Oxy-Imino Saccharidic Derivatives as a New Structural Class of Aldose Reductase Inhibitors Endowed with Anti-oxidant Activity

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Biological assays

The biological assays were performed following a previously described protocol\(^1\)

661w cell culture.

The 661W cell line, derived from immortalized cone photoreceptors (provided by Muayyad Al-Ubaidi, University of Oklahoma), was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, ThermoFisher Scientific, Waltham, MA) containing 4.5 g/l (24.5 mM) glucose and supplemented with 10% (v/v) heat-inactivated FBS, 1% L-glutamine and 1% pen/strep. For the experiments, confluent cells were maintained in 24.5 mM glucose (normoglycemic condition) or cultured in 55 mM glucose (hyperglycemic condition) during 24 h in the presence or absence of the test compound (Z)-8.

In vitro ALR2 enzymatic inhibition.

Briefly, cells were seeded in 24-well tissue culture plates and allowed to stabilize overnight in a 5% CO\(_2\) incubator at 37 °C. Then, they were cultured in 24.5 mM (NG) or 55 mM (HG) glucose-containing medium in the absence or presence of (Z)-8 (50 and 100 \(\mu\)M) for 24 h. After that, cells were mechanically lysate and the supernatant were collected by centrifugation and incubated with Aldo-Keto Reductase (AKR) Activity Assay Kit (AbCam) in a 96-well for 2 h, then absorbance was measured with a spectrophotometer (NanoQuant) at 450 nm.

Analysis of cellular viability and detection of apoptosis.

Briefly, quantification of cellular viability and apoptosis were performed using flow cytometry methods. Cells were seeded in 24-well tissue culture plates and allowed to stabilize overnight in a 5% CO\(_2\) incubator at 37 °C. Then, they were cultured in 24.5 mM (NG) or 55 mM (HG) glucose-containing medium in the absence or presence of (Z)-8 (50 and 100 \(\mu\)M) for 24 h. After the induction of HG condition, adherent and non-adherent cells were collected by centrifugation and incubated with Guava\textsuperscript{\textregistered} ViaCount\textsuperscript{\textregistered} Reagent or Muse\textsuperscript{TM} Annexin V & Dead Cell Kit (Millipore) which, thanks to the presence of specific DNA-binding dyes, are able to distinguish viable, apoptotic, and dead cells. Data collected were analyzed with Muse 1.5 Software (Millipore).

Immunocytochemistry.

Briefly, cells were directly washed with PBS and fixed in PAF for 1 min, then washed with PBS twice for 10 min and incubated with PBS containing 0.03% Triton and 5% of bovine serum
albumin (1 h at room temperature), and the primary antibody anti-Nrf2 (1:50, overnight at 4°C). Samples were then washed three times with PBS and subsequently incubated with the secondary goat anti-rabbit immunoglobulin G (IgG; 1:1000; Vector Laboratories) 488-Alexa flour conjugated for 2 h at room temperature. After washing with PBS, samples were incubated with Ethidium Bromide (1:5000, Sigma-Aldrich) for 5 min. Finally, after washing twice with PBS, the chambers were detached from the slide and mounted with Vectashield mounting medium (Vector Laboratories). This immunolabeling protocol was modified respect to Wang et al., 2019. The slides were examined with a fluorescent microscope (Nikon E-Ri). Images were processed with ImageJ software.

**Western blot.**

Levels of Sod1 and Sod2 proteins were normalized by the levels of their corresponding total proteins by using the Stain Free Technology (BioRad). The chemiluminescence was analyzed by using the Chemidoc Quantified (Bio-Rad) and the images obtained analyzed with ImageLAB Software, with the normalization of protein levels versus the total protein content (Bio-Rad Technologies).

**References**

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$^3$H NMR spectra of compound 1 (400 MHz, CDCl$_3$)

$^{13}$C NMR spectra of compound 1 (100 MHz, CDCl$_3$)
COSY spectra of compound 1 (400 MHz, CDCl₃)

HSQC spectra of compound 1 (400 MHz, CDCl₃)
$^1$H NMR spectra of mixture of two C-5 anomeric 6a (400 MHz, CDCl$_3$)

$^{13}$C NMR spectra of mixture of two C-5 anomeric 6a (100 MHz, CDCl$_3$)
DEPT-135 spectra of mixture of two C-5 anomeric 6a (100 MHz, CDCl₃)

HSQC spectra of mixture of two C-5 anomeric 6a (400 MHz, CDCl₃)
$^1$H NMR spectra of (Z)-8 (250.13 MHz, CD$_3$CN)

$^1$H NMR spectra of (E)-8 (250.13 MHz, CD$_3$CN)

$^1$H NMR spectra of mixture (E)-8+(Z)-8 (250.13 MHz, CD$_3$CN)
$^1$H NMR spectra of (E)-8 (250.13 MHz, CD$_3$CN)

$^{13}$C NMR spectra of (E)-8 (62.9 MHz, CD$_3$CN)
COSY spectra of (E)-8 (250.13 MHz, CD3CN)

HSQC spectra of (E)-8 (250.13 MHz, CD3CN)
$^1$H NMR spectra of (Z)-8 (250.13 MHz, CD$_3$CN)

$^{13}$C NMR spectra of (Z)-8 (62.9 MHz, CD$_3$CN)
COSY spectra of (E)-8 (250.13 MHz, CD$_3$CN)

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$^{13}$C NMR spectra of mixture of (E)-10+(Z)-10 (100 MHz, CD$_3$CN)
DEPT-135 spectra of mixture of (E)-10+(Z)-10 (100 MHz, CD$_3$CN)

$^1$H NMR spectra of mixture of (E)-11+(Z)-11 (400 MHz, CD$_3$OD)
$^{13}$C NMR spectra of mixture of (E)-11+(Z)-11 (100 MHz, CD$_3$CN)

DEPT-135 spectra of (E)-11+(Z)-11 (100 MHz, CD$_3$CN)
$\text{H NMR spectra of compound 12 (400 MHz, CD$_3$CN)}$

$\text{COSY spectra of mixture of (E)-11+(Z)-11 (400 MHz, CD$_3$OD)}$
$^{13}$C NMR spectra of compound 12 (100 MHz, CD$_3$CN)

DEPT-135 spectra of compound 12 (100 MHz, CD$_3$CN)
COSY spectra of compound 12 (400 MHz, CD$_3$CN)

HSQC spectra of compound 12 (400 MHz, CD$_3$CN)