



HAL
open science

N-acetylcysteine potentiates diclofenac toxicity in Saccharomyces cerevisiae: stronger potentiation in ABC transporter mutant strains

Houssein Al-Attrache, Hala Chamieh, Monzer Hamzé, Isabelle Morel, Samir
Taha, Ziad Abdel-Razzak

► **To cite this version:**

Houssein Al-Attrache, Hala Chamieh, Monzer Hamzé, Isabelle Morel, Samir Taha, et al.. N-acetylcysteine potentiates diclofenac toxicity in *Saccharomyces cerevisiae*: stronger potentiation in ABC transporter mutant strains. *Drug and Chemical Toxicology*, 2018, 41 (1), pp.89-94. 10.1080/01480545.2017.1320404 . hal-01681146

HAL Id: hal-01681146

<https://univ-rennes.hal.science/hal-01681146v1>

Submitted on 31 Jan 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

N-acetylcysteine potentiates diclofenac toxicity in *Saccharomyces cerevisiae*: stronger potentiation in ABC transporter mutant strains

Houssein Al-Attrache^{1,2,3}, Hala Chamieh¹, Monzer Hamzé⁵, André Guillouzo^{2,3}, Isabelle Morel^{2,3,4}, Samir Taha¹ and Ziad Abdel-Razzak^{1*}

¹Laboratory of applied biotechnology: biomolecules, LBA3B - AZM Center –Tripoli, Lebanon

²Inserm U991, Faculty of Pharmacy, Rennes, France

³Rennes 1 University, Rennes, France

⁴Laboratory of Emergency and Intensive Care, Pontchaillou Hospital, Rennes, France

⁵Laboratory of medical microbiology - AZM Center -Tripoli, Lebanon

*corresponding author: Pr. Ziad ABDEL-RAZZAK, ziad.abdelrazzak@ul.edu.lb; Lebanese University – Faculty of sciences I – Rafic Hariri Campus - Po. Box: 14-6573, Hadat - Beirut – Lebanon. Phone 00961 3 48 69 15

Abbreviations

Bpt1, Bile Pigment Transporter 1; DCF, Diclofenac; LC, L-Cysteine ; MPT, Mitochondrial Permeability Transition; NAC, N-acetylcysteine; OD, optical density; Pdr, Pleiotropic drug resistance; PKC, Protein Kinase C; ROS, Reactive Oxygen Species; Vmr1, Vacuolar Multidrug Resistance; Yap1, Yeast Activator Protein 1; YNB, Yeast Nitrogen Base; Yor1, Yeast Oligomycin Resistance; YPD, Yeast extract Peptone Dextrose; Zap1, Zinc-responsive Activator Protein.

Abstract

Diclofenac (DCF) adverse reactions involve diverse mechanisms in different models. We recently demonstrated that DCF-induced toxicity in HepaRG decreases as they express DCF-metabolizing enzymes. [DCF metabolism promotes toxicity in *Saccharomyces cerevisiae*, expressing heterologous cytochromes-P450](#). N-acetylcysteine (NAC) is used to treat diverse medical conditions due to its multiple properties (antioxidant, metal chelator, thiol-disulfide disruption). The latter property accounts for its mucolytic effects and broadens its potential molecular targets to signal transduction proteins, ~~and~~ ABC transporters and others. Interaction of NAC with DCF effects depends on the experimental model.

This study aims to investigate NAC/DCF interaction and the involvement of ABC transporters ~~in~~ in wild type and mutant *Saccharomyces cerevisiae*. DCF inhibited yeast growth in a dose and time-dependent manner and the cells started adapting to DCF 24 hours post-treatment. NAC potentiated DCF-induced toxicity if added prior or parallel to DCF. Pre-treatment with NAC increased its potentiation effect and compromised cells adaption to DCF. Post-treatment with NAC potentiated DCF toxicity without compromising adaptation. Moreover, mutant strains in ABC transporters Pdr5, Yor1, Bpt1 or Pdr15, were more sensitive to DCF; while mutant strains in Pdr5, Vmr1 or Pdr12 were more sensitive to NAC/DCF interaction. DCF±NAC elicited on the mutant strain in Yap1, an oxidative stress-related protein, the same effects as on the wild type. Therefore, oxidative stress does not seem to be key actor in DCF toxicity in our model. Our hypothesis is that NAC potentiation effect is at least due to its ability to disrupt disulfide-bridge in proteins required to overcome DCF toxicity in yeast.

Keywords: Diclofenac, N-acetylcysteine, oxidative stress, disulfide bridge, ABC transporters, *Saccharomyces cerevisiae*.

Introduction

Administration of diclofenac (DCF), a non-steroidal anti-inflammatory drug, has been associated with adverse drug reactions in some patients (Banks et al., 1995; Breen et al., 1986; Watanabe et al., 2007). DCF adverse effects have been related to different factors including its metabolism into 4'-OH-DCF, 5-OH-DCF, and acyl glucuronides (Bort et al., 1999; Kretz-Rommel and Boelsterli, 1993; Wang et al., 2004). We recently demonstrated that DCF-induced toxicity in hepatic HepaRG cell lines decreases as the cells differentiate and express DCF-metabolizing and detoxifying enzymes (Al-Attrache et al., 2016). An opposite effect was reported in *Saccharomyces cerevisiae* where heterologous expression of DCF-metabolizing cytochrome P450 potentiates DCF toxicity and reactive oxygen species (ROS) generation (van Leeuwen et al., 2011c). DCF-induced ROS generation originates in the mitochondrion and was shown to be respiratory chain-dependent in both mammalian and yeast cells (Masubuchi et al., 2002; van Leeuwen et al., 2011a). Furthermore, in several cellular systems, including human hepatocytes, DCF induces ROS formation, causing opening of the mitochondrial permeability transition (MPT) pore, cytochrome c release, caspase activation and apoptosis (Gomez-Lechon et al., 2003; Inoue et al., 2004; Lim et al., 2006). The ROS-mediated DCF apoptotic effects are counteracted by N-acetylcysteine (NAC) (Inoue et al., 2004).

Moreover, in *Saccharomyces cerevisiae* DCF toxic effects and cells adaptation to this toxicant involved pleiotropic drug resistance (Pdr) 5 gene and some components of the protein kinase C (PKC) and MAPK signaling pathways. Indeed, adaption and tolerance of yeast cells to DCF has been associated with induction of Pdr genes and Rlm1p-controlled genes; Rlm1p is a transcription factor in the PKC pathway. Moreover, increased DCF toxicity occurs after deletion of genes of components of the cell wall stress-responsive PKC pathway or the Zinc (Zn)-responsive transcription factor Zap1p (Zn-responsive activator protein). In addition, Zn-chelator increases DCF toxicity (van Leeuwen et al., 2011b).

NAC has been used in clinical practice for mucolytic purpose in lung diseases that are associated with mucus hypersecretion, treatment of pulmonary oxygen toxicity, adult respiratory distress syndrome, acetaminophen intoxication, doxorubicin cardiotoxicity, ischemia-reperfusion cardiac injury, chemotherapy-induced toxicity, heavy metal toxicity and psychiatric disorders (Rushworth and Megson, 2014; Samuni et al., 2013). However, NAC causes adverse drug reactions such as pruritus, rash and gastrointestinal symptoms (Koppen et al., 2014).

This diversity of NAC pharmacological applications and adverse effects is due to the multi-sided chemical features of its cysteinyl thiol group including nucleophilicity, ROS scavenging (antioxidant properties, *in vitro* and *in vivo*), heavy metal chelation and its ability to undergo thiol-disulfide (di-S) exchange reactions with other thiol redox couples leading to di-S bridge disruption as in the case of mucous proteins (Zafarullah et al., 2003). NAC is more efficient than other thiol-containing molecules (e.g. cysteine and glutathione) in terms of di-S bridge disruption due to its lower molecular weight (in comparison with glutathione) and might compete with

1
2
3 larger reducing molecules in sterically less accessible proteins cores. Moreover, NAC is a
4 precursor of cysteine formed by deacetylation of the latter. NAC also increases glutathione
5 levels.
6
7

8 NAC affects diverse molecular targets such as signal transduction proteins (e.g. NF κ B, c-Src,
9 STAT3, MAPK) through direct interaction with proteins and/or indirectly through ROS
10 neutralization (Hou et al., 2015; Parasassi et al., 2010; Ramudo and Manso, 2010; Samuni et al.,
11 2013). NAC affects cell division and differentiation causing change of expression of more than
12 900 genes including cytoskeleton- and cell cycle-related genes (Edlundh-Rose et al., 2005). NAC
13 is also likely to affect expression and activity of many di-S bridge-containing protein types
14 including ABC transporters as well as key proteins of transduction pathways in DCF-induced
15 toxicity.
16
17
18
19

20 Given the diverse toxic and therapeutic effects and targets of these two commonly used drugs,
21 NAC and DCF, and the common use of NAC as antioxidant in research, an important question
22 arises about their interaction and the possible involvement of ABC transporters in their
23 interaction in our experimental model. In fact, previous clinical reports as well as reports from
24 different experimental models showed diverging results in this respect. This is likely due to the
25 multiple mechanisms of actions of both drugs. NAC and DCF act in synergy as anti-
26 inflammatory drugs through cyclooxygenase inhibition (Parasassi et al., 2005), and inhibition of
27 TNF- α secretion (Mulhall et al., 2003). NAC was reported to reduce DCF-induced renal toxicity
28 (Efrati et al., 2007) and ROS-mediated DCF-induced toxicity and apoptosis (Al-Attrache et al.,
29 2016; Inoue et al., 2004). However, NAC has no effect on DCF-methyl phenyl pyridinium
30 potentiated toxicity (Morioka et al., 2004).
31
32
33
34
35

36 In the present study we investigated the interaction of NAC and DCF on wild type and ABC-
37 transporter mutant strains of *S. cerevisiae*. Our results showed that DCF inhibits cell growth in a
38 dose and time-dependent manner and that NAC potentiates DCF-induced growth inhibition of
39 yeast. Moreover, mutant strains in some ABC transporters are more sensitive to DCF, and to the
40 interaction of NAC with DCF.
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Materials and methods

Chemicals and reagents

Diclofenac sodium salt, N-acetylcysteine and YPD broth and agarose were purchased from sigma Aldrich (St. Quentin Fallavier, France). L-cysteine (LC) was from BDH (England).

Cell culture, treatment and growth assessment

Saccharomyces cerevisiae BY4741 and the mutants strains illustrated in Table 2 were supplied by Euroscarf (Germany). The mutant genes were disrupted by the kanamycin resistance gene (kanMX4) according to the supplier. Cells were seeded on YPD-agar (50 g/liter, 1.6 % agarose) for 48h at 30°C. Colonies are diluted in YPD broth (50 g/l) to an OD (optical density) at 600 nm between 0.1 and 0.2 and treated with drugs, or grown for 48h and then, diluted to obtain the same OD range and then treated with drugs for different periods of time. Culture shaking was performed only prior to growth assessment by OD measurement at 600 nm.

Wild type and mutant strains were treated with DCF and/or NAC at different concentrations and growth assessed at different time points by OD measurement. Results are expressed as percentage of the respective control untreated cells at each time point.

Statistical analysis

One-way ANOVA with Bonferroni's multiple comparison test (GraphPad Prism 5.00) was performed to compare data between DCF-, NAC-, DCF +NAC-treated cells and control cultures. The same test was applied to compare data obtained with mutant strains with those obtained with the wild-type strain. Differences were considered significantly different when $p < 0.05$.

Results

*Dose and time-dependent effect of DCF on growth of *S. cerevisiae* ~~BY4147~~ BY4741 strain*

In order to assess DCF effect on growth of *Saccharomyces cerevisiae* BY4741 strain, different doses and treatment time points were used. Yeast growth was inhibited by DCF in a time and dose-dependent manner (figure 1a and 1b) starting at the 3 hour-treatment time point and reaching a maximum after 9 hours. DCF effect appeared at the dose of 1000 μ M and was more efficient at 2000 μ M. This latter dose caused ~~22.6%~~ and ~~55.8%~~ inhibition at 3h and 6h treatment, respectively (Figure 1a). The inhibitory effect of DCF was partially abolished after 24 hour-treatment reflecting a previously described process of adaption of yeast cells to this drug (figure 1b (van Leeuwen et al., 2011b)).

*Interaction of NAC with DCF toxic effect on *S. cerevisiae* BY4741*

In order to assess interaction of NAC with DCF toxic effect, yeast cells (BY4741) were treated with DCF \pm NAC, simultaneously or successively with a period of 30 min delay. Growth was

1
2
3 assessed at the indicated time points (Table 1). Similarly to figure 1b, DCF maximal inhibitory
4 effect occurred after 9 hour-treatment (34.4% inhibition), and cells started to adapt at 24 hours
5 where DCF-mediated inhibition decreased. NAC, which had no statistically significant inhibitory
6 effect by itself, potentiated DCF toxicity in a significant manner regardless of whether drugs are
7 added simultaneously or successively. A dose of 1 mM NAC showed similar but mild
8 potentiating effects (data not shown). At 9h, NAC+DCF added together led to 64.7% growth
9 inhibition while DCF alone caused only 34.4% inhibition. At 24h, adaptation of cells to DCF in
10 the presence of NAC was decreased (19.4% versus 29.7% inhibition) but the difference was not
11 statistically significant.

12
13
14
15
16 Moreover, when cells were pre-treated with NAC, more potentiation of DCF toxicity occurred
17 (inhibition by 31.8%, 76.3%, 82.9% and 68% at 3, 6, 9 and 24h, respectively). Inhibition after
18 simultaneous addition of the two drugs was 10.7%, 39.6%, 64.7% and 29.7% at 3, 6, 9 and 24h,
19 respectively (Table 1). Therefore, cells are more sensitive to DCF-induced toxicity when NAC is
20 added prior to DCF than when they are added together. In addition, cells adaptation to DCF at 24
21 hours is seriously impaired by NAC pretreatment. However, when yeast cells were pretreated
22 with DCF, increase of DCF toxic effect by NAC was still evident, but NAC was no longer able
23 to impair their adaptation to DCF. This result suggests the involvement of diverse cellular targets
24 in NAC/DCF interaction.

25
26
27
28
29
30 *Investigation of DCF, NAC and LC effects and interactions on growth of wild type and mutant*
31 *strains of S. cerevisiae-growth*

32
33 We aimed here to assess the potential involvement of certain ABC transporters in terms of DCF
34 toxicity and its potentiation by NAC. Moreover, other amino acids effects were investigated to
35 check if the observed NAC/DCF interaction was relative to amino acids metabolic pathways,
36 osmotic stress, or mediated by other NAC-related mechanisms such as the antioxidant effect, di-
37 S bridge disruption, signal transduction alteration, metal ion chelation. Wild type and mutant
38 strains in some ABC transporters were used (illustrated in Table 2). We also used Yap1 mutant,
39 an AP-1 like transcription factor known to mediate oxidative stress response ([Kuge and Jones](#)
40 [1994](#)) ([Yano et al., 2009](#)).

41
42
43
44
45 DCF at 1000 μ M was toxic for all used strains causing growth inhibition ranging from 21.2% to
46 64.9%. The strains Y02409, Y05933, Y01503, Y04242 which are mutant in the genes Pdr5,
47 Yor1, Bpt1 and Pdr15 respectively, were more sensitive to DCF since growth inhibition
48 percentage was significantly higher (inhibition by 45.7%, 63.3%, 63.9% and 64.9%, for the
49 mutant strains respectively) in comparison with that of the wild type strain (34.4% inhibition)
50 (Table 2). Therefore each one of these ABC transporters partially protects yeast cells against
51 DCF. The other mutant strains showed no statistically significant change of sensitivity to DCF in
52 comparison with the wild type strain. Regarding yeast cells adaptation to DCF, all of the
53 investigated mutant strains were still able to adapt to the drug 24 hours post-treatment (data not
54 shown).

1
2
3 It is noteworthy emphasizing that the yeast strain Y00569, mutant in Yap1, had the same
4 sensitivity to DCF as the wild-type one, thereby suggesting that oxidative stress is not a key
5 factor in DCF-induced toxic effect in our experimental model and culture conditions. In addition,
6 ROS assessment, by the 2',7'-dichlorofluorescein di-acetate test, in DCF-treated cells showed up
7 to 30% ROS increase in our experimental conditions (data not shown). However, this increase
8 was not statistically significant.
9

10
11
12 In the presence of NAC, the mutant strains in Pdr5, Vmr1 and Pdr12 showed more potentiated
13 DCF toxicity (inhibition range from 84.8 to 93.9%) in comparison with the BY4741 wild type
14 strain (inhibition by 64.7%). Therefore, these mutant strains are more sensitive to NAC/DCF
15 interaction. Regarding interaction of DCF with the other amino acids, L-cysteine tended to
16 potentiate DCF toxicity but the results did not reach statistical significance neither with the wild
17 type nor with the mutant strains (Table 2), while L-lysine and L-isoleucine, individually, did not
18 cause any DCF toxicity potentiation (data not shown).
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Discussion

DCF induces adverse reactions in some patients (Boelsterli, 2003). Involvement of oxidative stress enzymes and drug metabolizing enzymes in this toxicity was reported in *in vitro* hepatic and non-hepatic models (Al-Attrache et al., 2016; Fredriksson et al., 2011; van Leeuwen et al., 2011c). DCF was reported to induce oxidative stress, apoptosis, cholestasis by diverse mechanisms (Al-Attrache et al., 2016; Fredriksson et al., 2011; Fredriksson et al., 2014; Maiuri et al., 2015) including deregulation of some ABC and SLC transporters (Al-Attrache et al., 2016), respiratory chain components and PKC signaling (van Leeuwen et al., 2011b).

Our study demonstrated that DCF inhibited yeast growth in a dose- and time-dependent and metabolism-independent manner. Our results are in accordance with previous studies (van Leeuwen et al., 2011c). Toxicity started with a DCF dose of 1000 μ M and was maximal after nine hours of treatment. Cells started to adapt to DCF and to overcome its toxicity at 24 hours, this is also in agreement with previous reports which showed that adaptation to DCF was due to up-regulation of several tens of genes such as the Pdr genes and genes under the control of Rlm1p (MADS-box transcription factor), a transcription factor in the PKC pathway. In addition, many other genes are down-regulated (van Leeuwen et al., 2011b).

The efficient DCF dose in our study was 1000 μ M, much higher than that reported in previous studies (van Leeuwen et al., 2011b) where DCF effects started at 50 μ M and reached a maximum at 200 or 500 μ M. Such high DCF concentration (>1000 μ M) is not aberrant and was already used with another yeast, *Candida albicans* (Ghalehnoo et al., 2010). The great difference of *S. cerevisiae* response to DCF between our study and that of Leeuwen *et al.* is probably due to use of the minimal medium YNB (yeast nitrogen base, supplemented with amino acids and glucose) while the complete YPD broth is used in our case. Indeed, YNB pH is usually more acidic (about 5, van Leeuwen et al., 2011a) than that of YPD (pH range 6.4 to 7). More acidic pH causes lower ratio of ionized/non ionized diclofenac thereby causing more efficient diffusion across the plasma membrane. In addition, YPD is a rich medium that contains many organic components that are likely to make complex with DCF thereby reducing its diffusion into cells. The different media used in our study and that of van Leeuwen et al., 2011c may also explain the difference in terms of DCF-generated ROS.

NAC properties and its diverse mechanisms of action and clinical applications were summarized in the introduction of this article. Though previous studies in other cell models reported protective effect of NAC against DCF-induced toxicity (Al-Attrache et al., 2016; Efrati et al., 2007; Inoue et al., 2004), the present study clearly showed an original interaction between NAC and DCF since toxicity of the latter on yeast was potentiated in the presence of NAC, and cells adaptation to DCF was compromised, especially when NAC is added first and DCF second, with a 30 minute-delay. Potentiation of DCF toxic effect by NAC was in agreement with the result we obtained using the Yap1 mutant strain (Y00569, Table 2) which showed sensitivity to DCF similar to that of the wild-type strain BY4741. This result, along with the lack of significant ROS

1
2
3 | increase by DCF (data not shown), enabled us to ~~hypothesize-suggest~~ that NAC effect was not
4 | dependent on its antioxidant properties here. Other previous studies had reported antioxidant-
5 | independent NAC effects. For instance, NAC potentiates effect of Cu_(II) H₂O₂ on the
6 | mitochondrial diaphorase (Gutierrez-Correa and Stoppani, 1997), interferes with transport of
7 | As₂O₃ (Vernhet et al., 2003) and inhibits mitochondrial autophagy (Deffieu et al., 2009). In our
8 | experimental model, NAC had a protective effect against toxicity of chlorpromazine
9 | (unpublished data) probably through its antioxidant property.
10
11
12

13
14 | However, ~~and~~-despite the lack of change of Yap1 mutant yeast (an oxidative stress-related
15 | transcription factor) sensitivity to DCF reported here and the lack of significant ROS induction
16 | by this drug, it is impossible to rule out involvement of the antioxidant properties of NAC in our
17 | context since ROS are known to trigger signaling pathways that could relief or alleviate toxicity
18 | (Hancock et al., 2001; Xu et al., 2002). The potential ~~is~~-early neutralization by NAC in the
19 | presence of DCF excess could make the cells vulnerable to DCF toxic effects due to lack of
20 | protective signaling pathways activation.
21
22
23

24 | In addition, NAC could potentiate DCF toxic effects through its ability to disrupt di-S bridges
25 | that occur in diverse proteins of signaling pathways, ABC efflux transporters, and house-keeping
26 | metabolic enzymes. Many ABC transporters such as Pdr5 and Yor1 (Egner et al., 1998; Pagant et
27 | al., 2008) have di-S bridge that are important to their stability and are potential targets that could
28 | be disrupted by NAC through its di-S bridge reducing effect. Our results demonstrated that
29 | mutant strains in these three ABC transporters made the cells more sensitive to DCF. This
30 | finding is supportive of the NAC-mediated di-S disruption hypothesis. The cells would become
31 | more vulnerable to DCF effect due to lack of drug efflux, especially because Pdr5 (ABCG, Pgp
32 | homolog), Yor1 (ABCC, MRP member) and Pdr15 which mediate efflux of a wide variety of
33 | toxicants-(Piecuch and Oblak, 2014). It is also possible that NAC/DCF interaction we described
34 | in this work was due to competition of both drugs to some efflux transporters since both NAC
35 | and DCF are weak organic acids.
36
37
38
39
40

41 | Di-S bridge disruption by NAC could occur for transcription factors that help the cell
42 | overcoming the toxic effects of DCF. It was reported that di-S bridge disruption occurs for
43 | signaling molecules such as NFκB (review by Samuni et al., 2013). Moreover, NAC could
44 | promote DCF toxicity by altering Zn-finger containing protein since NAC has metal ions
45 | chelator activity (Kelly, 1998). In fact, inactivation of the Zn-finger transcription factor Zap1 (by
46 | mutation or by Zn ion chelation) was reported to increase DCF toxicity in *S. cerevisiae* (van
47 | Leeuwen et al., 2011b). Many other Zn-finger containing proteins could be targets of NAC, such
48 | as Pdr1 and Pdr3 which are key transcription factors involved in the yeast pleiotropic drug
49 | resistance response. Their potential alteration by NAC would compromise cells response to
50 | chemical stress and aggravate toxicity. NAC could also disrupt key enzymes in the fermentation
51 | pathway, a preferred energetic mode in *S. cerevisiae*, thereby obligating cells to perform
52 | respiration-based metabolism. This latter situation is known to make the cells more sensitive to
53 | DCF (van Leeuwen et al., 2011a). All the previous hypothetical mechanisms of NAC action are
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

probable since pre-treatment of cells with NAC increases its ability to potentiate DCF toxic effects. ~~These previous~~ possible mechanisms of NAC effects are not mutually exclusive. Indeed, a contribution of different mechanisms is also likely since NAC pre-treatment impaired cells adaptation to DCF, a process involving nearly a hundred genes (van Leeuwen et al., 2011b).

It should be noted that yeast cells are sensitive to the osmotic ~~shock-ehoe~~-induced stress (Chant, 1999). Treatment of our strains with two other amino acids in combination with DCF, L-lysine and L-isoleucine, at the same concentrations as NAC, showed no change in the DCF toxic response amplitude (data not shown). ~~This result suggests that This helped us ruling out involvement of~~ osmotic stress and amino acid metabolic alteration are not involved in the observed NAC interaction with DCF.

By deacetylation, NAC is a precursor of LC, which could mediate the observed effects. Nevertheless, treatment of yeast cells with LC did not increase DCF toxicity in a significant manner in our study. This result showed that NAC acted by itself to increase DCF toxicity, and this was in agreement with the fact that NAC has higher redox potential than cysteine and is more efficient in terms of di-S bridge disruption (Rushworth and Megson, 2014; Samuni et al., 2013).

In conclusion, we demonstrated here for the first time that NAC, but not LC, potentiated DCF toxicity in *S. cerevisiae* cells. Some ABC-transporter mutant ~~strains~~ were more sensitive to DCF toxic effects and NAC/DCF interaction. Diverse possible mechanisms could explain the observed NAC/DCF interaction including di-S bridge disruption and Zn-chelating effect of NAC on key proteins (transporters, signaling proteins or transcription factors,...). Oxidative stress did not seem to ~~play a key role be involved~~ in the interaction NAC/DCF. Further experiments will be performed to unravel the involved mechanism(s).

Acknowledgments and financial support

This work was supported by the Lebanese University Research funds. We are grateful to the AZM center assistants for their cooperation. Houssein Al-Attrache was financially supported by a research grant from the Association AZM and the Lebanese University and the MIP-DILI project.

Declaration of interest statement

The authors declare that there are no conflicts of interest.

References

- Al-Attrache, H., Sharanek, A., Burban, A., Burbank, M., Gicquel, T., Abdel-Razzak, Z., Guguen-Guillouzo, C., Morel, I., Guillouzo, A., 2016. Differential sensitivity of metabolically competent and non-competent HepaRG cells to apoptosis induced by diclofenac combined or not with TNF-alpha. *Toxicology letters* 258, 71-86.
- Banks, A.T., Zimmerman, H.J., Ishak, K.G., Harter, J.G., 1995. Diclofenac-associated hepatotoxicity: analysis of 180 cases reported to the Food and Drug Administration as adverse reactions. *Hepatology* 22, 820-827.
- Boelsterli, U.A., 2003. Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. *Toxicology and applied pharmacology* 192, 307-322.
- Bort, R., Ponsoda, X., Jover, R., Gomez-Lechon, M.J., Castell, J.V., 1999. Diclofenac toxicity to hepatocytes: a role for drug metabolism in cell toxicity. *The Journal of pharmacology and experimental therapeutics* 288, 65-72.
- Breen, E.G., McNicholl, J., Cosgrove, E., McCabe, J., Stevens, F.M., 1986. Fatal hepatitis associated with diclofenac. *Gut* 27, 1390-1393.
- Chant, J., 1999. Cell polarity in yeast. *Annual review of cell and developmental biology* 15, 365-391.
- Deffieu, M., Bhatia-Kissova, I., Salin, B., Galinier, A., Manon, S., Camougrand, N., 2009. Glutathione participates in the regulation of mitophagy in yeast. *The Journal of biological chemistry* 284, 14828-14837.
- Edlundh-Rose, E., Kupersmidt, I., Gustafsson, A.C., Parasassi, T., Serafino, A., Bracci-Laudiero, L., Greco, G., Krasnowska, E.K., Romano, M.C., Lundeberg, T., Nilsson, P., Lundeberg, J., 2005. Gene expression analysis of human epidermal keratinocytes after N-acetyl L-cysteine treatment demonstrates cell cycle arrest and increased differentiation. *Pathobiology : journal of immunopathology, molecular and cellular biology* 72, 203-212.
- Efrati, S., Berman, S., Siman-Tov, Y., Lotan, R., Averbukh, Z., Weissgarten, J., Golik, A., 2007. N-acetylcysteine attenuates NSAID-induced rat renal failure by restoring intrarenal prostaglandin synthesis. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 22, 1873-1881.
- Egner, R., Rosenthal, F.E., Kralli, A., Sanglard, D., Kuchler, K., 1998. Genetic separation of FK506 susceptibility and drug transport in the yeast Pdr5 ATP-binding cassette multidrug resistance transporter. *Molecular biology of the cell* 9, 523-543.
- Fredriksson, L., Herpers, B., Benedetti, G., Matadin, Q., Puigvert, J.C., de Bont, H., Dragovic, S., Vermeulen, N.P., Commandeur, J.N., Danen, E., de Graauw, M., van de Water, B., 2011. Diclofenac inhibits tumor necrosis factor-alpha-induced nuclear factor-kappaB activation causing synergistic hepatocyte apoptosis. *Hepatology* 53, 2027-2041.
- Fredriksson, L., Wink, S., Herpers, B., Benedetti, G., Hadi, M., de Bont, H., Groothuis, G., Luijten, M., Danen, E., de Graauw, M., Meerman, J., van de Water, B., 2014. Drug-induced endoplasmic reticulum and oxidative stress responses independently sensitize toward TNFalpha-mediated hepatotoxicity. *Toxicological sciences : an official journal of the Society of Toxicology* 140, 144-159.

- 1
2
3 Ghalehnoo, Z.R., Rashki, A., Najimi, M., Dominguez, A., 2010. The role of diclofenac sodium in the
4 dimorphic transition in *Candida albicans*. *Microbial pathogenesis* 48, 110-115.
5
6 Gomez-Lechon, M.J., Ponsoda, X., O'Connor, E., Donato, T., Castell, J.V., Jover, R., 2003. Diclofenac
7 induces apoptosis in hepatocytes by alteration of mitochondrial function and generation of ROS.
8 *Biochemical pharmacology* 66, 2155-2167.
9
10 Gutierrez-Correa, J., Stoppani, A.O., 1997. Inactivation of yeast glutathione reductase by Fenton systems:
11 effect of metal chelators, catecholamines and thiol compounds. *Free radical research* 27, 543-555.
12
13 Hancock, J.T., Desikan, R., Neill, S.J., 2001. Role of reactive oxygen species in cell signalling pathways.
14 *Biochemical Society transactions* 29, 345-350.
15
16 Hou, Y., Wang, L., Yi, D., Wu, G., 2015. N-acetylcysteine and intestinal health: a focus on its mechanism
17 of action. *Frontiers in bioscience* 20, 872-891.
18
19 Inoue, A., Muranaka, S., Fujita, H., Kanno, T., Tamai, H., Utsumi, K., 2004. Molecular mechanism of
20 diclofenac-induced apoptosis of promyelocytic leukemia: dependency on reactive oxygen species, Akt,
21 Bid, cytochrome and caspase pathway. *Free radical biology & medicine* 37, 1290-1299.
22
23 Kelly, G.S., 1998. Clinical applications of N-acetylcysteine. *Alternative medicine review : a journal of*
24 *clinical therapeutic* 3, 114-127.
25
26 Koppen, A., van Riel, A., de Vries, I., Meulenbelt, J., 2014. Recommendations for the paracetamol
27 treatment nomogram and side effects of N-acetylcysteine. *The Netherlands journal of medicine* 72, 251-
28 257.
29
30 Kretz-Rommel, A., Boelsterli, U.A., 1993. Diclofenac covalent protein binding is dependent on acyl
31 glucuronide formation and is inversely related to P450-mediated acute cell injury in cultured rat
32 hepatocytes. *Toxicology and applied pharmacology* 120, 155-161.
33
34 Kuge, S., and N. Jones. 1994. "YAP1 Dependent Activation of TRX2 Is Essential for the Response of
35 *Saccharomyces Cerevisiae* to Oxidative Stress by Hydroperoxides." *The EMBO Journal* 13 (3): 655-64.
36
37 Lim, M.S., Lim, P.L., Gupta, R., Boelsterli, U.A., 2006. Critical role of free cytosolic calcium, but not
38 uncoupling, in mitochondrial permeability transition and cell death induced by diclofenac oxidative
39 metabolites in immortalized human hepatocytes. *Toxicology and applied pharmacology* 217, 322-331.
40
41 Maiuri, A.R., Breier, A.B., Gora, L.F., Parkins, R.V., Ganey, P.E., Roth, R.A., 2015. Cytotoxic Synergy
42 Between Cytokines and NSAIDs Associated With Idiosyncratic Hepatotoxicity Is Driven by Mitogen-
43 Activated Protein Kinases. *Toxicological sciences : an official journal of the Society of Toxicology* 146,
44 265-280.
45
46 Masubuchi, Y., Nakayama, S., Horie, T., 2002. Role of mitochondrial permeability transition in
47 diclofenac-induced hepatocyte injury in rats. *Hepatology* 35, 544-551.
48
49 Morioka, N., Kumagai, K., Morita, K., Kitayama, S., Dohi, T., 2004. Nonsteroidal anti-inflammatory
50 drugs potentiate 1-methyl-4-phenylpyridinium (MPP+)-induced cell death by promoting the intracellular
51 accumulation of MPP+ in PC12 cells. *The Journal of pharmacology and experimental therapeutics* 310,
52 800-807.
53
54
55
56
57
58
59
60

1
2
3 Mulhall, K.J., Curtin, W.A., Given, H.F., 2003. Comparison of different anti-inflammatory agents in
4 suppressing the monocyte response to orthopedic particles. *Orthopedics* 26, 1219-1223.

5
6 Pagant, S., Brovman, E.Y., Halliday, J.J., Miller, E.A., 2008. Mapping of interdomain interfaces required
7 for the functional architecture of Yor1p, a eukaryotic ATP-binding cassette (ABC) transporter. *The*
8 *Journal of biological chemistry* 283, 26444-26451.

9
10 Parasassi, T., Brunelli, R., Bracci-Laudiero, L., Greco, G., Gustafsson, A.C., Krasnowska, E.K.,
11 Lundeberg, J., Lundeberg, T., Pittaluga, E., Romano, M.C., Serafino, A., 2005. Differentiation of normal
12 and cancer cells induced by sulfhydryl reduction: biochemical and molecular mechanisms. *Cell death and*
13 *differentiation* 12, 1285-1296.

14
15 Parasassi, T., Brunelli, R., Costa, G., De Spirito, M., Krasnowska, E., Lundeberg, T., Pittaluga, E., Ursini,
16 F., 2010. Thiol redox transitions in cell signaling: a lesson from N-acetylcysteine.
17 *TheScientificWorldJournal* 10, 1192-1202.

18
19 Piecuch, A., Oblak, E., 2014. Yeast ABC proteins involved in multidrug resistance. *Cellular & molecular*
20 *biology letters* 19, 1-22.

21
22 Ramudo, L., Manso, M.A., 2010. N-acetylcysteine in acute pancreatitis. *World journal of gastrointestinal*
23 *pharmacology and therapeutics* 1, 21-26.

24
25 Rushworth, G.F., Megson, I.L., 2014. Existing and potential therapeutic uses for N-acetylcysteine: the
26 need for conversion to intracellular glutathione for antioxidant benefits. *Pharmacology & therapeutics*
27 141, 150-159.

28
29 Samuni, Y., Goldstein, S., Dean, O.M., Berk, M., 2013. The chemistry and biological activities of N-
30 acetylcysteine. *Biochimica et biophysica acta* 1830, 4117-4129.

31
32 van Leeuwen, J.S., Orij, R., Luttkik, M.A., Smits, G.J., Vermeulen, N.P., Vos, J.C., 2011a. Subunits Rip1p
33 and Cox9p of the respiratory chain contribute to diclofenac-induced mitochondrial dysfunction.
34 *Microbiology* 157, 685-694.

35
36 van Leeuwen, J.S., Vermeulen, N.P., Vos, J.C., 2011b. Involvement of the pleiotropic drug resistance
37 response, protein kinase C signaling, and altered zinc homeostasis in resistance of *Saccharomyces*
38 *cerevisiae* to diclofenac. *Applied and environmental microbiology* 77, 5973-5980.

39
40 van Leeuwen, J.S., Vredenburg, G., Dragovic, S., Tjong, T.F., Vos, J.C., Vermeulen, N.P., 2011c.
41 *Metabolism related toxicity of diclofenac in yeast as model system. Toxicology letters* 200, 162-168.

42
43 Vernhet, L., Allain, N., Le Vee, M., Morel, F., Guillouzo, A., Fardel, O., 2003. Blockage of multidrug
44 resistance-associated proteins potentiates the inhibitory effects of arsenic trioxide on CYP1A1 induction
45 by polycyclic aromatic hydrocarbons. *The Journal of pharmacology and experimental therapeutics* 304,
46 145-155.

47
48 Wang, A.G., Xia, T., Yuan, J., Yu, R.A., Yang, K.D., Chen, X.M., Qu, W., Waalkes, M.P., 2004. Effects
49 of phenobarbital on metabolism and toxicity of diclofenac sodium in rat hepatocytes in vitro. *Food and*
50 *chemical toxicology : an international journal published for the British Industrial Biological Research*
51 *Association* 42, 1647-1653.

1
2
3 Watanabe, N., Takashimizu, S., Kojima, S., Kagawa, T., Nishizaki, Y., Mine, T., Matsuzaki, S., 2007.
4 Clinical and pathological features of a prolonged type of acute intrahepatic cholestasis. *Hepatology*
5 research : the official journal of the Japan Society of Hepatology 37, 598-607.

6
7
8 Xu, Y.C., Wu, R.F., Gu, Y., Yang, Y.S., Yang, M.C., Nwariaku, F.E., Terada, L.S., 2002. Involvement of
9 TRAF4 in oxidative activation of c-Jun N-terminal kinase. *The Journal of biological chemistry* 277,
10 28051-28057.

11
12 ~~Yano, T., Takigami, E., Yurimoto, H., Sakai, Y., 2009. Yap1-regulated glutathione redox system curtails~~
13 ~~accumulation of formaldehyde and reactive oxygen species in methanol metabolism of *Pichia pastoris*.~~
14 ~~*Eukaryotic cell* 8, 540-549.~~

15
16 Zafarullah, M., Li, W.Q., Sylvester, J., Ahmad, M., 2003. Molecular mechanisms of N-acetylcysteine
17 actions. *Cellular and molecular life sciences : CMLS* 60, 6-20.

18
19
20
21
22 **Table 1: Interaction of NAC (5 mM) with DCF (1000 μ M) toxic effect on BY4741 growth as**
23 **a function of time (3, 6, 9 and 24 h) and of the drugs addition sequence, in which the drugs**
24 **are added to the cells.** Yeast colonies ~~are were~~ diluted in YPD to an OD between 0.1 and 0.2
25 and treated with NAC (5mM) and/or DCF (1000 μ M), simultaneously or after a pretreatment
26 delay. Pretreatment delay corresponds to addition of a drug 30 minutes before the second one.
27 The OD results were recorded after 3, 6, 9, and 24 hours and are expressed as percentage of the
28 control-untreated cells. Data and represent the means \pm SD of at least three independent
29 experiments. *P<0.05 compared with untreated cells. #P<0.05 compared to cells treated with
30 DCF. @P<0.05 compared with co-treatment. &P<0.05 compared with NAC pre-treatment.

31
32
33 **Table 2: Effect of DCF and NAC-DCF interaction on growth of ABC-transporter mutant**
34 **strains.** Wild type and mutant strains of *Saccharomyces cerevisiae* were pre-cultured and then
35 diluted to an OD between 0.1 and 0.2 and treated with DCF (1000 μ M) \pm NAC (5mM) or \pm L-
36 cysteine (LC, 5 mM) for 9h and growth was assessed by OD measurement. The results are
37 expressed as percentage of the control-untreated cells and represent the means \pm SD of at least
38 three independent experiments. *P<0.05 compared with untreated cells; #P<0.05 compared to
39 cells treated with DCF; \$P<0.05 compared with BY4741. For genes abbreviations refer to the
40 abbreviation list. ND, not determined.

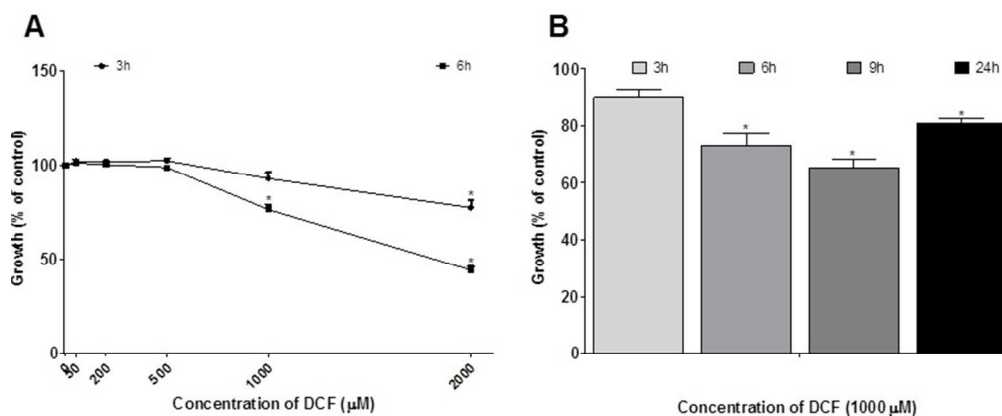


Figure 1: Dose- and time-dependent toxic effect of DCF on growth of wild-type yeast BY4741. Cells were treated with different DCF concentrations and growth was assessed by OD measurement at 3, 6, 9 and 24h. Results are expressed as %OD relative to the control untreated cells and represent means \pm SD of at least three independent experiments. *Statistically significant difference ($p < 0.05$) with respect to untreated cells. (A) Cells were treated with different concentrations of DCF for 3 and 6h after dilution of pre-culture for 48h (B) Cells from colonies are treated with DCF ($1000 \mu\text{M}$) for 3, 6, 9 and 24 hours without pre-culture.

216x97mm (96 x 96 DPI)

Table 1

Conditions Time (h)	DCF (1000 μM)	NAC (5 mM)	Co- treatment (NAC + DCF)	Pre-treatment with NAC, then NAC + DCF	Pre-treatment with DCF, then NAC + DCF
3	89.7±11.3	92.7±0.8	89.3±2.1	68.2±10.7 ^{*,#}	70.4±12.0 ^{*,#}
6	73.0±18.2 [*]	94.6±1.3	60.4±6.9 [*]	23.7±4.0 ^{*,#,@}	32.0±5.3 ^{*,#,@}
9	65.6±12.6 [*]	101.2±8.9	35.3±8.1 ^{*,#}	17.1±1.5 ^{*,#}	28.4±12.7 ^{*,#}
24	80.9±6.2 [*]	89.7±4.1	70.3±3.0 [*]	32.0±10.1 ^{*,#,@}	83.1±3.3 ^{&}

Table 2

Strain name	Mutant gene	DCF	NAC	LC	NAC+DCF	LC+DCF
BY4741	Wild type	65.6±12.6 [*]	101.2±8.9	90.5±11.4	35.3±8.1 ^{*,#}	71.7±4.3
Y02409	Pdr5	54.3±5.8 ^{*,§}	100.6±2.7	96.1±3.6	6.1±6.5 ^{*,#,§}	32.3±10.4 [*]
Y05933	Yor1	36.7±8.3 ^{*,§}	ND	ND	ND	ND
Y01503	Bpt1	36.1±4.6 ^{*,§}	ND	ND	ND	ND
Y04242	Pdr15	35.1±4.2 ^{*,§}	ND	ND	ND	ND
Y00569	Yap1	75.9±10.4 [*]	100.5±13.6	ND	39.2±21.8 ^{*,#}	ND
Y00928	Vmr1	76.5±3.1 [*]	98.5±0.7	97.1±3.4	15.2±7.7 ^{*,#,§}	65.1±7.5 [*]
Y02770	Pdr12	78.8±3.0 [*]	98.9±1.5	95.0±1.5	17.7±10.8 ^{*,#,§}	65.4±5.2 [*]