Autophagy, an evolutionarily conserved process by which components of the cell are degraded in lysosomes, may facilitate survival of cancer cells under stress conditions. 8-Azaguanine (8-AG), an inhibitor of purine nucleotide biosynthesis, shows antineoplastic activity in multiple tumor cells. However, chemoresistance has restricted its development as an anticancer agent, and the mechanism of 8-AG resistance is not fully understood. We report here that 8-AG induces a protective autophagy to eliminate its cytotoxicity, and inhibition of autophagy increases cellular sensitivity of cancer cells to 8-AG treatment. Using HepG2 or SMMC-7721 hepatic cancer cell lines, we found that 8-AG inhibited cell viability and induced intrinsic apoptosis, accompanied by the up-regulation of the pro-apoptotic protein BimS, one of Bim (also known as BCL-2-like protein 11, BCL2L11) isoforms.

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Furthermore, 8-AG treatment enhanced the autophagy flux by promoting the dephosphorylation and activation of Unc-51-like autophagy activating kinase 1 (ULK1) *via* Akt/mTORC1 (mammalian target of rapamycin complex 1) signaling inhibition. Depletion of autophagy-related gene 7 (*ATG7*) markedly enhanced the level of BimS, and promoted cell death in response to 8-AG. 8-AG in combination with autophagy inhibitor chloroquine (CQ) or bafilomycin A1 (Baf A1) promoted the 8-AG-induced apoptosis in hepatic cancer cells. Altogether, these findings suggest that autophagy promotes chemoresistance of cancer cells for 8-AG, and blocking autophagy increases cellular sensitivity of cancer cells to 8-AG treatment.

Key words: 8-azaguanine; chemoresistance; autophagy; Akt/mTORC1; apoptosis

8-氮鸟嘌呤 (8-azaguanine, 8-AG) 是鸟嘌呤的三唑类似物, 其作为嘌呤核苷酸生物合成的抑制剂能够干扰正常的生物合成途径[1]。8-AG具有抗肿瘤活性, 特别是对急性淋巴细胞白血病具有显著效果[2,3]。研究发现, 8-AG对多倍体 (high-ploidy) 乳腺癌细胞具有特异性杀伤作用[4], 也可增强自然杀伤细胞活力[5]。然而, 耐药性限制了8-AG作为抗癌药物的应用[2,3], 且其耐药机制尚未明确。

细胞自噬是真核生物进化保守的物质降解过程, 利用溶酶体将受损细胞器或蛋白聚集体降解再利用, 使细胞能够在营养缺乏或应激的条件下维持基本活性和生存能力[6]。自噬-溶酶体途径在细胞稳态中起关键作用, 其功能障碍与多种病理过程有关, 如神经元变性、衰老和肿瘤发生等[7]。哺乳动物自噬启动激酶ULK1 (Unc-51-like autophagy activating kinase 1) 是参与自噬膜成核的关键自噬蛋白, 在自噬诱导中发挥重要作用[8]。在营养充足情况下, 哺乳动物雷帕霉素靶蛋白复合物1 (mammalian target of rapamycin complex 1, mTORC1) 通过磷酸化ULK1丝氨酸757位点, 阻止ULK1活化并抑制自噬发生[9,10]; 在葡萄糖缺乏情况下, AMP激活蛋白激酶 (AMP activated protein kinase, AMPK) 通过磷酸化ULK1丝氨酸317和777位点激活自噬[10]。自噬可促进癌细胞在如营养限制、缺氧及抗癌药物治疗等应激条件下的存活, 因此, 抑制自噬可以提高肿瘤细胞对抗癌药物的敏感性[11-13]。

本研究通过细胞活力测定、流式细胞术检测细胞凋亡、Western blot法检测自噬标记蛋白 (LC3和p62) 及Akt (protein kinase B)/mTORC1信号通路蛋白等实验, 分析8-AG对肝癌细胞自噬及凋亡的影响, 旨在揭示8-AG的耐药机制, 为提高其临床治疗效果提供新策略。

材料与方法

细胞与试剂 肝癌细胞株HepG2、宫颈癌细胞株HeLa、人胚胎肾细胞株HEK-293T [购自ATCC (American type culture collection)] 以及肝癌细胞株SMMC-7721 (天津市第三中心医院高英堂研究员馈赠) 培养于含10%胎牛血清、100 u·mL^{-1}青霉素和0.1 mg·mL^{-1}链霉素的DMEM (Dulbecco's modified eagle medium) 培养基 (Gibco公司), 置于37 ℃、5% CO_2细胞培养箱; 8-AG (纯度99.82%, Selleckchem公司); 除特殊说明外, 所有化学品均购自Sigma Aldrich公司; phospho-Akt (p-Akt)、Akt和β-actin抗体 (Santa Cruz Biotechnology公司); caspase-9、caspase-3、phospho-p70S6K、phospho-ULK1、ATG7 (autophagy-related gene 7) 和p62抗体 (Cell Signaling Technology公司); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 抗体 (Biodragon公司); 辣根过氧化物酶标记山羊抗兔和抗小鼠二抗 (Sungene Biotech公司)。

质粒构建和稳定细胞系 GFP-LC3 (green fluorescent protein-LC3) 质粒构建参照已报道文章[14]。应用Lipofectamine 2000® (Invitrogen公司), 将pMD2.G和psPAX2 (CloneTech公司) 转染至HEK-293T细胞并生成慢病毒颗粒, 用pLVX-AcGFP-LC3慢病毒感染HeLa细胞获得稳定表达GFP-LC3的细胞, 并选择1 μg·mL^{-1}嘌呤霉素筛选至少2周。

Western blot实验 使用RIPA (radio immunoprecipitation assay) 裂解液提取细胞蛋白, 通过BCA (bicinchoninic acid) 检测试剂盒 (Beyotime公司) 对总蛋白进行定量。等量的蛋白质样品经SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) 处理后转移至PVDF (polyvinylidene fluoride) 膜 (Millipore公司)。经5%脱脂牛奶封闭后, 于4 ℃孵育抗体过夜, 与相应二抗孵育2 h后, 应用化学发光检测系统检测免疫印迹条带并应用ImageJ软件分析其灰度值。

细胞活力分析 细胞接种于96孔板, 每孔约8×10^{3}个细胞, 加入3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (20 μL, 5 mg·mL^{-1}) 于37 ℃继续孵育4 h, 应用酶标仪在490 nm检测吸光度。

细胞凋亡分析 采用Annexin V-FITC (fluorescein isothiocyanate) 凋亡分析试剂盒 (Sungene公司) 检测药物处理后细胞的凋亡情况。使用Accuri C6流式

细胞仪 (BD Biosciences公司) 评估细胞凋亡情况。

活性氧 (reactive oxygen species, ROS) 和线粒体膜电位 (mitochondria transmembrane potential, MMP) 检测 细胞内ROS含量测定采用DCFH-DA染色法, 将5×10^5个细胞接种于60 mm培养皿, 加入8-AG处理24 h。在37 ℃下, 用10 mmol·L^{-1} DCFH-DA染色30 min。收集细胞, 用荧光显微镜分析荧光。对于线粒体膜电位MMP检测, 将JC-1探针在37 ℃下与细胞孵育20 min, 洗涤后用荧光显微镜观察。

RNA干扰 构建表达*ATG7* shRNA (short hairpin RNA) 的pLKO.1-puro慢病毒载体 (靶定*ATG7* mRNA序列的互补序列为5'-CCGGCCCAGCTATTGGAACACTGTACTCGAGTACAGTGTTCCAATAGCTGGGTTTTT-3')。非靶shRNA对照载体 (SHC002) 由Sigma公司获得。用慢病毒感染细胞, 并选择1 μg·mL^{-1}的嘌呤霉素处理至少2周。

统计学分析 数据为3次独立实验结果的平均值 ± 标准差 ($\bar{x} \pm s$)。使用GraphPad Prism version 8.0软件的非配对student-*t*检验或单因素方差分析 (ANOVA) 进行统计学显著性分析。$P < 0.05$视为差异显著。

结果

1 8-AG抑制肝癌细胞活力并诱导凋亡

利用MTT法检测8-AG对HepG2和SMMC-7721细胞活力的影响 (图1A), 处理24或48 h后, 8-AG均以剂量依赖性方式抑制细胞活力, 对HepG2细胞的半数抑制浓度 (half maximal inhibitory concentration, IC_{50}) 值分别为10.78和5.32 μmol·L^{-1}, 对SMMC-7721细胞的IC_{50}值分别为9.99和5.11 μmol·L^{-1}。其中10 μmol·L^{-1}的8-AG处理48 h对肝癌细胞的活力有显著抑制作用, 抑制率约50%。Annexin V/PI (propidium iodide) 双染实验结果显示, 8-AG处理24 h后, HepG2和SMMC-7721细胞凋亡水平明显升高 (图1B)。使用荧光染料JC-1检测8-AG对线粒体膜电位的影响 (图1C), 结果显示, 8-AG处理可导致红色荧光减弱, 绿色荧光增强, 表明8-AG可增加线粒体膜通透性。此外, Western blot实验分析显示, 8-AG处理导致凋亡蛋白BimS、cleavage-caspase 9和cleavage-caspase 3蛋白水平显著升高 (图1D), 进一步说明8-AG可诱导细胞内源性凋亡。

2 8-AG促进肝癌细胞自噬

作者前期在HeLa细胞中证实8-AG可以启动细胞自噬 (数据未发表), 为评估8-AG对肝癌细胞自噬的影响, 本研究检测了8-AG处理后肝癌细胞HepG2和SMMC-7721中LC3-II和p62的水平。Western blot实验结果显示, 在HepG2和SMMC-7721细胞中, 8-AG处理可导致LC3-II水平显著增加, p62水平显著降低 (图2A、B)。此外, 与巴弗洛霉素A1 (bafilomycin A1, Baf A1) 单独处理相比, 8-AG和Baf A1共处理可导致LC3-II显著积累 (图2C、D)。上述结果表明, 8-AG促进肝癌细胞的自噬。

3 8-AG通过Akt/mTORC1/ULK1信号通路和ROS途径诱导自噬

研究表明, 哺乳动物自噬启动激酶ULK1在自噬诱导中具有重要作用, mTORC1通过磷酸化ULK1丝氨酸757位点阻止其激活。Western blot实验结果显示, 在HepG2和SMMC-7721细胞中, 8-AG处理导致磷酸化ULK1 (p-ULK1) 水平显著降低。此外, 8-AG也会明显抑制mTORC1底物核糖体蛋白激酶p70S6K的磷酸化水平 (图3A), 表明8-AG降低了mTORC1的活性。由于mTOR是Akt信号传导的主要效应蛋白, 本研究检测了8-AG对Akt活性的影响。如图3A所示, 8-AG显著抑制Akt磷酸化, 但未影响其总蛋白水平。这些数据表明, 8-AG通过Akt/mTORC1/ULK1信号诱导自噬发生。已有研究证明氧化应激能够负调控Akt信号通路, 因此本研究检测了8-AG对细胞ROS水平的影响。结果显示, 在8-AG处理的细胞中, ROS含量明显增加, 并且抗氧化剂*N*-乙酰半胱氨酸 (*N*-acetyl-*L*-cysteine, NAC) 能够阻止其产生 (图3B)。进一步实验结果显示, NAC阻断8-AG诱导的LC3-II积累 (图3C、D), 表明8-AG以ROS依赖性方式诱导自噬。

4 抑制自噬促进8-AG诱导的细胞凋亡

为评估自噬在8-AG诱导的细胞死亡中的作用, 本研究利用RNA干扰技术将HepG2和SMMC-7721细胞自噬相关蛋白*ATG7*基因敲低。如图4A所示, *ATG7*敲低有效降低8-AG引起的LC3-II积累, 表明自噬被抑制。MTT结果显示, *ATG7*敲低明显增加8-AG (10 μmol·L^{-1}, 24或48 h) 对细胞活力的抑制 (图4B)。此外, 流式细胞术实验结果显示, *ATG7*敲低显著增加Annexin V/PI双染阳性细胞的数量 (图4C)。Western blot实验结果进一步显示, *ATG7*敲低显著增加8-AG诱导的BimS、cleavage-caspase 9和cleavage-caspase 3的升高 (图4D)。这些结果表明, 8-AG引起的自噬能够削弱其细胞毒性。

5 自噬抑制剂促进8-AG诱导的肝癌细胞凋亡

利用自噬抑制剂氯喹 (chloroquine, CQ) 和Baf A1研究HepG2和SMMC-7721细胞对8-AG的敏感性。MTT实验结果显示, 与单独使用8-AG处理相比, CQ或Baf A1联合使用显著增加8-AG对细胞活力的抑制作用 (图5A)。流式细胞术实验结果显示, 8-AG和CQ或Baf A1联合用药显著增加两种肝癌细胞的凋亡率 (图

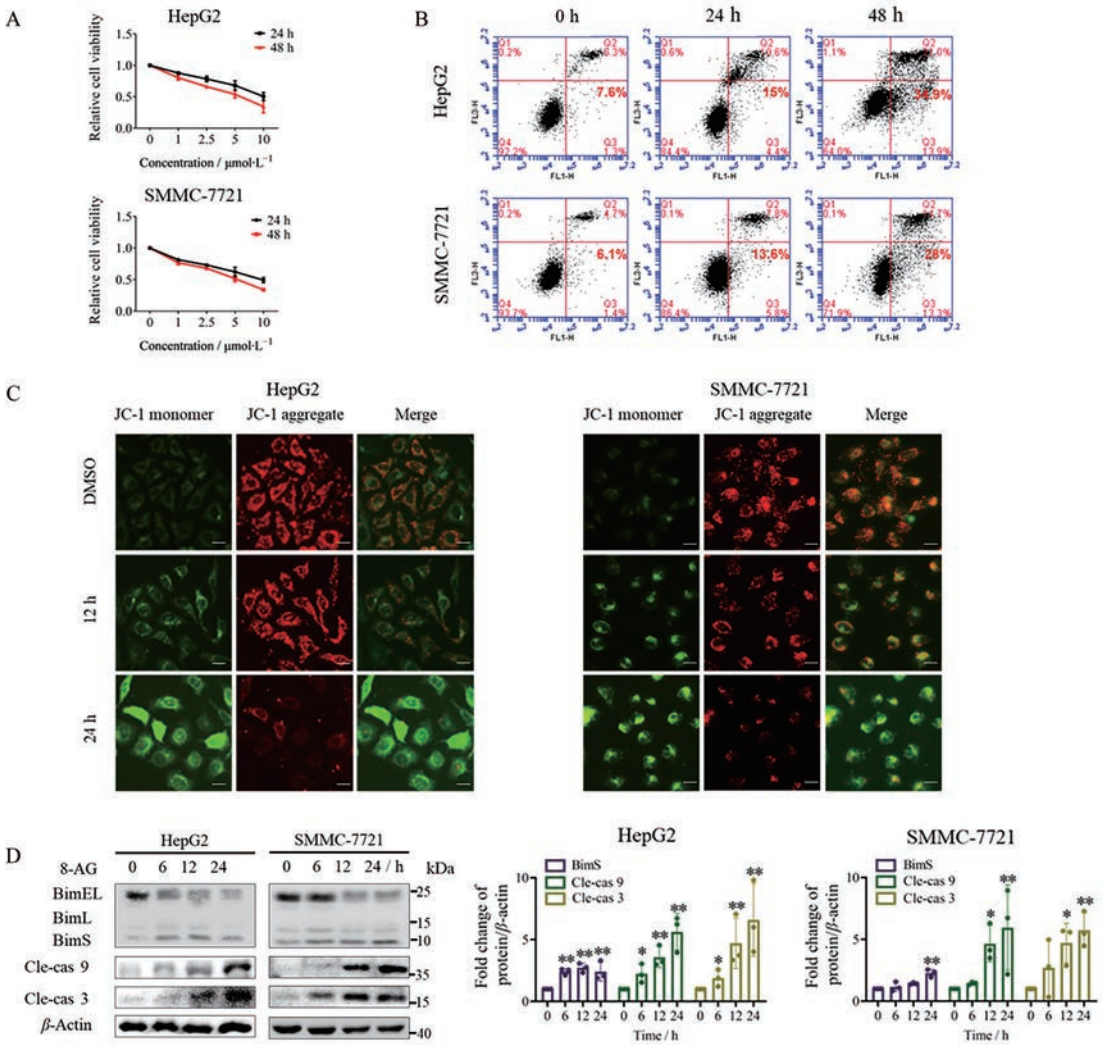

Figure 1　8-Azaguanine (8-AG) treatment induces cell viability inhibition and apoptosis in hepatic cancer cells. A: Effects of 8-AG on the viability of cancer cells. HepG2 or SMMC-7721 cells were incubated with increasing doses of 8-AG (1−10 μmol·L^{-1}) for 24 or 48 h. Cell viability was determined by MTT assay. The normalized value of cell viability from the untreated cells was arbitrarily set as 1.0. n = 3, $\bar{x} \pm s$; B: Effects of 8-AG on cancer cells apoptosis. HepG2 or SMMC-7721 cells were incubated with or without 10 μmol·L^{-1} 8-AG for 24 or 48 h. The percentage of cell apoptosis was determined by Annexin-V/propidium iodide (PI) staining and flow cytometry analysis; C: Cells treated with or without 10 μmol·L^{-1} 8-AG for 12 or 24 h were used to detect the mitochondrial membrane potential (MMP). The cells were stained with JC-1 and analyzed by fluorescence microscope. Mean JC-1 fluorescence intensity was measured by ImageJ software. Ratio of red/green fluorescence intensity from three view fields in three representative experiments is presented as $\bar{x} \pm s$; D: Cells treated with or without 10 μmol·L^{-1} 8-AG for indicated intervals were subjected to Western blot analysis using antibodies against the cleaved-caspase 9 (cle-cas 9), cleaved-caspase 3 (cle-cas 3), and BIM. β-Actin was used as a loading control. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control group. DMSO: Dimethyl sulfoxide; BIMEL: BCL-2-like protein 11 isoform BimEL; BIML: BCL-2-like protein 11 isoform BimL; BIMS: BCL-2-like protein 11 isoform BimS

5B)。此外, Western blot 实验结果显示, 与单独 8-AG 处理相比, CQ 或 Baf A1 联合使用显著增加 cleavage-caspase 9 和 cleavage-caspase 3 的水平 (图 5C)。上述结果表明抑制自噬增加肝癌细胞对 8-AG 的敏感性。

讨论

本研究结果表明, 8-AG 通过抑制 Akt/mTORC1 信号通路引起 ULK1 去磷酸化活化, 诱导保护性自噬从而削弱其对肝癌细胞的细胞毒性。因此, 抑制自噬可以增加癌细胞对 8-AG 治疗的敏感性。

8-AG 是一种核苷酸合成抑制剂, 作为抗代谢物干扰正常的生物合成途径, 从而抑制癌细胞特别是急性淋巴白血病细胞[2,3]的生长。然而, 耐药性限制了其作为抗肿瘤药物的临床使用[2,15,16]。已有多项研究探讨

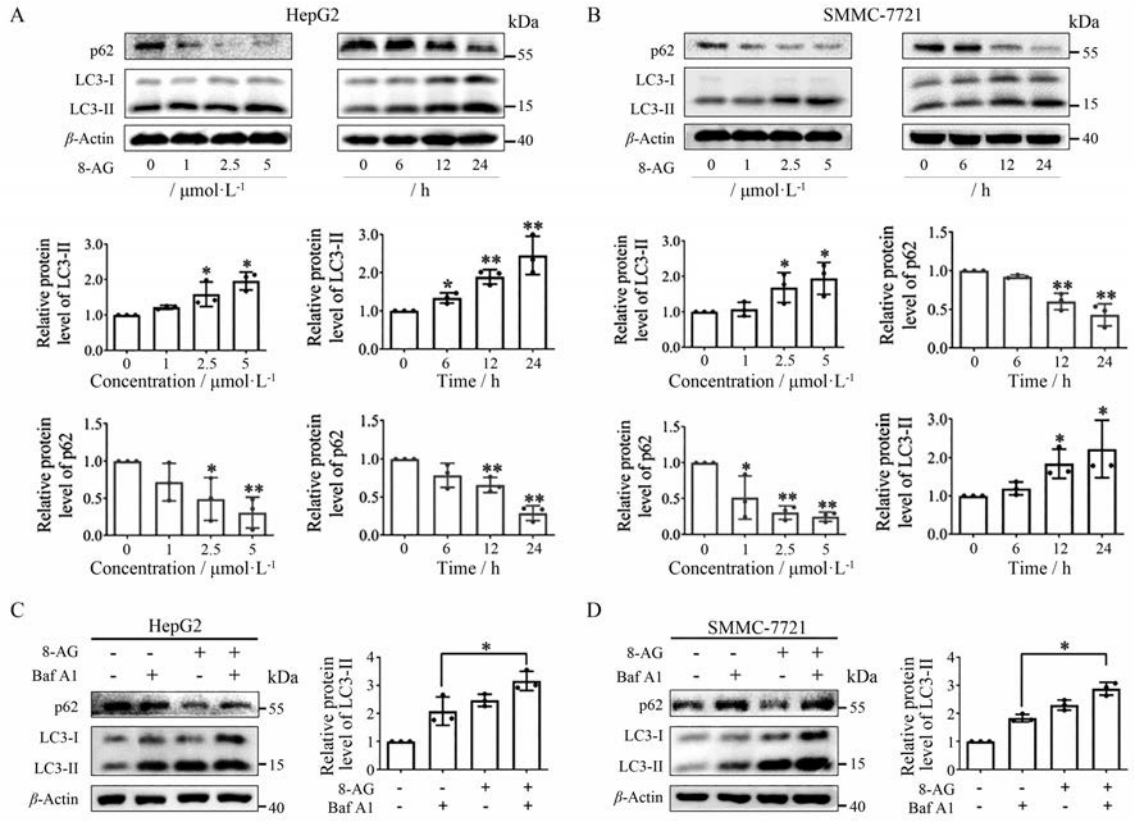

Figure 2 8-AG promotes autophagy in hepatic cancer cells. A and B: Effects of 8-AG on the abundance of LC3 and p62. HepG2 or SMMC-7721 cells were treated with the indicated concentration of 8-AG for 24 h or with 5 μmol·L^{-1} 8-AG for the indicated time points. Western blot analysis was used to analyze the abundance of LC3 and p62. *β*-Actin was used as a loading control; C and D: HepG2 or SMMC-7721 cells that were treated with or without 8-AG for 22 h were cultured in complete medium with or without 200 nmol·L^{-1} bafilomycin A1 (Baf A1) for 2 h. Corresponding changes in LC3 protein levels were measured by Western blot analysis. LC3-II or p62 levels were normalized to *β*-Actin. Densitometric analysis was performed using ImageJ. $n = 3$, $\bar{x} \pm s$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control group

了8-AG的潜在耐药机制, 例如, 在人急性白血病T细胞CEM和MOLT3中, 8-AG仅诱导MOLT3细胞凋亡, 这可能与其高表达表面抗原CD26有关[3], 而在CEM细胞中, 促凋亡蛋白Bax的缺少可能降低了对8-AG的敏感性[17]。此外, 8-AG可以被次黄嘌呤磷酸核糖转移酶1 (hypoxanthine phosphoribosyltransferase 1, HPRT1)激活, 因此高倍性乳腺癌中*HPRT1*的基因量增加可以提高肿瘤细胞对该药物的敏感性[4]。作者发现8-AG促进肝癌细胞的自噬, 并且抑制自噬能够促进8-AG诱导的细胞死亡。已有研究证实在化疗药物和放射治疗中, 自噬可促进肿瘤细胞的存活[18], 抑制自噬可促进癌细胞死亡并增强药物敏感性[11,12,19,20]。因此, 本研究结果揭示了8-AG耐药性的一种新机制, 为其临床应用提供新的策略, 尤其是对8-AG耐药的肿瘤治疗。

哺乳动物细胞自噬调节主要包括mTORC1和AMPK两种途径[9,10]。mTORC1复合体中的活性mTOR激酶磷酸化并抑制ULK1, 从而抑制自噬活性, 相反, mTORC1的失活导致ULK1在丝氨酸757位点去磷酸化激活[10]。此外, AMPK激活结节性硬化症肿瘤抑制因子TSC1/2以抑制应激条件下的mTORC1[21,22]。AMPK还可以通过磷酸化ULK1丝氨酸317位点直接激活自噬[10]。在本研究中, 8-AG处理导致mTORC1下游两个靶标蛋白p70S6K和ULK1的磷酸化水平减少, 表明8-AG抑制mTORC1活性。虽然AMPK途径也参与自噬启动的控制, 但磷酸化AMPK水平在8-AG处理后没有明显增加 (数据未显示)。这些结果进一步表明, 8-AG通过Akt/mTORC1/ULK1途径诱导自噬, 而非依赖AMPK信号通路。此外, 更多的研究需要揭示8-AG是否也通过直接与mTOR相互作用或间接激活TSC2来抑制mTORC1的活性。

此外, 作者结果表明8-AG能够显著增加细胞内ROS水平并降低线粒体的膜电位。NAC可以阻断8-AG诱导的LC3-II增加, 表明ROS是8-AG介导自噬的上游调节因子。已有研究报道氧化应激条件下, ROS能够对Akt信号产生负调节作用[23-25], 这为8-AG抑制Akt/mTORC1信号提供了一种可能机制, 更多研究需

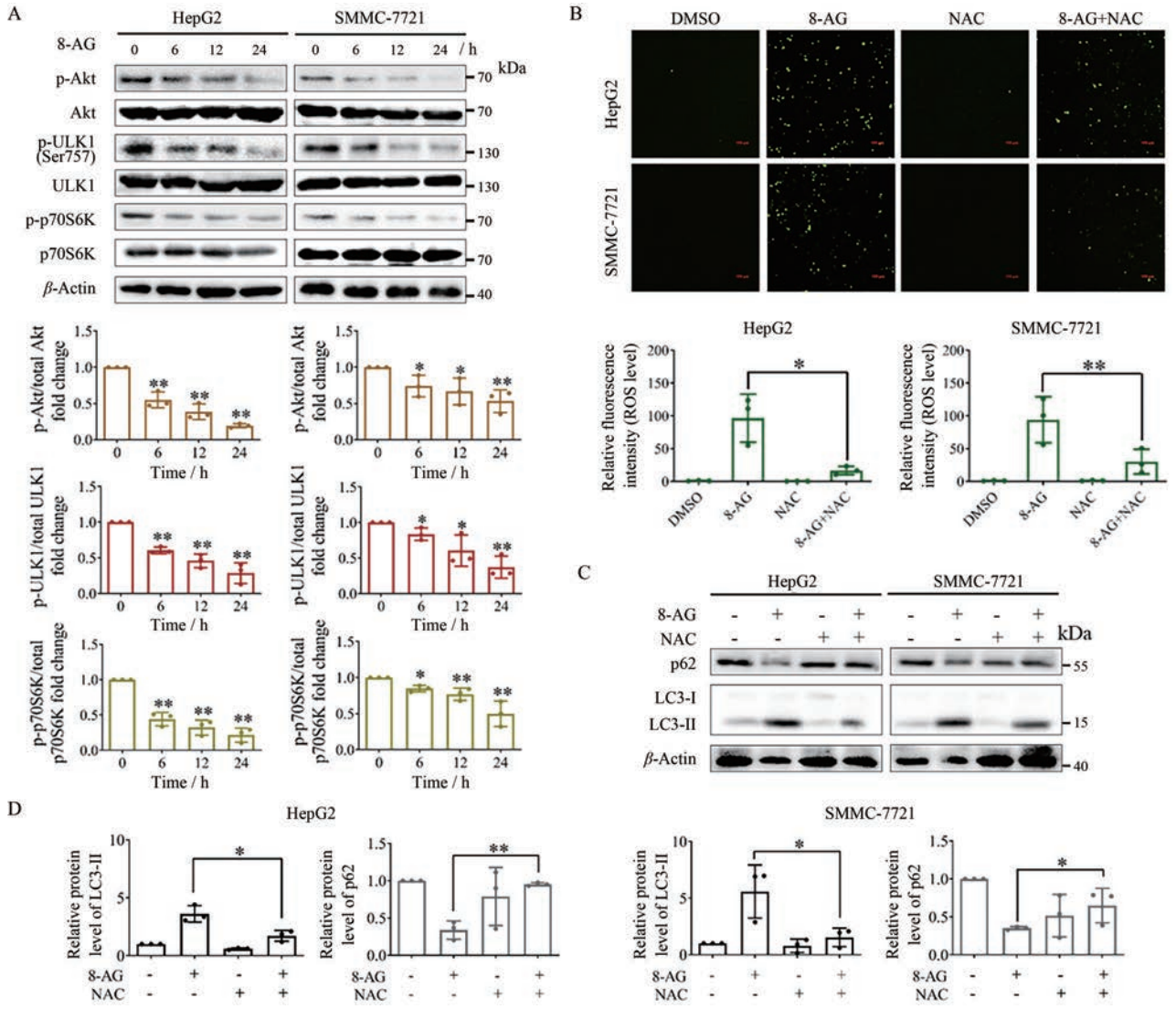

Figure 3 Effects of 8-AG on mammalian target of rapamycin complex 1 (mTORC1) signaling pathway and reactive oxygen species (ROS) levels. A: HepG2 or SMMC-7721 cells were treated with or without 5 μmol·L^{-1} 8-AG for indicated intervals. Cell lysates were subjected to Western blot analysis using the corresponding antibodies as shown in this figure. *β*-Actin was used as a loading control. p-Akt, p-ULK1, or p-p70S6K levels were quantified by densitometric analysis and normalized to total Akt, ULK1, or p70S6K, respectively. n = 3, $\bar{x} \pm s$. $^{*}P$ < 0.05, $^{**}P$ < 0.01 *vs* control group; B: HepG2 or SMMC-7721 cells were treated with or without 8-AG for 12 h, or treated with 10 mmol·L^{-1} NAC for 1 h followed by 8-AG for 12 h. NAC treatment was used as a negative control. ROS levels were measured by DCFH-DA staining assay and shown by quantitative bar graph measured as the fold change over DMSO-treated levels; C and D: Cells were treated with the same means as B. Corresponding changes in LC3 and p62 protein levels were measured by Western blot analysis. LC3 and p62 protein levels were normalized to *β*-actin. n = 3, $\bar{x} \pm s$. $^{*}P$ < 0.05, $^{**}P$ < 0.01

要进一步阐明8-AG如何增加细胞内ROS的水平。

促凋亡蛋白BIM是BCL2蛋白家族成员, 对细胞凋亡程序启动起关键作用[26]。选择性剪接产生3种BIM亚型, 包括BimEL、BimL和BimS, 其中BimS促凋亡活性最强[27]。BIM蛋白可以刺激线粒体释放细胞色素c, 从而激活caspase蛋白级联反应[27]。本研究发现8-AG还可以上调BimS蛋白的表达、caspase 9和caspase 3的激活。由于shRNA介导的*ATG7*敲低显著增加了BimS的蛋白水平, 并促进细胞凋亡, 因此, BimS通过自噬降解可能是8-AG凋亡阻滞的原因之一。有趣的是, 8-AG降低了BimEL蛋白的表达, 并且*ATG7*敲低不能恢复8-AG处理细胞中BimEL蛋白的丰度, 表明其他机制可能参与调节BIM稳定性以消除8-AG的细胞毒性。例如, 细胞外信号调节激酶 (extracellular signal-regulated kinase, ERK) 在丝氨酸55、65和73位点磷酸化BimEL, 通过泛素−蛋白酶体途径降解BimEL[28]。

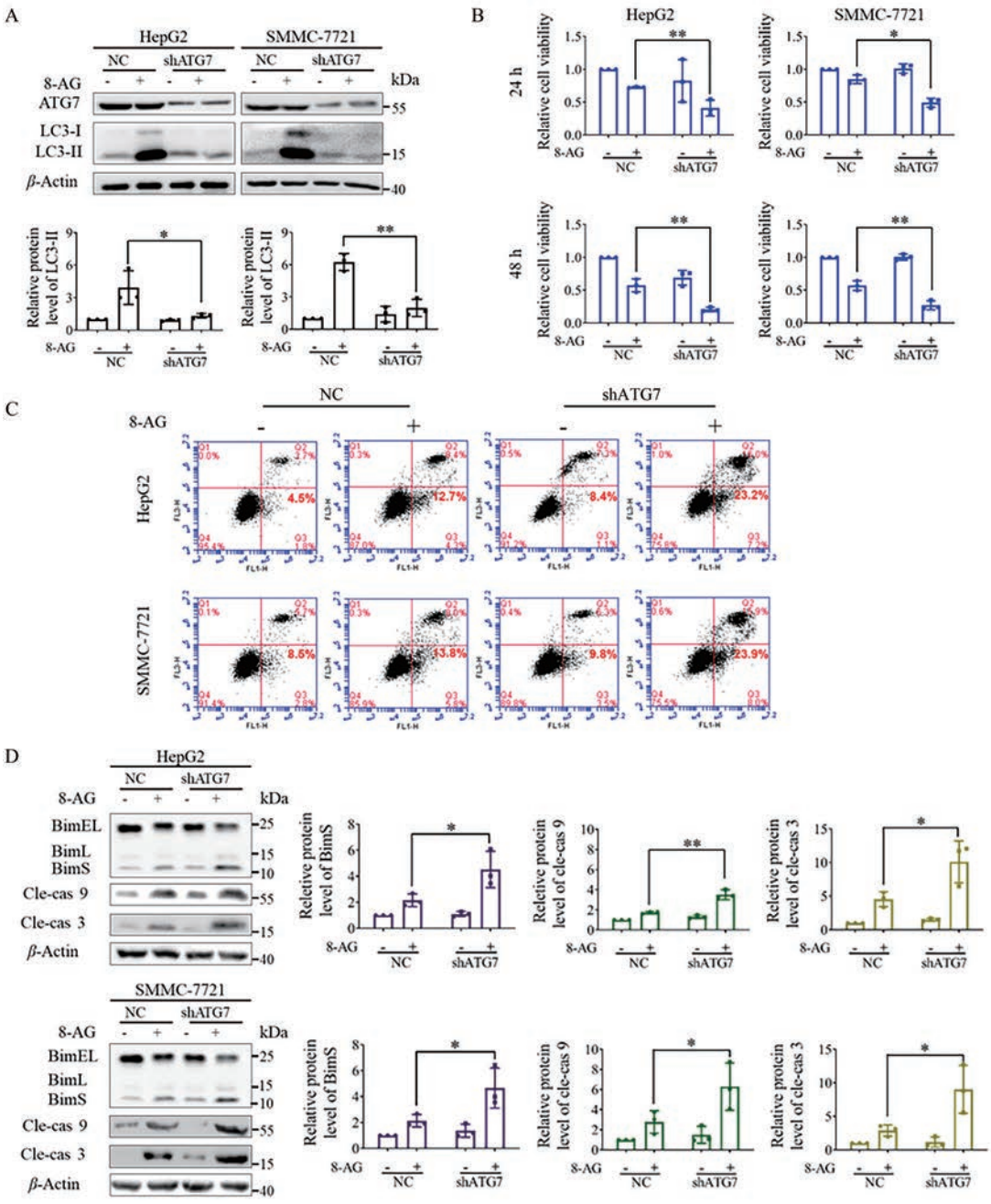

Figure 4 Depletion of autophagy-related gene 7 (*ATG7*) promotes 8-AG-induced cell death. A: HepG2 or SMMC-7721 cells stably expressing *ATG7* shRNA (shATG7) or negative control shRNA (NC) were treated with or without 8-AG (5 μmol·L^{-1}, 24 h). The protein levels of ATG7 and LC3 were measured by Western blot analysis. *β*-Actin was used as a loading control; B: Cell viability was analyzed by MTT assay at 24 and 48 h after 5 μmol·L^{-1} 8-AG treatment; C: HepG2 or SMMC-7721 cells stably expressing shATG7 or NC were treated with or without 5 μmol·L^{-1} 8-AG for 24 h. The percentage of apoptosis was determined by Annexin-V/PI staining and flow cytometry analysis; D: The same cells treated as C were used to measure the levels of caspase 9, caspase 3, and BIM by Western blot. All protein values were normalized to *β*-actin. $n = 3$, $\bar{x} \pm s$. $^{*}P < 0.05$, $^{**}P < 0.01$

更多的研究需要阐明8-AG是否也能增加ERK的活性, 或促进泛素–蛋白酶体途径。

本研究表明, 8-AG能够通过Akt/mTORC1/ULK1信号通路诱导保护性自噬, 抑制自噬可以增加肝癌细胞对其的敏感性。8-AG与自噬抑制剂联合使用为提高肿瘤治疗效果提供了新的策略。

作者贡献: 徐俊亭负责细胞培养及Western blot实验; 李殿龙负责RNA干扰及流式细胞实验; 王旭负责蛋白提取及

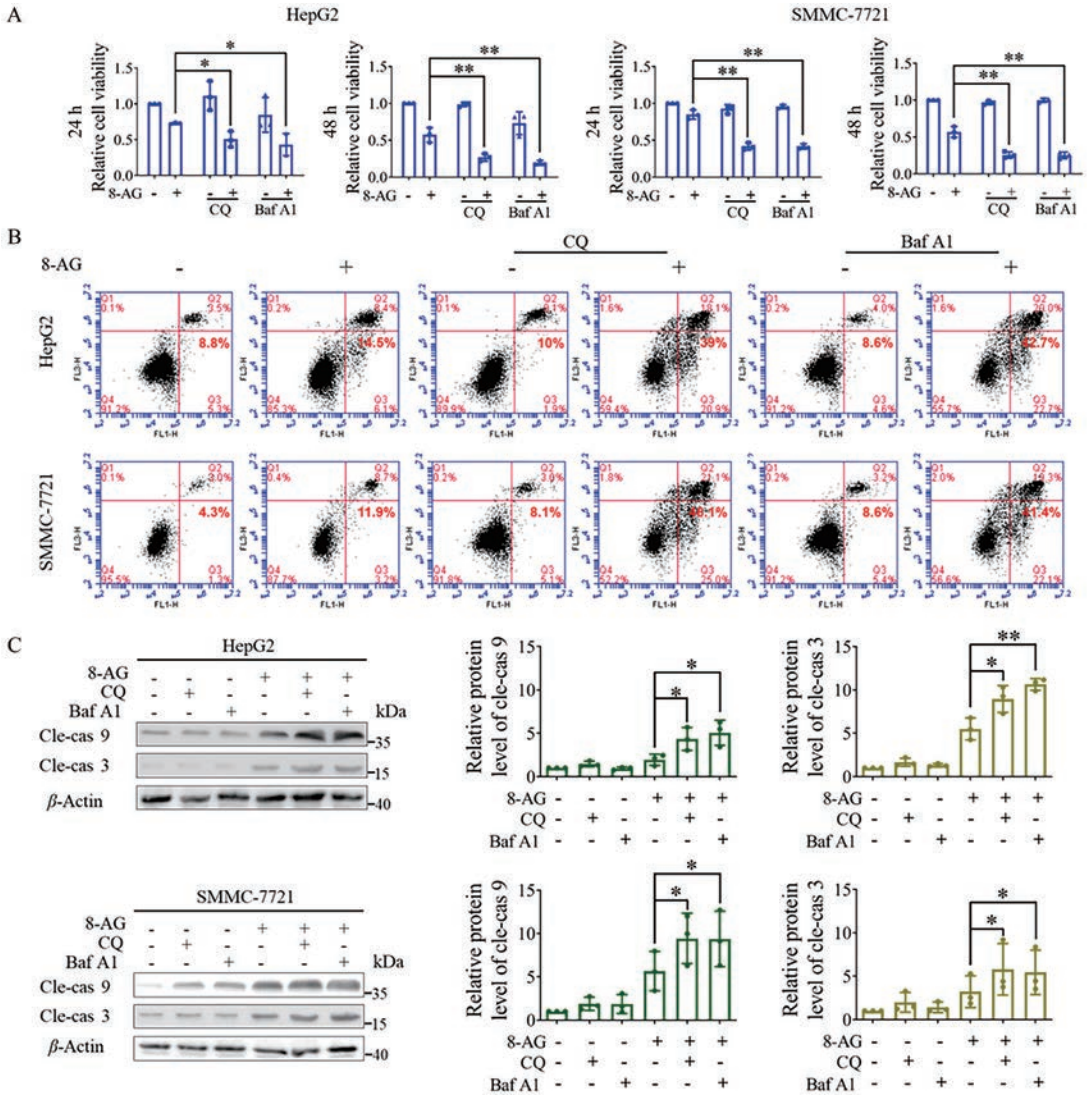

Figure 5 Combination treatment with 8-AG and autophagy inhibitors enhances the cytotoxicity of 8-AG. A: HepG2 or SMMC-7721 cells were treated with or without 5 μmol·L^{-1} 8-AG for 24 or 48 h, with a combination of chloroquine (CQ, 10 μmol·L^{-1}) or Baf A1 (200 nmol·L^{-1}) treatment. Cell viability was analyzed by MTT assay; B: Cells were co-treated with 8-AG and CQ or Baf A1 for 24 h. The percentage of apoptosis was determined by Annexin-V/PI staining and flow cytometry analysis; C: The same cells treated as B were used to detect the levels of caspase 9 and caspase 3 by Western blot. Protein expression was quantified by densitometric analysis and is represented as mean band intensity normalized to β-actin. $n = 3$, $\bar{x} \pm s$. $^{*}P < 0.05$, $^{**}P < 0.01$

ROS检测; 蔺洁茹负责质粒构建; 郝燕飞负责细胞免疫荧光; 张鑫朋负责MTT实验; 刁爱坡参与文章讨论; 刘振兴负责实验设计与论文撰写。

利益冲突: 所有作者均声明不存在利益冲突。

References

[1] Kidder GW, Dewey VC, Parks RE. Effect of lowered essential metabolite on 8-azaguanine inhibition [J]. J Biol Chem, 1952, 197: 193-198.

[2] Colsky J, Meiselas LE, Rosen SJ, et al. Response of patients with leukemia to 8-azaguanine [J]. Blood, 1955, 10: 482-492.

[3] Dourado M, Sarmento AB, Pereira SV, et al. CD26/DPPIV expression and 8-azaguanine response in T-acute lymphoblastic leukaemia cell lines in culture [J]. Pathophysiology, 2007, 14: 3-10.

[4] Choudhary A, Zachek B, Lera RF, et al. Identification of selective lead compounds for treatment of high-ploidy breast cancer [J]. Mol Cancer Ther, 2016, 15: 48-59.

[5] Kim N, Choi JW, Song AY, et al. Direct potentiation of NK cell cytotoxicity by 8-azaguanine with potential antineoplastic activity [J]. Int Immunopharmacol, 2019, 67: 152-159.

[6] Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy [J]. Dev Cell, 2004, 6: 463-477.

[7] Mizushima N, Levine B, Cuervo AM, et al. Autophagy fights disease through cellular self-digestion [J]. Nature, 2008, 451: 1069-1075.

[8] Mizushima N. The role of the Atg1/ULK1 complex in autophagy regulation [J]. Curr Opin Cell Biol, 2010, 22: 132-139.

[9] Jung CH, Ro SH, Cao J, et al. mTOR regulation of autophagy [J]. FEBS Lett, 2010, 584: 1287-1295.

[10] Kim J, Kundu M, Viollet B, et al. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1 [J]. Nat Cell Biol, 2011, 13: 132-141.

[11] Rubinsztein DC, Gestwicki JE, Murphy LO, et al. Potential therapeutic applications of autophagy [J]. Nat Rev Drug Discov, 2007, 6: 304-312.

[12] Degenhardt K, Mathew R, Beaudoin B, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis [J]. Cancer Cell, 2006, 10: 51-64.

[13] Apel A, Herr I, Schwarz H, et al. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy [J]. Cancer Res, 2008, 68: 1485-1494.

[14] Li W, Lin J, Shi Z, et al. Clomiphene citrate induces nuclear translocation of the TFEB transcription factor and triggers apoptosis by enhancing lysosomal membrane permeabilization [J]. Biochem Pharmacol, 2019, 162: 191-201.

[15] Rubin RC, Larson R, Rall DP. 8-Azaguanine (NSC-749). I. Preclinical toxicity studies and a preliminary report on intrathecal perfusion therapy for patients [J]. Cancer Chemother Rep, 1966, 50: 283-286.

[16] Brockman RW, Bennett LL,Jr., Simpson MS, et al. A mechanism of resistance to 8-azaguanine. II. Studies with experimental neoplasms [J]. Cancer Res, 1959, 19: 856-869.

[17] Sarmento-Ribeiro AB, Dourado M, Paiva A, et al. Apoptosis deregulation influences chemoresistance to azaguanine in human leukemic cell lines [J]. Cancer Invest, 2012, 30: 331-342.

[18] Yang ZJ, Chee CE, Huang S, et al. The role of autophagy in cancer: therapeutic implications [J]. Mol Cancer Ther, 2011, 10: 1533-1541.

[19] Paglin S, Hollister T, Delohery T, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles [J]. Cancer Res, 2001, 61: 439-444.

[20] Kanzawa T, Germano IM, Komata T, et al. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells [J]. Cell Death Differ, 2004, 11: 448-457.

[21] Hardie DG. The AMP-activated protein kinase pathway-new players upstream and downstream [J]. J Cell Sci, 2004, 117: 5479-5487.

[22] Sarkar S, Korolchuk VI, Renna M, et al. Complex inhibitory effects of nitric oxide on autophagy [J]. Mol Cell, 2011, 43: 19-32.

[23] Murata H, Ihara Y, Nakamura H, et al. Glutaredoxin exerts an antiapoptotic effect by regulating the redox state of Akt [J]. J Biol Chem, 2003, 278: 50226-50233.

[24] Shearn CT, Fritz KS, Reigan P, et al. Modification of Akt2 by 4-hydroxynonenal inhibits insulin-dependent Akt signaling in HepG2 cells [J]. Biochemistry, 2011, 50: 3984-3996.

[25] Makhov P, Golovine K, Teper E, et al. Piperlongumine promotes autophagy *via* inhibition of Akt/mTOR signalling and mediates cancer cell death [J]. Br J Cancer, 2014, 110: 899-907.

[26] Shukla S, Saxena S, Singh BK, et al. BH3-only protein BIM: an emerging target in chemotherapy [J]. Eur J Cell Biol, 2017, 96: 728-738.

[27] Akiyama T, Dass CR, Choong PF. Bim-targeted cancer therapy: a link between drug action and underlying molecular changes [J]. Mol Cancer Ther, 2009, 8: 3173-3180.

[28] Hubner A, Barrett T, Flavell RA, et al. Multisite phosphorylation regulates Bim stability and apoptotic activity [J]. Mol Cell, 2008, 30: 415-425.