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Identification of early gene expression changes in primary cultured neurons treated with topoisomerase I poisons

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ABSTRACT

Topoisomerase 1 (TOP1) poisons like camptothecin (CPT) are currently used in cancer chemotherapy but these compounds can have damaging, off-target effects on neurons leading to cognitive, sensory and motor deficits. To understand the molecular basis for the enhanced sensitivity of neurons to CPT, we examined the effects of compounds that inhibit TOP1—CPT, actinomycin D (ActD) and β-lapachone (β-Lap)—on primary cultured rat motor (MN) and cortical (CN) neurons as well as fibroblasts. Neuronal cells expressed higher levels of *Top1* mRNA than fibroblasts but transcript levels are reduced in all cell types after treatment with CPT. Microarray analysis was performed to identify differentially regulated transcripts in MNs in response to a brief exposure to CPT. Pathway analysis of the differentially expressed transcripts revealed activation of ERK and JNK signaling cascades in CPT-treated MNs. Immediate-early genes like *Fos*, *Egr-1* and *Gadd45b* were upregulated in CPT-treated MNs. *Fos* mRNA levels were elevated in all cell types treated with CPT; *Egr-1*, *Gadd45b* and *Dyrk3* transcript levels, however, increased in CPT-treated MNs and CNs but decreased in CPT-treated fibroblasts. These transcripts may represent new targets for the development of therapeutic agents that mitigate the off-target effects of chemotherapy on the nervous system.

1. Introduction

Topoisomerase I (TOP1) relaxes DNA supercoiling so that replication and gene transcription can proceed. TOP1 introduces single-strand DNA breaks, or nicks, in the supercoiled DNA by covalently binding to the DNA and forming a TOP1 cleavage complex (TOP1cc) [1]. Once the DNA is sufficiently unwound, the TOP1cc facilitates the religation of the nick and TOP1 is released from the DNA. TOP1ccs are normally very transient structures; camptothecin (CPT) and its derivatives act as interfacial inhibitors of TOP1 by reversibly stabilizing the complex. This stabilization prevents the religation of the double-stranded DNA [1] resulting in double-strand DNA breaks. As such, CPT-based compounds are sometimes referred to as TOP1 poisons as opposed to inhibitors [2]. Actinomycin D (ActD), in addition to its action as a transcription inhibitor [3], also stabilizes the TOP1cc and, therefore, acts as a TOP1 poison [4]. Alternatively, β-lapachone (β-Lap) is a direct inhibitor of TOP1 and does not interact with the TOP1cc [5].

Anti-cancer drugs like CPT and its derivatives can cause DNA damage and oxidative stress in non-target tissues like neurons [1,2]. The non-specific action of chemotherapy drugs on the central nervous system may contribute to chemotherapy-induced cognitive impairment, a phenomenon formerly known as “chemobrain” [6]. Dysarthria, which is an impairment of the motor component of speech, has been observed in cancer patients treated with the CPT

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prodrug irinotecan [7,8].

Neurons are exceptionally susceptible to DNA damaging agents like CPT because they maintain high levels of RNA synthesis and are more sensitive to oxidative stress [9]. We hypothesize that neurons differentially regulate the expression of genes that confer this increased susceptibility. In this study, we identified, by microarray, early transcripts differentially regulated in primary cultured rat neuronal (motor (MN) and cortical (CN) neurons) and non-neuronal (fibroblasts) cells after a brief exposure to the TOP1 inhibiting agents CPT, ActD and β-Lap.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted in accordance with the protocols described in the National Institutes of Health *Guide for the Care and Use of Animals* and were approved by the Nemours Biomedical Research Institutional Laboratory Animal Care and Use Committee.

2.2. Embryonic rat primary culture

Primary cultures of rat MNs, CNs and fibroblasts were obtained from embryonic day 15 (e15) Sprague-Dawley rat pups. Timed-pregnant dams (Charles River; Wilmington, MA) were deeply anesthetized using CO_2 and individual embryos were placed on ice in Hank's balanced salt solution (HBSS; Life Technologies, Grand Island, NY) after the uteri were remove. CNs and MNs were plated at a density of 5.0×10^4 cells/cm^2 on coverslips coated with 100 μg/mL poly-DL-ornithine (Sigma) and 2 μg/mL laminin (Millipore, Billerica, MA) for immunofluorescence or at a density of 2.5×10^6 cells/cm^2 on 10-cm dishes coated with poly-DL-ornithine and laminin for RNA extraction. Fibroblasts were plated at the same density but on gelatin-coated coverslips or 10-cm dishes.

For primary CNs and MNs, superficial segments of the forebrain or thoracolumbar spinal cords were dissected from embryos, rinsed in HBSS and incubated in 0.05% trypsin/EDTA (Life Technologies, Grand Island, NY) for 15 min at 37 °C. After trypsinization, the cells were triturated in Neuron Medium (Neurobasal medium supplemented with 1% B27, 2% horse serum, 500 μM L-glutamine, 1% penicillin/streptomycin (pen/strept), (all from Life Technologies, Grand Island, NY), 25 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and 1 ng/mL brain-derived neurotrophic factor (BDNF; R&D Systems, Minneapolis, MN)). MNs were separated from other spinal cord dissociated cells using Opti-Prep (Sigma-Aldrich; final concentration = 3%; 500*g* for 15 min) and then centrifuged (300*g* for 10 min) through a 4% bovine serum albumin (BSA; Sigma, St. Louis, MO) cushion. Both MNs and CNs were maintained in Neuron Medium.

Primary fibroblasts were harvested from the white tissues of the embryo by trypsinization. The pellets were resuspended in high glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% pen/strept. They were maintained in T-150 flasks for 2 passages prior to experiments.

These culturing conditions yielded high purities of the targeted cell types from embryonic rat tissues as shown using cell type-specific markers (β3-tubulin, neurofilaments and Fox3 for neurons, HB9 for MNs and fibronectin for fibroblasts; data not shown). In the primary CNs and MN cultures, there were very few (less than 5%) GFAP$^+$ glial cells present.

2.3. Drug response assays

Rat embryonic primary MNs, CNs and fibroblasts were treated with either DMSO (drug vehicle) or different concentrations of the following TOP1 inhibitors (n = 3/dose/drug): camptothecin (CPT; Tocris Biosciences, Bristol, UK; 10 nM–100 μM), β-lapachone (β-Lap, Sigma-Aldrich; 10 nM–10 μM), and actinomycin D (ActD, Sigma-Aldrich; 10 nM–10 μM). Primary cultures were treated for 24 h starting at either 2 days in vitro (DIV2) or 5 days in vitro (DIV5).

2.4. Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 15 min at room temperature. The cultures were then rinsed in PBS, permeabilized with 0.1% Triton-X100 (Sigma, St. Louis, MO) in PBS and then blocked with 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA) in PBS for 1 h at room temperature. Coverslips were then incubated with primary antibodies (see Supplementary Table 1) overnight at 4 °C, rinsed and incubated with combinations of AlexaFluor 488- and AlexaFluor 594-conjugated anti-rabbit and anti-mouse IgGs (Life Technologies; 1:500) for 60 min at room temperature. The cells were counterstained with Hoechst 33258 (1 μg/mL; Life Technologies) for 10 min at room temperature and mounted onto glass microscope slides with fluorescent mounting medium (DAKO, Carpenteria, CA). Cells were imaged using a DMRXA2 epifluorescence microscope (Leica Microsystems) with an ORCA-ER cooled CCD camera (Hamamatsu, Hamamatsu City, Japan) and OpenLab 5 software (Improvision Ltd, Lexington, MA).

Images from 5 representative fields (392 μm × 512 μm) were collected from each coverslip using a 20× objective. After the first field was selected, non-overlapping images were captured above, below, to the left and to the right of the first field. The non-pyknotic cells were counted using ImageJ (National Institutes of Health, Bethesda, MD). The cell counts were averaged for each coverslip. Relative viability was defined as the percentage of non-pyknotic cells in response to TOP1 inhibitor treatment relative to DMSO-treated cells.

2.5. RNA isolation

Primary cultures at DIV2 were treated with 1 μM CPT, 4 μM β-Lap, 1 μM ActD or DMSO for 2 h. Total RNA was isolated from rat embryonic primary MNs, CNs and fibroblasts using the RNeasy Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's directions. RNA integrity was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

2.6. Microarray analysis

Targets were prepared from 200 ng RNA using the Applause WT-Amp Plus ST System with the Encore Biotin Module (NuGEN, Inc., San Carlos, CA) following the manufacturer's protocol. Samples were hybridized to GeneChip Rat Gene 1.0ST Arrays (Affymetrix). These arrays were washed and stained in a GeneChip Fluidic Station 450 (Affymetrix) and then scanned with the GeneChip scanner 3000 7G (Affymetrix) following the manufacturer's protocols. The raw data were deposited into the NCBI Gene Expression Omnibus (GEO) under the accession number GSE67146.

2.7. Microarray data analysis

Expression values were calculated and LIMMA (linear models for microarray analysis) was performed as described previously [10]. The rank product non-parametric method RankProd was also used [11]. A false discovery rate (FDR) and an estimated percentage false positive (PFP) value less than 0.05 were considered significant. Identification of biological pathways and upstream regulators in rat