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Emerging Platform from Renewable Resources: Selection Guidelines for Human Exposure of Furfural-related Compounds

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5-hydroxymethylfurfural (HMF) is a precursor for the synthesis of potential chemical building blocks and biofuel products. Therefore, it is expected to be a very important bioplatform player in the future due to reduction of fossil resources. Controversial data exists about HMF toxicity and in addition, toxicological data of its derivatives is scarce. We evaluated the impact of several HMF derivatives in human skin fibroblast cells and data demonstrates that the dialdehyde **(10),** dihydroxymethyl **(12),** dimethyl **(18)** and the dimer **(20)** derivatives are potentially more harmful than the dicarboxylic acid derivative **(17)**. HMF was not cytotoxic whereas the reported derivative 5-sulfoxymethylfurfural (SMF) was weakly cytotoxic. Some examples of derivatives are presented which are considerably more toxic than SMF.

Introduction

The modern society is based on the use of energy resources and chemical based commodities that derived mainly from fossil supplies. With the reduction of non-renewable resources and growing needs of organic based diverse molecules by the emerging countries, the world is facing the need of changing the paradigm of current portfolio of chemical building blocks. In this context, extensive research is going on the creation of biorenewable derived chemical commodities that may gradually replace the current existing ones.[1-5] In this line, 5-hydroxymethylfurfural (HMF) **(1)**, a building block derived from carbohydrates, is called a "sleeping giant". This is due to the possibility of its preparation from cellulose and the potential rich chemistry, suitable by the presence of the hydroxyl, aldehyde and furan functionalities, which potentiate their use as precursor to biofuels and polymer building blocks that may replace the phthalate commodities.[6-8] Besides the intense ongoing research that already allowed the identification of potential building blocks and synthetic routes, it is extremely important to anticipate of their toxic impact in environment[9, 10] and humans.

Besides HMF **(1)** identification as a potential building block, special attention has been given to its formation during thermal treatment of carbohydrates-containing foods.[11] Studies in rats demonstrated that HMF can be a potent carcinogen, but in contrast, it was considered a weak carcinogen in mice.[12-13] HMF can be metabolized into 5-sulfoxymethylfurfural (SMF) **(2)** by sulfotransferases, which in some studies was seen as potential genotoxic and mutagenic[16-18]. Different sulfotransferase activity within the used experimental models was suggested as a possible explanation for the discrepant conclusions within the several reported studies.[11, 14, 15]

In this context we studied the toxic impact of HMF, SMF and a range of HMF derivatives in immortalized human skin fibroblast cells (CRL-1502) with the aim to provide key guidelines about the most human-friendly building blocks (Figure 1). CRL-1502 is a non-tumor cell line which was chosen to resemble human healthy tissue allowing to predict the impact of such compounds in humans. We assessed the viability as well as the ability of the compounds to generate oxidative stress in treated cells, which is known to be a player in many diseases. In addition, it was included in this study the already in use and established bioplatform furan-based molecules furfural (**11**) and furfuryl alcohol (**15**) and non-aromatic levulinic acid (**22**) as control that allows a comparison with the new potential emerging molecules containing the furan ring namely HMF and analogues.[19]

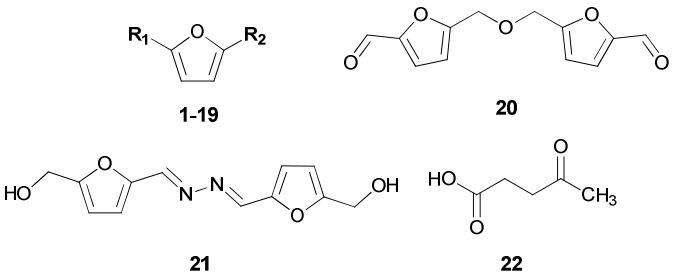

Figure 1: Tested Compounds. R_1 and R_2 are presented in Table 1.

Results and Discussion

Viability.CRL-1502 cells were incubated with 100-500 μM of the 22 tested compounds for 72 hours and correspondent toxicity curves can be found in the supplementary part. Treatment with SMF **(2)** induced a decline in the viability of about 30%, whereas HMF **(1)** was not seen to lead to detectable

variations. One-way ANOVA analysis demonstrated that these differences are statistically significant ($p<0.05$) and we may assume that SMF **(2)** constitutes a compound of a slightly higher probability to generate cytotoxicity (Table 1). Some derivatives of HMF **(1)** are suggested as promising candidates for polymer production and biofuel replacement such as compounds **22** (levulinic acid) and **17**, and **18**, respectively, which were also included in this study (Table 1). The first two did not affect significantly cell viability whereas compound **18** induced a viability decrease at a similar extent as SMF; and results were statistically significant ($p<0.05$) when compared to our control molecule (HMF – compound **1**, Table 1). CRL-1502 cells incubated with 500μM of compounds **1**, **2** and **18** had a viability of 94%, 68% and 65%, respectively, after 72 hours treatment. In the presence of CH_2Cl, CH_2OCOCH_3 or CH_2OCOPh as lateral R_2 groups to generate compounds **3**, and **9** (Figure 1), CRL-1502 cells experienced a weak decrease in viability at the maximum tested dose, which did not go below 60% (Table 1). Compounds unable to produce a decline of at least 50% of the cells viability at a 500μM dose were considered not cytotoxic. In contrast, they were classified as moderately cytotoxic or cytotoxic when 50% viability decline or more were achieved, respectively (Table 1).

Other studied compounds as **5** and **6** were also demonstrated to be not cytotoxic. However, in the presence of the more lipophilic compound **8,** induced cytotoxicity was undoubtedly enhanced producing 80% viability decline at a 500μM dose. The di-aldehyde of the furan ring (**10**) was the second most toxic compound following compound (**8**) (Table 1). Within the remaining compounds, compound **20**, which is composed by two furfural units (Figure 1), was considered moderately cytotoxic (Table 1). Data shows that the acid substituents (COOH) derivative – compound **17** and even compound **20** (Figure 1) are safer choices than the aldehyde substituents (CHO) – compound **10**. Furthermore, compounds **4**, **11**, **13**, **14**, **15**, **16**, **19**, and **21** were not seen to decrease viability of this model up to 500μM, whereas at the same concentration compounds **2**, **3**, **5**, **6**, **9**, **18**, and **20** did induce to some extent decrease in cell viability and were more toxic than HMF. Nevertheless, from this group, only compound **20** was considered moderately cytotoxic. The studied derivatives containing ether substituents were seen in this model not to affect cells viability (compounds **4**, **13** and **16**) up to a 500μM concentration.

Table 1. Percentage of viability and ROS determined for treated CRL-1502 cells. Viability was assayed after 72 hours incubation and presented values correspond to a 500μM concentration. For the determination of ROS, cells were incubated with 250μM of compound for 90 minutes.

	R_1	R_2	Viability (%)	ROS (%)
1	CHO	CH_2OH	94±3	106±7
2	CHO	CH_2OSO_3Na	68±5[a]	120±11
3	CHO	CH_2Cl	70±10[a]	**379±60**[a]
4	CHO	CH_2OEt	90±15	107±22
5	CHO	CH_2OCH_2Ph	72±13[a]	**177±14**[a]
6	CHO	$CH_2OSi(CH_3)_2{}^tBu$	75±24[a]	**158±36**[a]
7	CHO	CH_2OCOCH_3	86±11	108±34
8	CHO	CH_2OCO*n*-C_5H_{11}	**22±3**[a]	**370±44**[a]
9	CHO	CH_2OCOPh	62±7[a]	**153±17**[a]
10	CHO	CHO	**32±2**[b]	113±12
11[b]	CHO	H	95±8	101±30
12	CH_2OH	CH_2OH	96±10	**254±32**[a]
13	CH_2OH	CH_2OEt	95±10	116±7
14	CH_2OH	COONa	95±17	89±13
15[b]	CH_2OH	H	99±6	101±28
16	CH_2OEt	CH_2OEt	114±15	97±21
17	COOH	COOH	93±11	86±7
18	CH_3	CH_3	65±8[a]	**172±20**[a]
19	CH_3	H	93±6	84±20
20	See Figure 1		**51±6**[a]	134±19
21	See Figure 1		109±5[a]	109±19
22[b]	Levulinic acid		98±5	122±52

[a] $p<0.05$; [b] Tested for control purpose.

Reactive Oxygen Species (ROS). For the assessment of ROS, CRL-1502 cells were incubated with 250μM of the compounds for a period of 90 minutes. This concentration was used because we wanted to test all the compounds at similar conditions, and at 500 μM the cytotoxicity of some of the compounds could enable ROS determination. With exception of compound **2, 10** and **20**, data showed that some compounds that affect viability lead to considerably reactive oxygen species (ROS) generation. Within such compounds, the decreasing trend of oxidant potential is: compounds **3** ≈ **8** >> **5** ≈ **18** > **6** ≈ **9**, where the most toxic is a halogenated compound (Table 1). This effect was not detected in cells exposed to HMF (Table 1). But, increment of ROS does not seem to represent the key player for the inducing cytotoxicity effects, for the several reasons: 1) the highly and moderately toxic compounds **10** and **20** respectively, did not increment ROS that much; 2) weakly toxic compounds as **3**, **5** and **18** enhanced significantly ROS levels and 3) compound **12** which not led to detectable changes in viability was an important ROS generator.

Interestingly, if we compare compounds **12**, **14** and **15**, it is clear that the functionalization of the furan ring with methyl-hydroxyl groups increases the potentiality for the compound to cause harmful effects on a long term basis due to formation of oxidative stress. And the same occurs for the dimethylated compound, if we compare compounds **18** and **19** from Table 1. This information could not be concluded from the viability assay discussed previously, which demonstrates the importance of this assessment. Compounds **1**, **4**, **7**, **11**, **13**-**17**, **19**, **21** and **22** were not seen to affect oxidative status in addition to their lack of cytotoxicity in the neutral red assay meaning that these are very likely the safest studied compounds. (Table 1)

Experimental

Synthetic procedures. The protocols for preparation of the tested compounds, spectral data, HPLC and GC-MS chromatograms and copies of ^1H and ^{13}C NMR spectra is provided in the electronic supplementary information.

Cell Culture. Human skin fibroblasts (CRL-1502) were purchased from ATCC and cultivated in media RPMI-1640 with L- glutamine and supplemented with 10% fetal bovine serum (FBS), antibiotic and antimycotic solution and kept in a humidified atmosphere with 5% CO_2 and at 37ºC.

Toxicity Assays. Cells were plated in 96-well plates and grown until confluence to form a monolayer. Cells were incubated with the tested compounds pre-dissolved in ethanol or dimethylsulfoxide (percentage of organic solvent in contact with the cells ≤ 1%). Compounds were diluted in the same cell culture media with only 0.5% FBS and added to the cells that were kept for 72 hours in the incubator. Tested concentrations were 100, 200, 250 and 500μM. Afterwards media was removed, cells washed with phosphate buffer saline (PBS) and incubated with fresh media containing 50μg/ml neutral red. Three hours later, cell monolayers were washed with PBS and

the amount of neutral red retained by the cells extracted and dissolved with an organic solution (19.96 ml distilled water, 20 ml ethanol and 400µl glacial acetic acid). Absorbance of the wells was measured at 540 nm in a plate reader. Viability was determined by the ratio of absorbance of treated cells and control cells. Presented results are the average ± standard deviation (SD). Analysis of variance were performed in GraphPad Prism 5 and with One-way ANOVA and using the Dunnett´s Multiple Comparison Test with 95% confidence intervals, and data was compared with the control compound **1** (HMF).

Determination of Reactive Oxygen Species. Cells were plated in 96-well plates and grown until reaching approximately confluence. They were washed with Hank´s balanced salt solution (HBSS) supplemented with $CaCl_2$ (0.14g/L) and $MgSO_4$ (0.20g/L) and incubated for 15 minutes with 8µM of the probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) in supplemented HBSS. Cells were washed again with supplemented HBSS and incubated for 90 minutes with 250µM of the compounds diluted in the same buffer. Afterwards, compounds were removed, cells were washed and trypsinized (30µl of trypsin/EDTA per well). To neutralize trypsin, 100µl of buffer was added to each well. Plate was read for fluorescence in a Guava easyCyte HighThroughput Flow Cytometer (Millipore). Percentage of ROS was determined by the ratio of two fluorescence means (treated cells/control cells) x 100. Data was analyzed in GraphPad Prism 5. Presented results are the average ± standard deviation (SD). Analysis of variance were performed in GraphPad Prism 5 and with One-way ANOVA and using the Dunnett´s Multiple Comparison Test with 95% confidence intervals, and data was compared with the control compound **1** (HMF).

Conclusions

In conclusion, and within the studied furan derivatives, we can organize them into two different groups: one group constituted by compounds that did not decrease viability and did not enhance ROS levels (compounds **1**, **4**, **7**, **11**, **13**-**17**, **19**, **21**, **22**); and a second group formed by the remaining compounds. In this last group, we can distinguish three different types of compounds: 1) compounds not involved in ROS generation but weakly cytotoxic (compound **2**) or extremely cytotoxic (compound **10**); 2) others that induced both ROS and viability decrease (compounds **3**, **5**, **6**, **8**, **9**, **18** and **20**; and 3) compound **12** that was not seen to decrease viability but it was seen to form ROS in a significant way. This suggests other major players being responsible for the induced cytotoxicity rather than oxidative stress.

From all the data, just compounds **1**, **4**, **7**, **11**, **13**-**17**, **19**, **21** and **22** can be considered as the safest compounds within the studied array of compounds, since compounds that induce ROS may induce cell damage in a long term basis. The well known compounds furfural (**11**), furfuryl alcohol (**15**) and levulinic acid (**22**) were also included for comparison purpose.[19] Regarding the controversial data in the literature for HMF toxicological impact, we have not seen any significant cellular impact in our work. In addition, SMF was shown to be just a moderated cytotoxic compound in the tested model.

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Notes and references

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† Electronic Supplementary Information (ESI) available: experimental procedures for the synthesis of the tested compounds, spectral data, HPLC and GC-MS chromatograms and copies of 1H and 13C NMR. Toxicity curves of the several tested compound are also available in this document. See DOI: 10.1039/b000000x/

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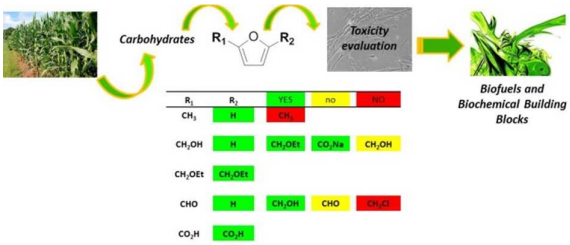

Toxicity of hydroxymethylfurfural and 21 derivatives was assessed. Important guidelines for selection of more human friendly building blocks were obtained.