**Supplementary Information**

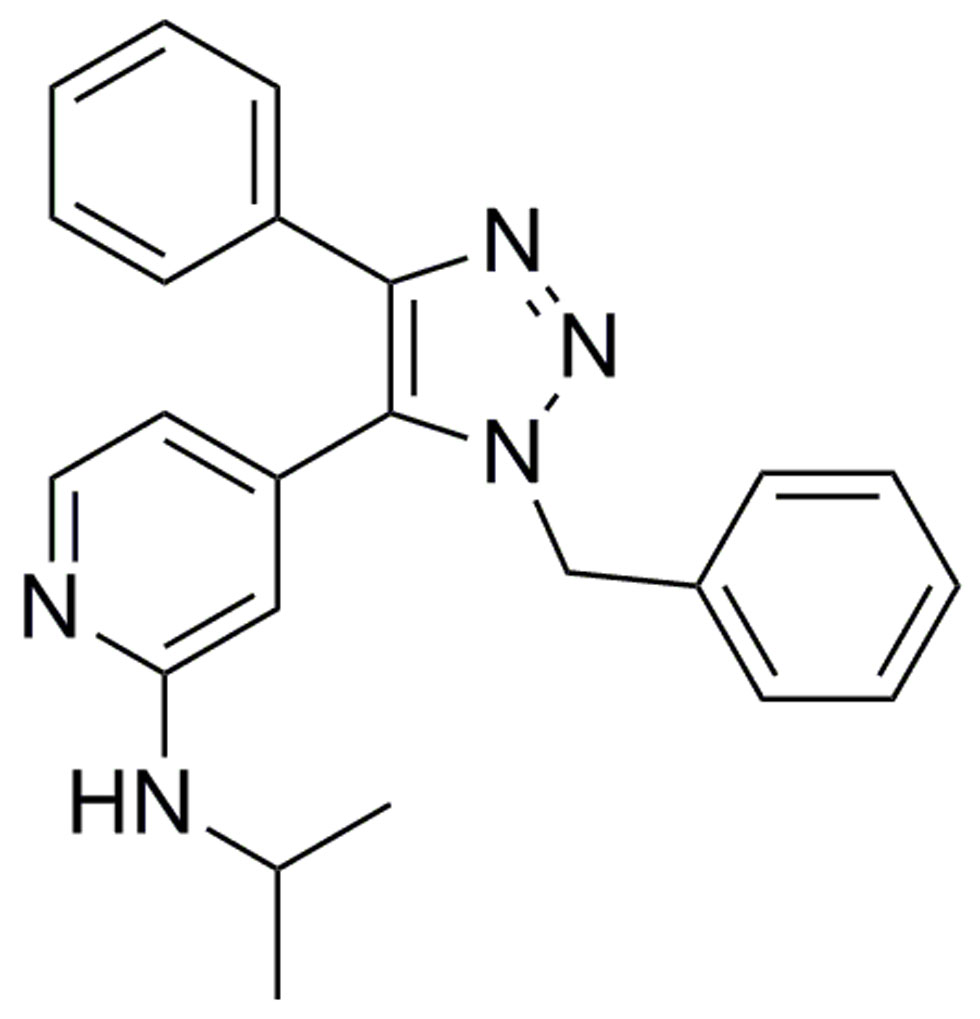
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**S1. Chemical Structure of Inhibitor**

Figure S1 shows the chemical structure of the Hog1 inhibitor.

**Figure S1.** Chemical structure of the selective Hog1 inhibitor: 4-(1-benzyl-4-phenyl-1H-1,2,3-triazol-5-yl)-*N*-isopropylpyridin-2-amine (M = 478.67 g/mol).



**S2. Comparing Measurements Using a Previously Developed Three-Inlet Microfluidic Chamber**

A tree-inlet system was used for making the comparison between the introduction of the inhibitor in a flow over an array of cells, and an inhibitor introduction in a stationary environment of cell suspension (*i.e.*, the four-inlet chamber). Using the previously developed three-inlet microfluidic chamber [1,2], cells were subjected to the stress agent (500 mM sorbitol) and fluorescently imaged during 45 min. The cells were introduced in one of the inlet channels neighbouring the middle inlet channel. There, to ensure that the cells were not exposed to untimely perturbation from the third inlet channel, the middle channel was kept neutral. (If the neutral channel instead had been used for administering the inhibitor, the flow in the cell channel would have to be higher than possible for optical trapping of the cells.)

As a result of the limited number of channels, the cells had to be pre-incubated with the inhibitor. The pre-incubation times varied between 16 and 42 min due to difficulties keeping the setup assembly time constant and the mean pre-incubation time was 29 ± 13 min. The temporal difficulties, present only in the case of pre-incubation, were caused by e.g., occasional obstructions in the inlet channels and tubing failure. The pre-treatment time corresponded to the time elapsed from adding the inhibitor to the cells in a test tube, until the imaging started. During this time, the syringes were filled, the tubing from the syringes to the inlet channels was connected, the microfluidic chamber was mounted on the microscope and cells were positioned with the optical tweezers in the array. In the constant flow exposure described in the main article (using four-inlet channels), this time could be perfectly controlled.

Computer simulations of two different flow configurations were performed for the three-inlet microfluidic chamber (Figure S2A):

1. The configuration used when catching cells with the OT and placing them in an array.
2. The configuration used when exposing the cells to 500 mM sorbitol.

These simulations coincided well with the practical evaluation of the device performed by   
Eriksson *et al*. [1], using fluorescein. Based on the simulated data of the concentration profiles, the location of the cell array was selected (Figure S2).

**Figure S2.** Temporal and spatial properties of the cell array in the three-inlet reference microfluidic chamber. (**A**) Simulations of the used flows and concentrations. The arrow length is proportional to the flow velocity (given at a distance of 13.5 μm, half of the channel height, from the bottom surface). The concentration distribution (given at a distance of 0.1 μm from the bottom surface) is represented by shades from blue (0 M) to red (500 mM). The green area represents the position of the yeast cells and the pump   
rates are shown in nl/min for each inlet channel. The first configuration (I) is used for positioning the cell array using optical tweezers and the second configuration (II) is used for sorbitol treatment and imaging; (**B**) Shows the channel junction and the cell array site. The coordinate system (*x*, *y*, *z*) of the microfluidic chamber has its origin (marked with +) at the junction between the wider inlet channel and its adjacent neighbour. The array is placed with the uppermost left yeast cell at (40, −25, 0). Cells are not drawn to scale.

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Upon pre-treatment of the cells with increased concentrations of the Hog1 inhibitor, the Hog1 translocation was impaired (Figure S3). The amplitude of the mean ratio values (described in equation 1 in the main article) decreased with increased Hog1 inhibitor concentration in a dose dependent way: At most, the Hog1-GFP nuclearization was inhibited by 88% (from the mean ratio values at *t* = 150 s) when treated with 25 µM Hog1 inhibitor compared to cells only experiencing the sorbitol without exposure to the inhibitor. The IC50-value was established to 180 nM, in comparison to the earlier determined *in vitro* value of 7.4 ± 0.41 nM [3].

Comparing the Hog1 translocations during 10 nM Hog1 inhibitor treatment and 1 µM treatment, it is seen that the higher inhibitor concentration gives the greatest inhibition of Hog1 translocation. However, there was no significant ratio change when decreasing the concentration of the Hog1 inhibitor from 1 µM to 100 nM.

**Figure S3.** Dose response curves for Hog1-GFP nuclearization after static Hog1 inhibitor treatments in three-inlet microfluidic chambers show that the nuclearization is dose dependent and that a Hog1 inhibitor concentration of 25 µM results in the largest suppression of Hog1-GFP nuclearization. Hog1 inhibitor pre-treatment (of indicated concentration) was followed by 500 mM sorbitol stress treatment in the microfluidic chamber. Data were acquired from *t* = −30 s to *t* = 2700 s and the sorbitol-inhibitor mixture was introduced into the microfluidic chamber at *t* = 0 s. The curves show mean *R*-values from two to four different experiments for each concentration (one for the no sorbitol measurement). The number of cells analysed in each experiment, is indicated within brackets in the legend.



Figure S4 shows single cell data acquired using the three-inlet chamber: When the cells are treated with a 5 µM Hog1 inhibitor solution and subsequently a sorbitol-inhibitor mixture, their   
Hog1-GFP nuclearization response is decreased compared to the response in cells treated with only sorbitol (no inhibitor). As seen in the main article, increasing the inhibitor concentration introduced by a flow in the four-inlet microfluidic chamber to 25 µM, leads to similarly the same extent of   
Hog1-GFP nuclearization inhibition as the 5 µM (and 25 µM) pre-treatment approach generated (Figure S4).

Since the inhibitor treatment time seems crucial, the results could to some extent explain the differences seen between the mean ratio values obtained in the three- and four-inlet chamber experiments. It is probable that the most dramatic effect of the inhibitor in the flow condition takes place in a time window of less than the averaged 29 min of the three-inlet inhibitor pre-incubation.

**Figure S4.** A single-cell dose-response comparison performed in a three-inlet microfluidic chamber. Pre-incubation of the cells with the inhibitor is used, since there is no inlet channel for the inhibitor. Hence, the temporal control of the inhibitor treatment is poor and incubation times vary between 16 and 42 min for the pre-incubation setup. From the left: sorbitol stress only, two different inhibitor concentrations, and the inhibitor only. Increasing the inhibitor concentration from 5 to 25 µM in the setup resulted in similar Hog1-GFP nuclearization inhibition as the 5 µM constant flow exposure approach generated (see Figure 5 in main article), but displayed more intercellular variation.   
The curves show *R*-values from two to four different experiments for each concentration (one for the no sorbitol measurement). The total number of cells used was from left to right: 68, 103, 37 and 27.



**S3. Video of the Fluorescent Stress Response of Hog1-GFP and Msn2-GFP**

Figure S5 shows the protein migration of Hog1-GFP and Msn2-GFP in cells subjected to 500 mM sorbitol and 5 µM Hog1 inhibitor.

**Figure S5.** Movie showing partial nuclearization of Hog1-GFP and Msn2-GFP in cells subjected to 500 mM sorbitol and 5 µM Hog1 inhibitor. The images are acquired every   
30 s for 5 min; every minute for 10 min, every other minute for yet another 10 min and every tenth minute for 20 min. The combined stress and inhibitor solution is administrated at *t* = 0 s. A slow delocalization of the reporter proteins is initiated at approximately   
ten minutes. The rightmost column of cells consists of control cells expressing Msn2-GFP (as well as all cells in the bottom row except the first two). The video is also available at the supplementary file.



References

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