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reports

Diflunisal targets the HMGB1/CXCL12 heterocomplex and blocks immune cell recruitment

Federica De Leo, Giacomo Quilici, Mario Tirone, Francesco De Marchis, Valeria Mannella, Chiara Zucchelli, Alessandro Preti, Alessandro Gori, Maura Casalgrandi, Rosanna Mezzapelle, Marco E. Bianchi and Giovanna Musco

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 8th Mar 2019

Thank you for submitting your manuscript for consideration by EMBO Reports. It has now been seen by three referees whose comments are shown below.

As you can see, all referees express interest in your study revealing a mechanism of diflunisal action through HMGB1-CXCL12 targeting. However, they also raise concerns that need to be addressed in full before we can consider publication of the manuscript here.

Given these constructive comments, I would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO Reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS:

Referee #1:

What is known:
This project is a follow-up of a story that has been started in 2012 by some of the authors and since then resulted in the publication of several highly cited, high impact articles.
Key players in this are the mediators HMGB1 and CXCL12 that form heterocomplexes. The cellular effects of extracellular HMGB1 (three cysteines) depend on its oxidative state. Both, the reduced

and the disulfide form have been shown to be released. Reduced HMGB1 binds CXCL12 and synergistically enhances cell migration induced by CXCL12 via CXCR4 and oxidized HMGB1 directly induces cytokine expression b via TLR4 (Schiraldi, 2012).
Glycyrrhizin is a natural HMGB1-binding protein that inhibits synergistic effects on CXCL12 (Schiraldi, 2012).
Screening for binding-proteins of salicylic acid (SA) revealed reduced and oxidized HMGB1 as partner for SA. SA inhibited both, HMGB1-induced chemotaxis and induction of cytokine expression (Choi, 2015)

What is new:
Diflusan (DFL), t is a more potent functional inhibitor of HMGB1-CXCL12 than any other tested SA-variant and is shown to disrupt preformed HMGB1-CXCL12 complexes by binding to both, fully reduced but not disulfide HMGB1 and CXCL12.
Based on NMR experiments the authors provide structure models of DFL binding to HMGB1 and CXCL12.DFL does not inhibit chemotaxis that is mediated by CXCL12 alone.
DFL is a selective inhibitor for synergistic chemotactic effects of the heterocomplex but not other activities of HMGB1.

General comment: The formation of heterodimers between distinct species of inflammatory mediators that inhibit or enhance functional activity is an emerging field that offers further opportunities to address specific targets that are important e.g. in inflammation. In contrast to molecular pockets for receptor antagonists and enzyme inhibitors the large contact area renders the development of protein interaction inhibitors more difficult. Here the authors report how a small molecule can inhibit the formation of chemotactic hetero-complexes without affecting other biologic activities of the single partners such as the increase of cytokine expression. Beneficial effects will depend on the scenario, as inhibition of the HMGB1-CXCL12 complex might dampen inflammation or prevent healing. The Introduction is quite short and does not present any discussion/literature about other chemokines that have been reported to form hetero-complexes resulting in biological effects.
1) It is not always clear how many independent chemotaxis experiments were carried out. It is for instance confusingly written in Fig. 1B, 2E "Data represent the average {plus minus} standard deviation (avg {plus minus} sd, n=3) of one representative experiment". The independent number of experiments should be clearly indicated and significant differences should be always visualized in the figure.
2) Fig. 1 B, E. In contrast to results by Choi et al., it seems that the chemotactic effect of the mutated HMGB1 (R23AK27A is R24AK28A) is significantly different from the effect of wild type HMGB1 (Fig. 1 B and E). If so, how do the authors explain the different activities? Does the mutation affect the interaction with CXCL12?
3) The authors reason that they used DFL for its superior activity to block HMGB1/CXCL12-induced chemotaxis. However, the reported apparent Kds of DFL binding to HMGB1 (~1.6 mM by MST) and CXCL12 (~0.8 mM by NMR) are very weak and are in striking contrast to the reported high affinity of 3AESA (a related NSAID) to HMGB1 (Choi et al.) that was measured by SPR with a Kd of 1.48 nM. This mismatch in affinity and biologic activity should be discussed as potentially additional mechanisms have to be taken into account.
4) The authors state that the NMR exchange time scale is fast-intermediate exchange, and yet their e.g. MST-derived Kd values are 0.8 - 1.6 mM, which are not consistent with intermediate exchange, but are with fast exchange. Is it possible that observed line broadening results from changes in internal motions (vis-à-vis exchange dynamics) modulated by ligand binding?
5) CXCL12 is known to dimerize with Kd in the mM range, and thus fast exchange on the NMR time scale. However, the extent of dimer formation (i.e. monomer-dimer equilibrium) can be followed by monitoring CXCL12 chemical shift changes (vs concentration, pH, etc...) as reported (Veldkamp, 2005). This would be a good way for the authors to follow what is occurring from the perspective of CXCL12 itself. This is especially crucial when delineating the mechanism of action with HMGB1 and DFL. Perhaps the authors already have these data. They should be shown and discussed. Along this line the authors conclude that "DFL promotes CXCL12 self-association." Here it should be stated to what extend dimerization occurs as CXCL12 homodimers are chemotactically inactive.
6) The authors discuss binding of HMGB1 to CXCL12 and provide a Kd value of ~4 μM. Did the authors take into account that the stoichiometry is not 1:1, but rather 2:1, i.e. (CXCL12)2(HMGB1), as published by Schiraldi et al., 2012? And how might this affect their Kd value?

7) Relatedly, how can the authors explain that DFL binding is so much weaker (0.8 - 1.6 mM), and yet can apparently dissociate the HMGB1:CXCL12 complex?
8) In Fig 3F, the MST data were with 50 nM HMGB1 and 50 µM CXCL12. In this exp, 6-His tagged HMGB1 was used with a non-covalently linked fluorescence dye. Surprisingly, only 10-100 µM DFL induced dissociation, yet DFL binds to either much more weakly. How do the authors explain this?
9) Also, the authors note aggregation at higher concentrations of DFL in this MST experiment. This indicates formation of large oligomers/aggregates induced by the presence of DFL, yet the concentrations of CXCL12 and HMGB1 are much lower. How is this explained? And might this affect this MST experiment and its analysis?
Small details:
10) Typically fluorine is green in the CPK coloring convention which may be a bit confusing when looking at the space filling models in figures 1D and 2C.
11) The clone of the inhibitory CXCL12 antibody is not mentioned
12) It would facilitate reading the figures if a clear distinction between reduced and disulfide HMGB1 is always denoted (e.g. in Fig. 3H)

Comments to the text/writing:
"Cell migration experiments, however, showed that DFL was unable to inhibit CXCL12-induced chemotaxis at concentrations where it inhibited chemotaxis induced by HMGB1 (Fig. 2D). Thus, neither binding to HMGB1 alone or CXCL12 alone can justify the inhibition of chemotaxis." The causal link of these two sentences is not logically correct as reduced HMGB1 alone could bind and stimulate CXCR4-mediated chemotaxis.
"we suggest that it (DFL) should not be given to patients recovering from injury or trauma." Is this sentence really necessary or appropriate in a basic science report without any patient data?

Overall, this is a very interesting study, and methodologically there are no major concerns.

Referee #2:

The paper by Musco and coworkers reports on a very intriguing finding related to a potential novel molecular mechanism for an established and well-known NSAID drug, i.e. Diflunisal. In particular, they provide convincing evidences on a potential protein-protein disruption mechanism, which may be at the basis, or at least contribute to, the HMGB1-induced cell migration. The authors provide several data using both NMR and MST to fully prove the new putative molecular mechanism. Additionally, they study the compound at cellular level, and eventually provide in vivo data too. All this goes in the direction of discovering a new mechanism for this NSAID. The paper is well written, reads well, and reports on real new findings, which is not always the case for the time being when many "me too" papers are often submitted for publication. In light of this consideration, the manuscript well deserves publication in EMBO report. Only one minor concern the present reviewer would like to raise is related to the multitarget mechanism of action, which has slightly been touched by the authors in the discussion. Indeed, there might be a little discrepancy between the molecular data (micromolar) and the cellular data (nanomolar), pointing to the involvement of other potential targets responsible for the phenotypic profile due to the drug treatment. Indeed, Diflunisal has previously been reported to bind to P300 as well as to CREB-binding protein, besides its "conventional" mechanism of inhibition of COX enzymes. All these molecular interactions, and possibly others, may contribute to the complex mechanism of action of the drug and may be at the basis of its ultimate therapeutic profile. These aspects should be better considered by the authors, discussed more in depth in the text, and taken frankly into considerations for other mechanistic studies the authors may conceive for the present or subsequent papers.

Referee #3:

This manuscript described an interesting mechanism for the anti-inflammatory effects of diflunisal, an aspirin-like NSAIDs. The authors show that diflunisal binds to the two "box domains" of

HMGB1 and to CXCL12 by MST, NMR and functional studies using chemotaxis. HMGB1 exists in an oxidized state with a disulfide between Cys22-Cys44 that does not form a heterocomplex with CXCL12 and activates TLR4 receptor, and in a reduced form that forms a heterodimer and activates CXCR4. Diflunisal disrupts this heterocomplex leading to its anti-inflammatory effect. However, CXCL12 alone should also activate CXCR4, counteracting the effects of the heterocomplex. What effect the heterocomplex has in activating CXCR4 that is different from CXCL12 is not discussed. It is interesting to note that diflunisal interacts with both box domains (with Kd of ~1.8 mM measured by MST- with an inability to reach saturation due to solubility issues) and with CXCL12 (with Kd of ~ 0.8 mM - based on titrations of two NMR peak perturbations). It would be good to use both techniques with each protein, so that the Kd's can be compared.

Although the authors published papers on salicylate acid and HMGB1, there is a serious problem with using 3T3 fibroblasts. There is some controversy as to whether they express CXCR4 (PMID: 22048734, among others). Since CXCR4 is crucial to this study, I would recommend that the authors perform FACS to validate the expression of CXCR4. The compound Diflunisal is very hydrophobic and may have cellular targets that can confound these results, if the expression of CXCR4 is not verified.

The Figure 2C, which shows the HADDOCK models based on some NMR data, should be zoomed in to highlight the interactions of interest. It is difficult with the current figure to understand what residues and interactions is the author trying to highlight.

The authors should have done experiments R23 and R109 mutants, which were identified from the HADDOCK models as important for the electrostatic interactions. The use of the R23A/K29A mutant, which was part of another study, is not justified by itself. It would have been acceptable if it was followed by cell migration studies with the double mutant R23A/R109A as well as R23A and R109 to determine if there are varying effects with the single mutants and the mutations combined.

In figure 2D, the concentrations of Diflunisal tested for effects on cell migration with CXCL12 go from 0-30nM. However, the apparent KD for Diflunisal binding to CXCL12 is around 800µM as shown in Figure S6. The authors should provide a rationale as to why such low concentrations of the drug were tested since not even half of CXCL12 would be bound to Diflunisal at such low concentrations.

In Figure 2E, where DFL is tested for inhibition of HMGB1/CXCL12 complex, the concentrations of CXCL12 added for complex formation is 1.5nM, which is much lower than the Kd values for CXCL12 binding to HMGB1, which has been calculated as approx. 4µM, as shown in Figure 3C. Please provide a rationale for using such low concentrations of CXCL12 for complex formation. Is there evidence of complex formation at such low concentrations? Otherwise, the experiment does not make sense.

1st Revision - authors' response 7th Jun 2019

We thank the Editor and the Reviewers for their appreciation and constructive comments, which have certainly helped us improve our manuscript.
We have extensively rewritten the text, and reformatted it from *Report* to *Full Article*. Thus, we only direct the reviewer to the pages containing the new text.

Referee #1:

What is known:
This project is a follow-up of a story that has been started in 2012 by some of the authors and since then resulted in the publication of several highly cited, high impact articles.
Key players in this are the mediators HMGB1 and CXCL12 that form heterocomplexes. The cellular effects of extracellular HMGB1 (three cysteines) depend on its oxidative state. Both, the reduced and the disulfide form have been shown to be released. Reduced HMGB1 binds CXCL12 and synergistically enhances cell migration induced by CXCL12 via CXCR4 and oxidized HMGB1 directly induces cytokine expression b via TLR4 (Schiraldi, 2012).

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General comment: The formation of heterodimers between distinct species of inflammatory mediators that inhibit or enhance functional activity is an emerging field that offers further opportunities to address specific targets that are important e.g. in inflammation. In contrast to molecular pockets for receptor antagonists and enzyme inhibitors the large contact area renders the development of protein interaction inhibitors more difficult. Here the authors report how a small molecule can inhibit the formation of chemotactic hetero-complexes without affecting other biologic activities of the single partners such as the increase of cytokine expression. Beneficial effects will depend on the scenario, as inhibition of the HMGB1-CXCL12 complex might dampen inflammation or prevent healing. The Introduction is quite short and does not present any discussion/literature about other chemokines that have been reported to form hetero-complexes resulting in biological effects.
We thank the reviewer for the suggestion. Following her/his advice we have expanded the introduction and discussed the biological relevance of other chemokine hetero-complexes (see pages 2 and 3)

1) It is not always clear how many independent chemotaxis experiments were carried out. It is for instance confusingly written in Fig. 1B, 2E "Data represent the average {plus minus} standard deviation (avg {plus minus} sd, n=3) of one representative experiment". The independent number of experiments should be clearly indicated and significant differences should be always visualized in the figure.

We have clarified the number of independent chemotaxis experiment in the figure legend. Significant differences have been also visualized.

2) Fig. 1 B, E. In contrast to results by Choi et al., it seems that the chemotactic effect of the mutated HMGB1 (R23AK27A is R24AK28A) is significantly different from the effect of wild type HMGB1 (Fig. 1 B and E). If so, how do the authors explain the different activities? Does the mutation affect the interaction with CXCL12?

The differences in number of cells/field observed between this work and Choi et al. is due to the different methods used to count the cells. The method applied in the present work, relies on a new semi-automatized counting procedure routinely applied now in our laboratory. In the Choi et al paper cell count was still manual. Thus, there is no discrepancy between the results on R23AK27A obtained in this paper and in Choi et al. Please also note that in this revised version of the manuscript, to avoid confusion, we have removed the R23AK27A mutant, and have generated 3 new mutants, as suggested by reviewer 3 (R23A, R109A and R23A/R109A). None of these mutations affected cell migration and binding to CXCL12 (Fig 1E, EV2A,B; Appendix Fig S2A,B).

3) The authors reason that they used DFL for its superior activity to block HMGB1/CXCL12-induced chemotaxis. However, the reported apparent Kds of DFL binding to HMGB1 (~1.6 mM by MST) and CXCL12 (~0.8 mM by NMR) are very weak and are in striking contrast to the reported high affinity of 3AESA (a related NSAID) to HMGB1 (Choi et al.) that was measured by SPR with a Kd of 1.48 nM. This mismatch in affinity and biologic activity should be discussed as potentially additional mechanisms have to be taken into account.

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We thank the reviewer for suggesting to comment results previously obtained on 3ASA. Following his/her advice, we have mentioned in the Results section the inhibitory effect of 3ASA on HMGB1 elicited cell-migration, pointing out that the high metabolic instability of this molecule discourages its pharmacological application (page 4). This instability has prompted us to search for more stable salicylate derivatives with similar or even better activity.
We agree with the reviewer that the Kd of DFL is in striking difference with the low affinity of 3ASA measured by SPR (nanomolar range). However, the Kd of DFL has been measured in solution (NMR and MST) whereas the Kd of 3ASA has been measured with SPR with 3ASA immobilized on the sensor chip. A nanomolar affinity in solution should have resulted in a slow-exchange regime in NMR titrations of 3ASA into HMGB1. However, this was not observed in Choi et al., in contrast, addition of 3ASA induced in the ^{15}N HSQC spectra of HMGB1 only very small chemical shifts in the fast exchange regime, suggesting a µM/mM affinity in solution (similar to DFL). The weak affinity in solution was also confirmed by the presence of STD effects, that in principle are not observable with high affinity ligands. Thus, we suspect that immobilization of the 3ASA on the SPR sensor surface might influence/contribute to additional interactions, that do not seem to be present when both the ligand and the protein are free in solution, like in NMR titrations. These discrepancies between affinity measured in solution or on an immobilized surface are not unusual (1,2).
The authors state that the NMR exchange time scale is fast-intermediate exchange, and yet their e.g. MST-derived Kd values are 0.8 - 1.6 mM, which are not consistent with intermediate exchange, but are with fast exchange. Is it possible that observed line broadening results from changes in internal motions (vis-à-vis exchange dynamics) modulated by ligand binding?
Indeed, the fast exchange is in agreement with the mM range affinity measured by MST, that is on turn in line with the affinity measured by NMR monitoring the CSP as function of DFL concentration (2.8 ± 1.4 mM) (now reported in the manuscript, page 6) and with the observed STD effects. The intermediate exchange observed for some residues, might be due to changes in internal motions as correctly pointed out by the reviewer. We have thus removed the "intermediate" exchange definition, and we have commented the observed line broadening effects for discrete amide resonances, hypothesizing that these effects are due to changes in internal motion occurring upon ligand binding. See page 5.

4) CXCL12 is known to dimerize with Kd in the mM range, and thus fast exchange on the NMR time scale. However, the extent of dimer formation (i.e. monomer-dimer equilibrium) can be followed by monitoring CXCL12 chemical shift changes (vs concentration, pH, etc...) as reported (Veldkamp, 2005). This would be a good way for the authors to follow what is occurring from the perspective of CXCL12 itself. This is especially crucial when delineating the mechanism of action with HMGB1 and DFL. Perhaps the authors already have these data. They should be shown and discussed. Along this line the authors conclude that "DFL promotes CXCL12 self-association." Here it should be stated to what extend dimerization occurs as CXCL12 homodimers are chemotactically inactive.

As suggested by the reviewer, along the line of Veldkamp et al,. we have followed the extent of dimer formation monitoring CXCL12 chemical shift changes as a function of CXCL12 concentration in the absence and in the presence of 1 mM DFL. We obtained an apparent dimerization constant of 5.6 ± 0.4 and 2.1 ± 0.8 mM, respectively (now reported in Figure EV4). This indicates that in a solution containing 0.1 mM CXCL12 and 1 mM DFL the percentage of CXCL12 dimer increases from 3 to 8%. This relatively small increase in dimer formation should be negligible in cell migration experiments. As a matter of fact DFL does not inhibit CXCL12 induced chemotaxis, as shown in Figure 2D. Data related to the percentage of CXCL12 dimer formation and comments on this issue have been added in the manuscript. See pages 7-8 and Fig. EV4.
The authors discuss binding of HMGB1 to CXCL12 and provide a Kd value of ~4 µM. Did the authors take into account that the stoichiometry is not 1:1, but rather 2:1, i.e. (CXCL12)2(HMGB1), as published by Schiraldi et al., 2012? And how might this affect their Kd value?
We considered that the stoichiometry is most likely 1:2, however we observed the typical Langmuir isotherm, which is indistinguishable from binding isotherms with multiple equivalent binding sites. That is, the affinity of each site for ligand is the same as all other specific binding

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sites (identical K_d values). We have now clarified this issue in Material and Methods session under " MST measurements ", pages 26-27.

Relatedly, how can the authors explain that DFL binding is so much weaker (0.8 - 1.6 mM), and yet can apparently dissociate the HMGB1:CXCL12 complex?

This was indeed an important issue, and we have now devoted a significant part of the Discussion to it. See pages 13-14.

5) In Fig 3F, the MST data were with 50 nM HMGB1 and 50 µM CXCL12. In this exp, 6-His tagged HMGB1 was used with a non-covalently linked fluorescence dye. Surprisingly, only 10-100 µM DFL induced dissociation, yet DFL binds to either much more weakly. How do the authors explain this?

This was also an important issue, and we have devoted another part of the Discussion to it. See pages 14-15.

6) Also, the authors note aggregation at higher concentrations of DFL in this MST experiment. This indicates formation of large oligomers/aggregates induced by the presence of DFL, yet the concentrations of CXCL12 and HMGB1 are much lower. How is this explained? And might this affect this MST experiment and its analysis?

For samples preparation for MST experiments special care was dedicated to avoid the presence of any aggregate. To this aim after 15 minutes incubation, all the 16 samples have been centrifuged at 15,000 g for 10 minutes before loading them into the capillaries. Herewith we removed large aggregates and guaranteed good sample quality and homogeneity. Indeed, the binding curve presented in Figure 3F (resulting from three different measurements) is highly reproducible, has small standard deviations and good signal/noise ratio. Nevertheless at high DFL concentration (the last 2 titration points), we observed bumpiness of the MST traces suggestive of aggregation. We also observed the presence of "shoulders" on the typical gaussian shape of the capillaries, that suggest sticking of the sample on the capillaries. Thus, we excluded the 2 highest concentrations from the fitting. It is not unusual to observe aberrant MST traces due to protein aggregation/denaturation at elevated DFL concentration as (3) *Why this happens is difficult to explain, we cannot exclude that at higher concentrations aspecific phenomena might occur that then induce aggregations. Of note, the fluorescent label used for MST experiment provides high sensitivity so that by using nM concentrations of fluorescently label protein small changes in thermophoretic movement induced either by ligand binding (up to mM affinity) or aggregation can be detected.*

To test whether exclusion of points at high concentrations might affect the results, we also excluded the point at 0.63 mM, and we obtained an EC_{50} of 514 mM, which was quite similar to the one obtained including the point at 0.63 mM. Notably, in both cases the curve is fitted showing a transition from HMGB1-CXCL12 bound state at 950 to an unbound state (complex breakage) at ~ 925 Fnorm (‰), which are the values observed in the reference titration HMGB1-CXCL12 in Figure 3C. Thus, we are confident that the 14 data points in Figure 3F represent a good data set for a reliable fitting, and having more points at higher concentrations would not significantly change the fitting results.

In the "Material and Methods" under "MST measurements" we have now specified that "All samples were incubated for 15 minutes and centrifuged at 15,000 g for 10 minutes before measurements."

Small details:

7) Typically fluorine is green in the CPK coloring convention which may be a bit confusing when looking at the space filling models in figures 1D and 2C.

We have changed Figures 1D, 2C and EV5 as suggested.

8) The clone of the inhibitory CXCL12 antibody is not mentioned

We have now added this info, see page 18.

9) It would facilitate reading the figures if a clear distinction between reduced and disulfide HMGB1 is always denoted (e.g. in Fig. 3H)

Following the reviewer's advice in the figures showing migration experiments we have clearly stated whether fully reduced (fr-HMGB1) or disulfide protein (ds-HMGB1) was used.

Comments to the text/writing:
"Cell migration experiments, however, showed that DFL was unable to inhibit CXCL12-induced chemotaxis at concentrations where it inhibited chemotaxis induced by HMGB1 (Fig. 2D). Thus, neither binding to HMGB1 alone or CXCL12 alone can justify the inhibition of chemotaxis." The causal link of these two sentences is not logically correct as reduced HMGB1 alone could bind and stimulate CXCR4-mediated chemotaxis.
We have removed this sentence, as the causal link was difficult to follow. See page 8.
"we suggest that it (DFL) should not be given to patients recovering from injury or trauma." Is this sentence really necessary or appropriate in a basic science report without any patient data?
We have removed this sentence.
Overall, this is a very interesting study, and methodologically there are no major concerns.
We thank the reviewer for her/his appreciation.

Referee #2:

The paper by Musco and coworkers reports on a very intriguing finding related to a potential novel molecular mechanism for an established and well-known NSAID drug, i.e. Diflunisal. In particular, they provide convincing evidences on a potential protein-protein disruption mechanism, which may be at the basis, or at least contribute to, the HMGB1-induced cell migration. The authors provide several data using both NMR and MST to fully prove the new putative molecular mechanism. Additionally, they study the compound at cellular level, and eventually provide in vivo data too. All this goes in the direction of discovering a new mechanism for this NSAID. The paper is well written, reads well, and reports on real new findings, which is not always the case for the time being when many "me too" papers are often submitted for publication. In light of this consideration, the manuscript well deserves publication in EMBO report. Only one minor concern the present reviewer would like to raise is related to the multitarget mechanism of action, which has slightly been touched by the authors in the discussion. Indeed, there might be a little discrepancy between the molecular data (micromolar) and the cellular data (nanomolar), pointing to the involvement of other potential targets responsible for the phenotypic profile due to the drug treatment. Indeed, Diflunisal has previously been reported to bind to P300 as well as to CREB-binding protein, besides its "conventional" mechanism of inhibition of COX enzymes. All these molecular interactions, and possibly others, may contribute to the complex mechanism of action of the drug and may be at the basis of its ultimate therapeutic profile. These aspects should be better considered by the authors, discussed more in depth in the text, and taken frankly into considerations for other mechanistic studies the authors may conceive for the present or subsequent papers.
We thank the reviewer for her/his appreciation, and prompted by her/his suggestion and by the comments of the other reviewers, we have expanded the discussion section and we have commented more in depth the discrepancy between the biophysical and cellular data.

Referee #3:

This manuscript described an interesting mechanism for the anti-inflammatory effects of diflunisal, an aspirin-like NSAIDs. The authors show that diflunisal binds to the two "box domains" of HMGB1 and to CXCL12 by MST, NMR and functional studies using chemotaxis. HMGB1 exists in an oxidized state with a disulfide between Cys22-Cys44 that does not form a heterocomplex with CXCL12 and activates TLR4 receptor, and in a reduced form that forms a heterodimer and activates CXCR4. Diflunisal disrupts this heterocomplex leading to its anti-inflammatory effect. However, CXCL12 alone should also activate CXCR4, counteracting the effects of the heterocomplex. What effect the heterocomplex has in activating CXCR4 that is different from CXCL12 is not discussed.
The synergic effect of the heterocomplex in CXCR4 induced chemotaxis has been better discussed both in the Introduction and in the Discussion.

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It is interesting to note that diflunisal interacts with both box domains (with Kd of ~1.8 mM measured by MST- with an inability to reach saturation due to solubility issues) and with CXCL12 (with Kd of ~ 0.8 mM - based on titrations of two NMR peak perturbations). It would be good to use both techniques with each protein, so that the Kd's can be compared.

Following the reviewer's suggestion we have measured the affinity of DFL to CXCL12 and to HMGB1 with both MST and NMR. Both techniques indicate that the affinity is in the millimolar range. These data have been included in the manuscript, see page 6 and Fig. EV1, and page 7 and Fig. EV3.

Although the authors published papers on salicylate acid and HMGB1, there is a serious problem with using 3T3 fibroblasts. There is some controversy as to whether they express CXCR4 (PMID: 22048734, among others). Since CXCR4 is crucial to this study, I would recommend that the authors perform FACS to validate the expression of CXCR4. The compound Diflunisal is very hydrophobic and may have cellular targets that can confound these results, if the expression of CXCR4 is not verified.

HMGB1 does not induce the migration of Cxcr4 KO MEFs (4), *and CXCR4 is detectable in WT MEFs. Regarding 3T3 cells, we verified that our 3T3 clone expresses CXCR4 by querying a transcriptome analysis that we had performed for different reasons, and found that CXCR4 mRNA is present at quite decent levels. This leaves the possibility that the protein is not adequately expressed, and indeed anti-CXCR4 antibodies give weak signals by flow cytometry. Then we tested whether AMD3100, which is a clinically used specific CXCR4 inhibitor, would inhibit the chemotactic activity of HMGB1 on 3T3 cells. It clearly inhibits 3T3 cell migration, and we attach here the results.*

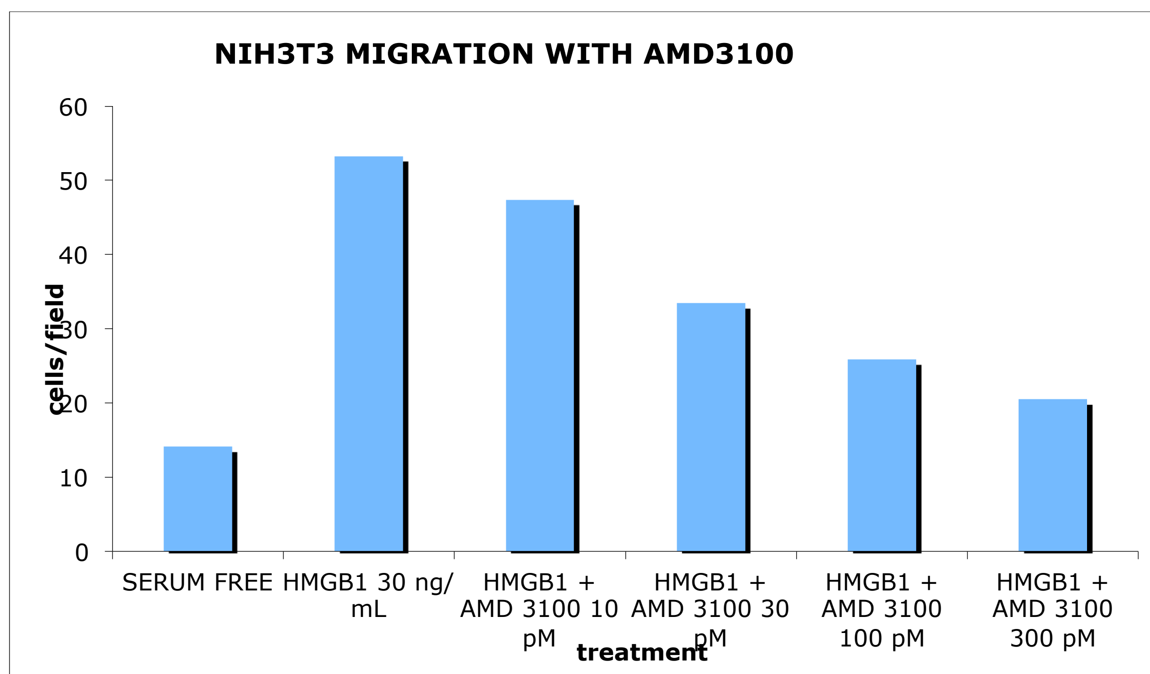

Figure for reviewer only

The Figure 2C, which shows the HADDOCK models based on some NMR data, should be zoomed in to highlight the interactions of interest. It is difficult with the current figure to understand what residues and interactions is the author trying to highlight.

We have modified Figure 2C according to the referee's comment.

The authors should have done experiments R23 and R109 mutants, which were identified from the HADDOCK models as important for the electrostatic interactions. The use of the R23A/K29A mutant, which was part of another study, is not justified by itself. It would have been acceptable if it was followed by cell migration studies with the double mutant R23A/R109A as well as R23A and R109 to determine if there are varying effects with the single mutants and the mutations combined.

Following the reviewer's advice we have generated three new mutants: R23A, R109A and the double mutant R23A/R109A. These mutations do not compromise the ability of HMGB1 to induce cell migration, however their chemotactic activity is not hampered by the presence of DFL, thus

indicating that these residues are important for DFL binding. These data have been now included in Figure 1E and Fig EV2A,B. Sea also Appendix Fig. S2A,B.

In figure 2D, the concentrations of Diflunisal tested for effects on cell migration with CXCL12 go from 0-30nM. However, the apparent KD for Diflunisal binding to CXCL12 is around 800µM as shown in Figure S6. The authors should provide a rationale as to why such low concentrations of the drug were tested since not even half of CXCL12 would be bound to Diflunisal at such low concentrations.

The rationale was to test whether DFL would inhibit CXCL12 at concentrations where it inhibits the heterocomplex. If it did, one could have argued that DFL was really inhibiting CXCL12 alone, which is not what we found.

In Figure 2E, where DFL is tested for inhibition of HMGB1/CXCL12 complex, the concentrations of CXCL12 added for complex formation is 1.5nM, which is much lower than the Kd values for CXCL12 binding to HMGB1, which has been calculated as approx. 4µM, as shown in Figure 3C. Please provide a rationale for using such low concentrations of CXCL12 for complex formation. Is there evidence of complex formation at such low concentrations? Otherwise, the experiment does not make sense.

We have added a whole section in the Discussion to speculate why the heterocomplex is active on cells at nanomolar concentrations, while its Kd is in the micromolar range (see pages 13-15)

1. Zega A. NMR Methods for Identification of False Positives in Biochemical Screens. J Med Chem. 2017;60:9437–47.
2. Davis BJ, Erlanson DA. Learning from our mistakes: The “unknown knowns” in fragment screening. Bioorganic Med. Chem. Lett. 2013.
3. Linke P, Amaning K, Maschberger M, Vallee F, Steier V, Baaske P, et al. An Automated Microscale Thermophoresis Screening Approach for Fragment-Based Lead Discovery. J Biomol Screen. 2016;21:414–21.
4. Schiraldi M, Raucci A, Muñoz LM, Livoti E, Celona B, Venereau E, et al. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. J Exp Med. 2012;209:551–63.

2nd Editorial Decision — 4th Jul 2019

Thank you for submitting the revised version of your manuscript. It has now been seen by all of the original referees.

As you can see, all referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address the below minor/editorial points:

REFEREE REPORTS:

Referee #1:

The manuscript has considerably improved. I have got two requests/remarks regarding Figure 1:
It is unusual to provide chemotaxis data from one representative experiment (Fig 1B)and calculate statistics from the three replicates. Instead, as the authors state that three independent experiments have been done, the means +- sd of the independent experiments should be used.
Fig 1E (chemotaxis towards R23A/R109A) and EV2 B(chemotaxis towards R109A) appear to be identical. Please check whether one of the data sets has to be replaced by the correct data.

Referee #2:

The authors have adequately responded to my point and the paper is now suitable for publication

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Referee #3:

The manuscript aims to describe mechanism for the anti-inflammatory effects of a well known drug i.e. Diflunisal. The authors have utilized MST and NMR to demonstrate that Diflunisal disrupts the HMGB1-CXCl12 heterocomplex for its anti-inflammatory effects. The authors have successfully answered all the concerns with the manuscript. With the additional data related to the suggested mutants and validation of the affinity data by both techniques, the quality of the manuscript is suitable for publication in EMBO. The findings of the paper are very interesting and will be appealing to the scientific audience.

2nd Revision - authors' response 10th Jul 2019

To address the comment of Reviewer 1, we have provided in Appendix Figure S1 the results emerging from independent experiments performed at different times, which prove the reproducibility of the inhibitory activity of DFL in cell migration experiments.

We are indebted to Reviewer 1 for having pointed out the problem with Figure EV2 B, which has now been replaced with the correct one.

The authors performed the requested editorial changes.

3rd Editorial Decision 23rd Jul 2019

Thank you for submitting your revised manuscript. I have now looked at everything and all looks fine. Therefore I am very pleased to inform you that your manuscript has been accepted for publication in EMBO Reports.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Giovanna Musco

Journal Submitted to: EMBO Reports

Manuscript Number: EMBOR-2019-47788

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In cell migration experiments n=3 biological replicates is standard practice.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We have used n=4 because it is the minimal sample size which can can give statistically significant results in Kruskal-Willis tests.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples/animals were excluded from the analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	The mice were randomly assigned to experimantal groups
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	The data were blinded
5. For every figure, are statistical tests justified as appropriate?	YES
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The cell migration data are normally distributed according to Kologorov-Smirnov test (not shown here). Animal tests did not assume a normal distribution, and non- parametric tests were used.
Is there an estimate of variation within each group of data?	Yes, standard deviation is always reported
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All the antibodies used have been previously validated, and are in Antibodypedia
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines is ATCC and cells were recently authenticated. They are routinaly tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Eight-week old wild type male C57Bl6 mice were purchased from Charles River, Calco, Italy, and housed in the San Raffaele animal house for 3 days before experimentation
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Protocol 838 was approved by the San Raffaele IACUC and by the Italian Istituto Superiore di Sanità.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	we confirm compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n.a.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	n.a.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n.a.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n.a.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n.a.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n.a.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n.a.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	n.a.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We have provided the PDB files of the models
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n.a.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n.a.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No