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Short Communication

The SGLT2-inhibitor dapagliflozin improves neutropenia and neutrophil dysfunction in a mouse model of the inherited metabolic disorder GSDIb

Roberta Resaz [a,1], Federica Raggi [a,1], Daniela Segalerba [a,1], Chiara Lavarello [b], Alessandra Gamberucci [c], Maria Carla Bosco [a], Simonetta Astigiano [d], Antonia Assunto [e], Daniela Melis [f], Mariavittoria D'Acierno [g,h], Maria Veiga-da-Cunha [i,j], Andrea Petretto [b], Paola Marcolongo [c], Francesco Trepiccione [g,h,2], Alessandra Eva [a,*,2]

[a] Laboratory of Molecular Biology, IRCCS Istituto Giannina Gaslini, Via Gerolamo Gaslini 5, 16147 Genova, Italy
[b] Core Facilities-Clinical Proteomics and Metabolomics, IRCCS, Istituto Giannina Gaslini, Via Gerolamo Gaslini 5, 16147 Genova, Italy.
[c] Department of Molecular and Developmental Medicine, Università degli Studi di Siena, Siena, Italy
[d] Animal Facility, IRCCS Ospedale Policlinico San Martino, Genova, Italy
[e] Medical, Surgical, and Dental Department, Università degli Studi di Salerno, Salerno, Italy.
[f] Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana," Section of Pediatrics, Università degli Studi di Salerno, Via Salvador Allende, 43 84081, Baronissi, Salerno, Italy
[g] Biogem Research Institute "Gaetano Salvatore", Ariano Irpino, Italy
[h] Department of Translational Medical Sciences, Università degli Studi della Campania "L. Vanvitelli", Naples, Italy
[i] Walloon Excellence in Lifesciences and Biotechnology, B-1200 Brussels, Belgium
[j] Groupe de Recherches Metaboliques, de Duve Institute, UCLouvain, (Université Catholique de Louvain), B-1200 Brussels, Belgium

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ABSTRACT

Glycogen Storage Disease type 1b (GSDIb) is a genetic disorder with long term severe complications. Accumulation of the glucose analog 1,5-anhydroglucitol-6-phosphate (1,5AG6P) in neutrophils inhibits the phosphorylation of glucose in these cells, causing neutropenia and neutrophil dysfunctions. This condition leads to serious infections and inflammatory bowel disease (IBD) in GSDIb patients. We show here that dapagliflozin, an inhibitor of the renal sodium-glucose co-transporter-2 (SGLT2), improves neutrophil function in an inducible mouse model of GSDIb by reducing 1,5AG6P accumulation in myeloid cells.

1. Introduction

GSDIb is an inherited rare disease caused by a defect in glucose-6-phosphate (G6P) translocase,(G6PT/SLC37A4). GSDIb is characterized by severe hypoglycemia, growth retardation, osteoporosis, and long-term risk of liver tumors and renal failure (1). In addition, GSDIb patients are affected by neutropenia and myeloid dysfunctions that predispose to inflammatory bowel disease (IBD) and recurrent bacterial infections sometimes leading to life-threatening complications. Treatment with granulocyte colony stimulating factor improves neutropenia and reduces the frequency of infections and severity of IBD but increases the risk to develop myeloid neoplasm (2,3).

The cause of neutropenia and neutrophil dysfunction in GSDIb was recently ascribed to the lack of transportation of the glucose-6-phosphate analog 1,5-anhydroglucitol-6-phosphate (1,5AG6P) by G6PT into the endoplasmic reticulum, to be dephosphorylated by the phosphatase G6PC3. It was proposed that the failure to destroy this non-canonical metabolite causes its accumulation in neutrophil cytosol,

Abbreviations: GSDIb, Glycogen Storage Disease type 1b; 1,5AG, 1,5-anhydroglucitol; 1,5AG6P, 1,5-anhydroglucitol-6-phosphate; SGLT2, sodium-glucose co-transporter-2; G6PT, glucose-6-phospate translocase; G6PC3, glucose-6-phosphatase C3; TM, tamoxifen; CFU, colony forming units; BM, bone marrow; G-CSF, granulocyte colony stimulating factor; M-CSF, macrophage colony stimulating factor; NET, neutrophil extracellular trap; PMA, phorbol myristate acetate; fMLP, N-formyl-L-methionyl-L-leucyl-phenylalanine; PRM, parallel reaction monitoring.

* Corresponding author.
E-mail address: alessandreva@gaslini.org (A. Eva).
[1] Roberta Resaz, Federica Raggi, and Daniela Segalerba contributed equally to this work.
[2] Francesco Trepiccione and Alessandra Eva share the last authorship.

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inhibiting glucose phosphorylation by hexokinases and thus glycolysis in these cells (4). Gliflozins are drugs currently used for treating type 2 diabetes mellitus, since they reduce the renal threshold for glucose reabsorption by blocking the renal sodium-glucose co-transporter-2, SGLT2, and by causing glycosuria (5). Since it was shown that glucose competes with the renal re-uptake of 1,5-anhydroglucitol (1,5AG) (6,7), treatment with gliflozins should ameliorate neutrophil dysfunction by increasing urine excretion of 1,5AG. In fact, in recent studies, it was shown that treatment with empagliflozin improves neutrophil function in G6PC3-deficient mice and in a few G6PC3- or G6PT- deficient patients (4,8).

We have addressed the problem of neutrophil dysfunction in a mouse model of GSDIb (9). We report here that dapagliflozin treatment significantly improves myeloid cells' function in these mice.

2. Material and methods

2.1. TM-G6PT$^{-/-}$ mice

6–8-week-old G6PT$^{lox/lox}$.creER/wt mice, hereafter referred to as G6PT$^{+/+}$, were injected intraperitoneally with 1 mg/10 g body weight/day of tamoxifen (TM) for five consecutive days to obtain TM-G6PT$^{-/-}$ mice, as described (9). Dapagliflozin was obtained from AstraZeneca (NCR-16-12,171) and administered to adult mice in drinking water (1.5 mg/kg/day) for 10 days, starting three days before TM induction. All animal experiments were reviewed and approved by the internal Review Board (OPBA) and authorized by the Italian Ministry of Health, accordingly with the current National and European regulations and guidelines for the care and use of laboratory animals (D.L. 26/2014; 86/609/EEC Directive).

2.2. Evaluation of myeloid cell functions

Bone marrow (BM) cells were harvested from femoral and tibia bones by flushing with 3 ml of PBS. Neutrophils were harvested as previously described (9). Analysis of neutrophil functional activity and hematopoietic progenitor cell assays were performed with standard techniques, as described (9). Tunel assay was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Cat. No. 11684795910) according to the manufacturer's instructions. Quantification of neutrophil extracellular trap (NET) was done using the membrane-impermeable DNA binding dye SYTOX green (10). Calcium fluxes were measured as previously reported (9). Three or four mice were used per group.

2.3. 1,5AG6P determination

Exactive Orbitrap mass spectrometer coupled to UHPLC Vanquish Horizon were used to quantify 1,5AG and 1,5AG6P. BM neutrophils were prepared according to the previously published protocol (4). Extracted metabolites were separated with hydrophobic interaction liquid chromatography columns using A (10 mM ammonium formate in H_2O, 0.1% formic acid) and B (acetonitrile) eluents. Data were obtained in PRM by acquiring the accurate *m/z* of 243.0275 and 163.0612 Da. Data were processed with MSDIAL software (11) using a customized database. The extracted areas were normalized by the total ion current.

2.4. Statistics

Animal experiments were performed with 3 mice per group. Results are reported as means ± SEM of three different experiments. Unpaired Student's *t*-test was performed using the GraphPad Prism program, version 5 (GraphPad Software, San Diego, CA, USA), and Perseus software (12). Values were considered statistically significant at p value ≤ 0.05.

3. Results

We have previously characterized the activity of the neutrophils purified from BM of TM-G6PT$^{-/-}$ mice and demonstrated that they were affected by the same dysfunctions as GSDIb patients, but with a milder phenotype (9). Specifically, we found that an inactive G6PT leads to neutrophil apoptosis, hampered chemotaxis and phagocytic activity, and that the colony forming units (CFU) of BM precursor cells in response to G-CSF and M-CSF was impaired in these mice. In the present study we evaluated the functional recovery of neutrophils in TM-G6PT$^{-/-}$ mice as a consequence of dapagliflozin treatment. We first showed that the number and maturation state of neutrophils was markedly improved in mice treated with dapagliflozin (Fig. 1a). Neutrophils of treated TM-G6PT$^{-/-}$ mice had a higher phagocytic activity (Fig. 1b) and migratory capacity (Fig. 1c). In addition, the CFU of BM precursor cells in response to G-CSF (Fig. 1d) improved upon treatment with dapagliflozin. The CFU of BM precursor cells also improved in response to M-CSF (Fig. 1e), consistent with monocyte/macrophage dysfunction in GSDIb patients. Neutrophils of treated mice displayed less apoptosis (Fig. 1f) and increased formation of NETs in response to PMA (Fig. 1g), an indication of functional recovery of a specific antimicrobial activity. Moreover, mobilization of Ca^{2+} in neutrophils from dapagliflozin-treated mice increased in response to fMLP compared to untreated TM-G6PT$^{-/-}$, though not in a statistically significant manner, suggesting a partial restoration of calcium fluxes (Fig. 1h). Analysis of 1,5AG6P confirmed the reduction of this metabolite in neutrophils of dapagliflozin-treated mice. In fact, while 1,5AG was barely detectable in neutrophils, independently of the source of the cells, significant accumulation of 1,5AG6P was observed in TM-G6PT$^{-/-}$ mice in comparison with G6PT$^{+/+}$ mice. This accumulation was prevented by treatment with dapagliflozin (TM-G6PT$^{-/-}$ + DAPA). (Fig. 1i),

4. Discussion

The identification of the metabolite 1,5AG6P exerting toxic effects in neutrophils has led to therapeutic perspectives for GSDIb (4). Evidence of the positive effects of gliflozins in reducing 1,5AG6P accumulation and thus improving neutrophil counts and function has been shown for G6PC3-deficient mice. These results have consequently led to preliminary off-label use of empagliflozin in few GSDIb patients. Despite the rather low number of treated patients, the results obtained are very encouraging, demonstrating an approximate 6-fold decrease in the concentration of 1,5AG in plasma and of 1,5AG6P in neutrophils which leads to a good recovery of neutrophil activity and an improvement of GSDIb-associated symptoms (4,8,13,14,15). Here, we report the results obtained in the GSDIb mouse model we have generated in terms of neutropenia and neutrophils' functional activity recovery upon treatment with the SGLT2 inhibitor dapagliflozin, an empagliflozin analog. We provide statistically significant evidence of the positive role of dapagliflozin in improving neutropenia and restoring neutrophil functions in GSDIb. Moreover, we demonstrate that dapagliflozin prevents 1,5AG6P accumulation in neutrophils and improves response to M-CSF of monocyte/macrophage, another cell type that shows metabolic dysfunction and signaling defects in GSDIb (16). The use of animal models is the cornerstone for understanding the pathophysiology and for progressing in the quest for treatment of "neglected" diseases, such as GSDIb, and is one of the best ways to thoroughly evaluate a new therapy in terms of efficacy and potential risks. We have for now experimented and reported here the efficacy of this drug. Based on pharmacokinetic, pharmacodynamic, and pharmacologic properties, dapagliflozin has a similar affinity for SGLT2 receptor but has a faster onset and a longer half life than empagliflozin (17), possibly ensuring a more effective treatment of GSD1b neutropenia and neutrophil dysfunction. On the other hand, the assessment of potential risks for both drugs will have to wait other experimental approaches and long-term evaluation.

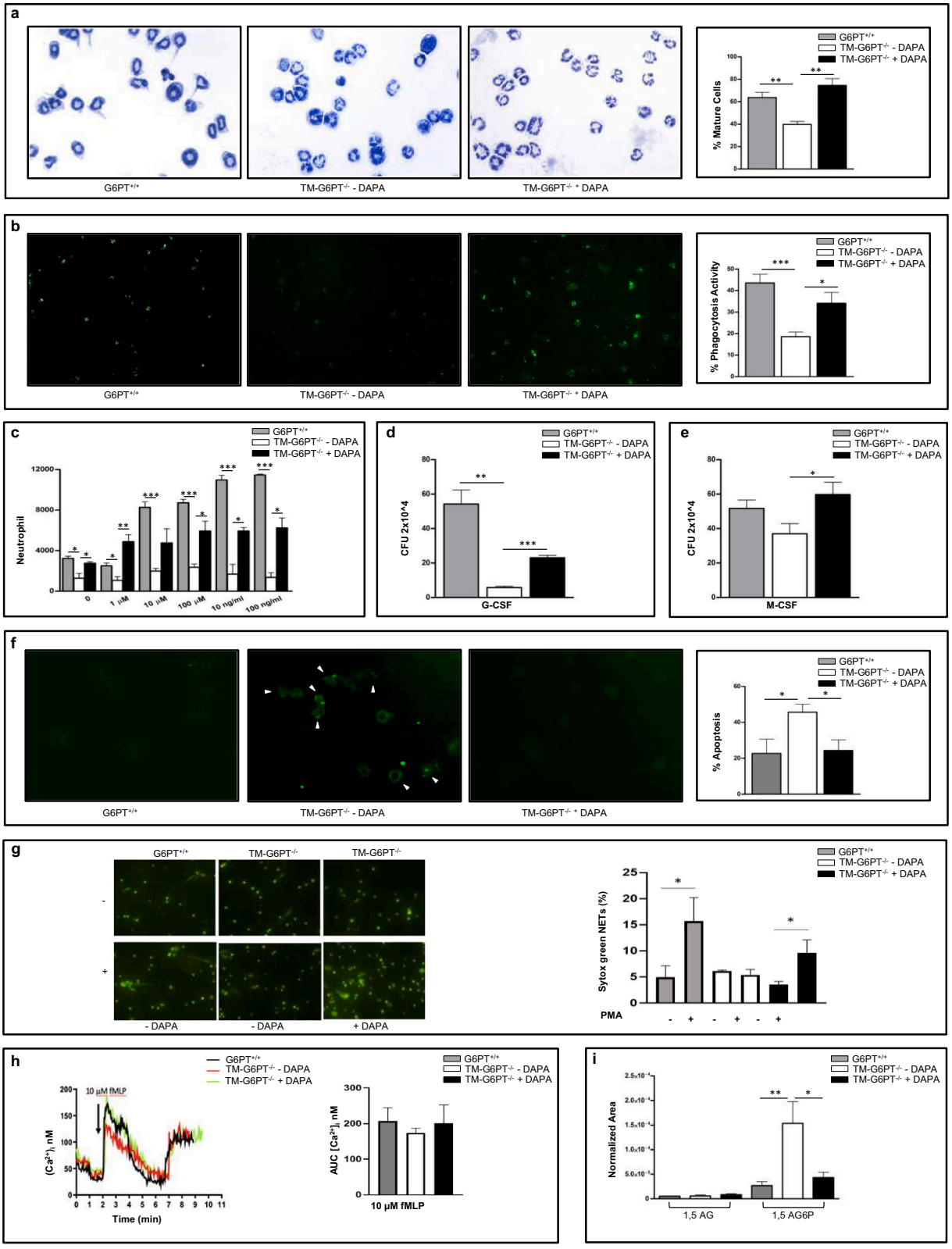

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Fig. 1. Evaluation of the impact of dapagliflozin on myeloid cell functions in a GSD1b mouse model.
(a) Morphological evaluation of neutrophils and their precursors. Left panel: neutrophil maturation state was determined by microscopy examination (200× magnification). Right panel: results are shown as bar graphs. *p*-Values of TM-G6PT$^{+/+}$ or dapagliflozin treated (+DAPA) relative to untreated (-DAPA) TM-G6PT$^{-/-}$ mice: $^{**}p \leq 0.01$.
(b) Phagocytosis activity of neutrophils and their precursors. Left panel: uptake of *E. coli* by neutrophils was evaluated by immunofluorescence after incubation at 37 °C for 60 min. Right panel: results are shown as a bar graph and expressed as percentages of cells internalizing *E. coli*. *p*-Values of TM-G6PT$^{+/+}$ or dapagliflozin treated (+DAPA) relative to untreated (-DAPA) TM-G6PT$^{-/-}$ mice: $^{*}p \leq 0.05$; $^{***}p \leq 0.001$. **(c)** Chemotactic activity of neutrophils and their precursors measured in response to fMLP and CXCL2 by microscopy examination (40× magnification). Results are shown as a bar graph by counting the number of labelled cells on plate-well membrane. *p*-Values of TM-G6PT$^{+/+}$ or dapagliflozin treated (+DAPA) relative to untreated (-DAPA) TM-G6PT$^{-/-}$ mice: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$ $^{***}p \leq 0.001$ **(d-e).** Colony-forming unit (CFU) was evaluated following the stimulation of bone marrow cells with G-CSF (**d**) or M-CSF (**e**). Results are shown as a bar graph and expressed as number of scored colonies after 7–10 days of culture. *p*-Values of TM-G6PT$^{+/+}$ or dapagliflozin treated (+DAPA) relative to untreated (-DAPA) TM-G6PT$^{-/-}$ mice $^{*}p \leq 0.05$; $^{**}p \leq 0.01$ $^{***}p \leq 0.001$. **(f)** Apoptosis of neutrophils and their precursors. Left panel: cells were stained with TUNEL (green) to reveal apoptotic cells and nuclei were stained with DAPI (blue). Apoptotic cells are indicated by the arrowheads. Right panel: results are shown as a bar graph and expressed as percentages of apoptotic cells. *p*-Values of TM-G6PT$^{+/+}$ (CTR) or dapagliflozin treated (+DAPA) relative to untreated (-DAPA) TM-G6PT$^{-/-}$ mice: $^{*}p \leq 0.05$. **(g)** NETs formation of neutrophils and their precursors. Left panel: neutrophils were treated with the membrane-impermeable DNA binding dye SYTOX green to reveal NET formation. Right panel: NETs were evaluated by counting the percentage of NETs positive cells versus the total number of cells following a 2 h stimulation with 100 nM PMA. p-Values of PMA stimulated cells relative to unstimulated cells derived from TM-G6PT$^{+/+}$ and treated (+DAPA) or untreated (-DAPA) TM-G6PT$^{-/-}$ mice: $^{*}p \leq 0.05$. **(h)** Calcium release in neutrophils and their precursors. Left panel: representative traces of calcium release in Fura-2-loaded-cells derived from TM-G6PT$^{+/+}$ and dapagliflozin treated (+DAPA) or untreated (-DAPA) TM-G6PT$^{-/-}$ mice and stimulated with fMLP. Right panel: results are shown as a bar graph and expressed as quantification of calcium release. The mean ± SEM of the AUC of three different experiments is shown. **(i)** quantification of 1,5AG and 1,5AG6P in neutrophils and their precursors. Results are shown as a before-after graph and expressed as quantification of both metabolites. The mean ± SEM of the AUC of three different experiments is shown. *p*-Values of TM-G6PT$^{+/+}$ or dapagliflozin treated (+DAPA) relative to untreated (-DAPA) TM-G6PT$^{-/-}$ mice: $^{*}p \leq 0.05$;$^{**}p \leq 0.01$. Images shown are representative of three experiments. Each value represents the mean of the measurement of three or four mice.

Declaration of Competing Interest

The authors declare no conflict of interest.

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