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Synthesis and toxicity assessment of 3-oxobutanamides against human lymphocytes and isolated mitochondria

Nima Razzaghi-Asl[a], Enaytollah Seydi[b], Radin Alikhani[a], Saba Rezvani[a], Ramin Miri[c], Ahmad Salimi[d,*]

[a] Department of Medicinal Chemistry, School of Pharmacy, Ardabil University of Medical Science, Ardabil, Iran
[b] Research Center for Health, Safety and Environment (RCHSE), Department of Occupational Health Engineering, Alborz University of Medical Sciences, Karaj, Iran
[c] Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Science, Shiraz, Iran
[d] Department of Pharmacology and Toxicology, School of Pharmacy, Ardabil University of Medical Science, Ardabil, Iran

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ABSTRACT

To reduce costly late-phase compound scrubbing, there has been an increased focus on assessing compounds within in vitro assays that predict properties of human safety liabilities, before preclinical in vivo studies. The aim of our study was to answer the questions that whether the toxicity risk of a series of 3-oxobutanamide derivatives could be predicted by using of human lymphocytes and their isolated mitochondria. Using biochemical and flow cytometry assessments, we demonstrated that exposure of lymphocytes and isolated mitochondria to five 3-oxobutanamide derivatives (**1–5**) did not exhibit remarkable toxicity at low concentrations (50–500 μM) but toxicity could be observed at high concentrations (1000 and 2000 μM), particularly for *N*-(5-(4-bromophenyl)-3-isoxazolyl)-3-oxobutanamide (**4**) and *N*-(2-benzothiazolyl)-3-oxo butanamide (**5**). Compounds **4**, **5** and partly *N*-(5-methyl-3-isoxazol yl)-3-oxo butanamide (**1**) also showed a marked cellular and mitochondrial toxicity while compound **5** displayed superior toxicity. Compound **5** induced cytotoxicity on human blood lymphocytes which was associated with the generation of intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP) collapse, lysosomal membrane injury, lipid peroxidation and depletion of glutathione. Our results suggested that among assessed compounds, increased toxicity of compound **5** compared to other compounds could be likely attributed to the presence of bromine substituent in **5**. Finally our findings proposed that using of antioxidants and mitochondrial/lysosomal protective agents could be beneficial in decreasing the toxicity of **5**.

1. Introduction

Newly, there has been an increased centralizing on the field of predictive toxicology not only as a measure to limit critical adverse effects associated with drugs (Krewski et al., 2010), but also as an answer to a more challenging drug development where the percentage of compound attrition owing to safety-related reasons is at least 30% and the cost for launching a drug on the market is approximately $1 billion (Porceddu et al., 2012). The rapid growth of predictive toxicology has led to the development of a type of in vitro methods that are planned to predict toxicity, particularly hepatotoxicity since it is a major reason for late stage attrition. For instance, isolated organelle techniques that discover mitochondrial toxicity have been used as a predictor of liver injury (Porceddu et al., 2012). At the cellular level, high specificity in predicting organelle injury has been demonstrated (Porceddu et al., 2012).

It is supposed that the number of the chemicals exceed 10^{60} molecules, and it is impossible for man to make all of those molecules. Up to now, only about 27 million compounds have been recorded (Li and Vederas, 2009). Among these compounds drugs are a big family since many natural and synthetic compounds are introduced for their pharmacological applications every year while developing new drugs is more expensive and more difficult (Li and Vederas, 2009). According to previous studies the poor drug efficacy, toxicology, safety, and physicochemical characteristics are responsible for most of the drug attritions (Wang and Hou, 2009). The physical, chemical and toxicological properties of a compound

* Corresponding author at: Toxicology and Pharmacology School of Pharmacy, Ardabil University of Medical Science, P.O. Box: 56189-53141, Ardabil, Iran.
E-mail addresses: salimikd@yahoo.com, a.salimi@pharmacy.arums.ac.ir (A. Salimi).

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are all related to the unique molecular structure. The biological activity of a compound depends on two factors. First it must be transported from its site of administration to its site of action; then it must bind to or react with a receptor or target (Wang and Hou, 2009).

3-oxobutanamides or β-keto amides are privileged dicarbonyl compounds with suitable applications in the synthesis of bioactive heterocycles as initial or intermediate reactants (Synthesis, 2015; Iman et al., 2015). Besides their important role in producing varied pharmaceutical scaffolds, a few biological effects have been reported for 3-oxobutanamides and their hydrazone derivatives. Some derivatives of 3-oxobutanamides seemed promising for reducing glucose uptake in malignant cells via interaction with the intramembrane channel of glucose transport protein GLUT1. Such effect is therapeutically valuable since tumor cells may be more susceptible to the intracellular variations of glucose (Wood et al., 2008).

Our aim in this study was to evaluate 3-oxobutanamide derivatives by two experimental techniques, an organelle-based method using mitochondria and a cell-based method using human lymphocytes, to predict toxicity of these compounds.

2. Materials and methods

2.1. Chemistry

All the reagents used in this study were purchased from Sigma-Aldrich Company and used without further purification. Melting points were obtained with a Reichert-Jung hot-stage microscope and were uncorrected. FT-IR (KBr) spectra were recorded on a Perkin-Elmer Spectrum two spectrometer. The completion of the reactions was monitored by thin-layer chromatography (TLC) on precoated silica gel 60 F254 aluminum plates (Merck, Germany).

2.1.1. General procedure for the synthesis of N-(aryl)-3-oxobutanamides(1–5)

For further information on synthesis, purification and characterization procedure of 3-oxobutanmaides, readers are referred to our previous reports Compound **3** was obtained from Sigma-Aldrich and its purity was checked by TLC and FT-IR. Chemical properties and characteristics of the 3-oxobutanamides under study are summarized in Table 1 (Razzaghi-Asl et al., 2013; Miri et al., 2015).

2.2. Cellular assessments

2.2.1. Blood samples

All blood samples (n = 10) were acquired and approved by Blood Administration Center. The studies were performed at the school of Pharmacy, Shahid Beheshti University of Medical Sciences, under the guidance of an expert physician. This study was approved by the Shahid Beheshti University of Medical Sciences research ethics committee, and all healthy individuals signed an informed consent form.

2.2.2. Lymphocytes isolation

Lymphocytes were collected from healthy individuals at age range 25–35 years old. Blood was obtained from 10 healthy, non-smoking volunteers, who showed no signs of infection disease symptoms at the time that the blood samples were collected. Lymphocytes were isolated using Ficoll Paque Plus according to the manufacturer's instructions. The obtained lymphocytes were suspended in RPMI1640 medium with L-glutamine and 10% FBS. The final lymphocytes account used in the experiments was 1×10^6 cells/ml. The viability of the lymphocytes was over 95% (Salimi et al., 2016a).

2.2.3. Lymphocytes treatment

3-Oxobutanamide derivatives were dissolved in DMSO 0.05%. The final concentrations of compounds were in the range of 100–2000 μM. The cells were incubated with compounds for 24 h to analyze cell viability, and oxidative stress mechanistic parameters were studied within 12 h of incubation. The incubation was performed at 37 °C in 5% CO_2 atmosphere (Salimi et al., 2016a).

2.2.4. Cell viability

Lymphocytes (1×10^4 cells/well) were incubated in 96 well plates with and without 3-oxobutanamides for 24 h in a final volume of 10 μl. At the end of the treatment, 25 μl of MTT (5 mg/mL in RPMI) was added to each well and incubated for an additional 1 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO, and the absorbance was measured at 570 nm with ELISA reader. Each concentration was tested in three different experimental runs in three replicates for each sample (Salimi et al., 2016a).

2.2.5. Determination of ROS

To determine the rate of lymphocytes ROS generation induced by 3-oxobutanamide derivatives, dichlorofluorescein diacetate (DCFH-DA, 1.6 μM) was added to the lymphocytes. It penetrates lymphocytes and becomes hydrolyzed to non-fluorescent dichlorofluorescein (DCFH). The latter then reacts with ROS to form the highly fluorescent dichlorofluorescein (DCF), which effluxes the cell. The fluorescence intensity of DCF was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 nm and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (Salimi et al., 2016a).

2.2.6. MMP assay

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123 (1.5 μM), has been used for the estimation of mitochondrial membrane potential. The amount of rhodamine 123 remaining in the incubation medium was measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to take up the rhodamine 123 was calculated as the difference (between control and treated cells) in rhodamine 123 fluorescence (Salimi et al., 2016a).

2.2.7. Lysosomal membrane integrity assay

Lymphocytes lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange. Aliquots of the cell suspension (0.5 ml) that were previously stained with acridine orange (5 μM) were separated from the incubation medium by 1 min centrifugation at 1000 rpm. Cell washing process was carried out twice to remove the fluorescent dye from the media. Acridine orange redistribution in the cell suspension was then measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths (Salimi et al., 2016a, 2015; Seydi et al., 2015).

2.2.8. Lipid peroxidation

Evaluation of lipid peroxidation in lymphocytes was conducted by determining the amount of thiobarbituric acid reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532 nm in a Beckman DU-7 spectrophotometer (Salimi et al., 2016a).

2.2.9. GSH and GSSG

GSH and GSSG were determined according to the spectrofluorometric method. Each sample was measured in quartz cuvettes