Non-epigenetic induction of HEXIM1 by DNMT1 inhibitors and functional relevance

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We have been studying the role of Hexamethylene bisacetamide (HMBA) Induced Protein 1 (HEXIM1) as a tumor suppressor whose expression is decreased in breast and prostate cancer. The anti-cancer actions of HEXIM1 in melanomas and AML have been reported by other groups. Previous studies have shown that 5-Aza-2’-deoxycytidine (5-AzadC), a DNMT1 inhibitor, induces re-expression of tumor suppressor genes by removing/erasing methylation marks from their promoters. Our studies highlighted another mechanism wherein 5-AzadC induced DNA damage, which then resulted in enhanced occupancy of NF-κB, P-TEFb, and serine 2 phosphorylated RNA Polymerase II on the HEXIM1 gene. As a consequence, 5-AzadC induced HEXIM1 expression in prostate cancer cell lines and triple negative breast cancers. 5-AzadC-induced DNA damage enhanced P-TEFb occupancy via a mechanism that involved activation of ATR and ATM and induction of NF-κB recruitment to the HEXIM1 promoter. Downregulation of NF-κB attenuated 5-AzadC-induced HEXIM1 expression in prostate and breast cancer cells. The functional relevance of 5-AzadC-induced HEXIM1 expression is revealed by studies showing the HEXIM1 is required for the induction of apoptosis. Collectively, our findings support a non-epigenetic mechanism for 5-AzadC-induced re-expression of HEXIM1 protein, and may contribute to the clinical efficacy of 5-AzadC.

Cancer development is strongly correlated with progressive accumulation of multiple genetic and epigenetic events, which may eventually lead to genetic instability and subsequently to the progressive disease. The most common epigenetic change in cancers is hypermethylation of the CpG islands within the promoters of tumor suppressor genes due to either increased activity or overexpression of DNA-methyltransferase 1 (DNMT1). Two nucleoside (cytosine) analogs 5-aza-2’-cytidine (5-AzaC) and 5-aza-2’-deoxycytidine (5-AzadC) have been characterized as DNMT inhibitors. These inhibitors have been approved by the US Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML)1. The FDA approval of these “epidrugs” supports the therapeutic potential of DNMT1 inhibitors.

Despite the widely accepted demethylating activity of DNMT1 inhibitors2–4, another proposed basis for their cytotoxicity is the formation of irreversible, covalent enzyme–DNA adducts and the ensuing response to DNA damage5,6. The responses to 5-AzaC-induced DNA double-strand breaks (DSBs) are mediated predominantly via the Phosphatidylinositol 3-kinase-related kinase (PIKK) family member sensor proteins, Ataxia Telangiectasia, Mutated (ATM) and Ataxia Telangiectasia and Rad3-related protein (ATR)7. ATR and ATM then phosphorylate downstream Checkpoint Kinase 1 and 2 (CHK1 and CHK2) proteins, respectively, to further activate downstream targets. In particular, DNA-damage triggered ATM directly activates IKK and the NF-κB pathway. NF-κB has been reported to be involved in the recruitment of Positive Transcriptional Elongation Factor B (P-TEFb) to the promoter regions of genes involved in innate and adaptive immunity8. P-TEFb is a heterodimer between the cyclin-dependent kinase 9 (Cdk9) and its regulatory subunit Cyclin T1.

One of the promoter regions wherein P-TEFb is recruited to is the gene encoding the tumor suppressor Hexamethylene bisacetamide (HMBA) Induced Protein 1 (HEXIM1)9. HEXIM1, in turn, inhibits P-TEFb activity and thereby transcriptional elongation of HEXIM1 target genes9. Studies from our laboratory demonstrated that HEXIM1 is a co-repressor of the androgen and estrogen receptors, and is required for the inhibition of the activity of these receptors by anti-hormones10,11. HEXIM1 expression is decreased in hormone resistant breast and prostate cancer. HEXIM1 expression is also decreased in metastatic breast cancer, and re-expression of HEXIM1 resulted in the inhibition of mammary tumor growth and metastasis12. Together these studies support the re-expression of HEXIM1 as a therapeutic goal.
In this report, we show that 5-AzadC induced DNA damage and recruitment of CDK9 and serine 2 phosphorylated (S2P) RNAPII to the HEXIM1 promoter and HEXIM1 coding region, respectively. Occupancy of HEXIM1 gene by P-TEFb results in increased HEXIM1 transcription. The resulting increase in HEXIM1 expression resulted in upregulation of p21, likely mediated by HEXIM1 upregulation of p53 stability. Thus, the induction of the tumor suppressor protein HEXIM1 is part of the cellular response to DNA damage and the resulting inhibition of cell cycle progression or apoptosis. Our findings also have important implications for the development of small molecules or other strategies to induce the expression of HEXIM1 as therapeutic options against cancer.

Results

5-Aza-2′ deoxycytidine induced HEXIM1 expression. Because of the well-known role of DNMT1 in the inhibition of the expression of tumor suppressor genes, we determined if DNMT1 inhibitors could be utilized to re-express HEXIM1 in cancer cells. To determine the optimal dose and duration for 5-AzadC-induced HEXIM1 re-expression, C4-2 and LNCaP cells were treated with 5-AzadC at different time points and doses (Fig. 1 and Supplemental Fig. 1B). The optimal dose of 5 µM for induction of HEXIM1 expression (Supplemental Fig. 1A) is similar to the dose others have reported as required for 5-AzadC inhibition of DNMT1 and the ensuing demethylation of promoter regions. While 5-AzadC induced HEXIM1 mRNA and protein expression by 8 h, maximum induction was evident at 48 h (Fig. 1 and Supplemental Fig. 1A). The level of induction of HEXIM1 expression was higher in C4-2 due to lower basal HEXIM1 expression in these cell lines, as we have previously reported. 5-AzadC treatment did not result in alterations in DNMT1 expression (Supplementary Fig. 1C). No significant increase in HEXIM expression was evident after treatment with other DNMT1 inhibitors, Fludarabine and Cladribine (Supplementary Fig. 1D). As a measure of the functional relevance of the induction of HEXIM1 expression by 5-AzadC, we examined the expression of p21, which was upregulated by HEXIM1 during HEXIM1-induced cancer cell differentiation. 5-AzadC induced p21 expression by 8 h, and the maximum induction of p21 expression was observed 24–48 h after treatment in C4-2 and LNCaP cell lines (Fig. 1). Based on these results, the 48-h time point after 5-AzadC treatment was selected for subsequent experiments.

Non-epigenetic mechanism for 5-Aza-2′ deoxycytidine-induced HEXIM1 expression. While there is evidence for methylation of the HEXIM1 promoter by DNMT1, involvement of other mechanisms for DNMT1 inhibitor-induced HEXIM1 expression has not been reported. Previous studies have shown that 5-AzadC-induced DNA damage is associated with the phosphorylation of CHK1 and CHK2 by ATR and ATM kinases, respectively. Involvement of a non-epigenetic mechanism should provide novel insight into the regulation of HEXIM1 expression in cancer cells. Consistent with 5-AzadC-induced SSB and/or DSB, 5-AzadC treatment resulted in increased levels of phosphorylated histone H2A.X, CHK1, and CHK2 proteins in C4-2 and LNCaP cells (Fig. 2A and Supplemental Fig. 1E). The time course for induction of pCHK1 and pCHK2 by 5-AzadC was similar to that observed for the induction of HEXIM1 expression. Involvement of the DNA damage response pathway in the upregulation of HEXIM1 expression by 5-AzadC was validated by downregulating the expression or activity of ATM or ATR. 5-AzadC-induced HEXIM1 expression was attenuated by downregulation of ATM by shRNAs (Fig. 2B and Supplemental Fig. 2A). Because of the modest attenuation of 5-AzadC-induced HEXIM1 expression by downregulating either ATR or ATM individually, we also utilized a
pharmacological inhibitor of ATR (VE-822) and a pharmacological inhibitor of ATM (caffeine). While VE-822 and caffeine exhibit selectivity towards ATR and ATM, respectively, these compounds also exhibit some activity on the other PIKK family member sensor protein [ref.20,21 and Supplemental Fig. 2B]. 5-AzadC-induced HEXIM1 expression was attenuated by either VE-822 or caffeine (Fig. 2C). Decreases in the levels of pCHK1/CHK1 and pCHK2/CHK2 in the presence of either ATR and ATM inhibitors are shown in Supplemental Fig. 1B. These findings suggest that activation of the DNA damage response pathway by 5-AzadC is a contributing factor in the activation of downstream transcriptional factors that facilitates re-expression of the HEXIM1 protein. 5-Aza-2′deoxycytidine induced CDK9 recruitment and S2P RNAPII occupancy on the HEXIM1 promoter. We then determined how 5-AzadC-induced DNA damage results in upregulation of HEXIM1 gene transcription. DNA damage has been reported to induce the release of P-TEFb from the promoter-bound 7SK snRNP complex[20,22] and RNAPII pause release, resulting in transcriptional elongation and activation[8,23,24]. 5-AzadC has been reported to be a P-TEFb releasing agent[25], but the mechanism was not clearly defined. Release of P-TEFb from the 7SK snRNP complex has been reported to result in activation of HEXIM1 transcription[3]. Thus, we determined whether 5-AzadC-induced HEXIM1 re-expression can be attributed to enhanced recruitment of CDK9 to the HEXIM1 promoter and the ensuing increase in S2P RNAPII occupancy on the HEXIM1

![Figure 2](https://www.nature.com/scientificreports/)
Coding region in C4-2 and LNCaP cells. As shown in Fig. 3, 5-AzadC induced CDK9 recruitment to the HEXIM1 promoter, and this was attenuated in VE-822- or caffeine-treated cells. Similarly, 5-AzadC treatment resulted in enhanced occupancy of S2P RNAPII serine phosphorylation on the HEXIM1 coding region, which can also be inhibited by VE-822 or caffeine (Fig. 3). CDK9 levels were not altered by treatment with 5-AzadC in the absence or presence of VE-822 or caffeine (Supplemental Fig. 3A). Collectively, our data suggest that 5-AzadC-induced DNA damage promotes binding of transcription factors associated with transcriptional elongation to the HEXIM1 promoter, resulting in increased HEXIM1 expression.

Nuclear factor-kappa B (NF-κB) is a mediator in 5-Aza-2′deoxycytidine-induced HEXIM1 expression. It has been reported that following DNA damage, NF-κB is directly or indirectly involved in the dissociation of P-TEFb from an inhibitory complex with 7SK snRNP, resulting in transcriptional activation of certain genes. NF-κB is phosphorylated at Ser 276 in response to DSB, a process that requires ATM. Consistent with 5-AzadC-induced DSB and activation of ATM, we observed upregulation of phospho-Ser 276 NF-κB in response to 5-AzadC treatment (Fig. 4A).

We then examined the relative role of NF-κB in 5-AzadC-induced recruitment of CDK9 to the HEXIM1 promoter. In C4-2 and LNCaP cells, 5-AzadC induced NF-κB recruitment to the HEXIM1 promoter, which is attenuated by VE-822 or caffeine (Fig. 4B). NF-κB recruitment to the HEXIM1 coding region was not altered by 5-AzadC (Supplemental Fig. 3B). NF-κB levels were not altered by treatment with 5-AzadC in the absence or presence of VE-822 or caffeine (Supplemental Fig. 3C).

The critical role of NF-κB in 5-AzadC-induced HEXIM1 expression is supported by attenuation of 5-AzadC-induced HEXIM1 expression upon knockdown of NF-κB in LNCaP and C4-2 cells (Fig. 4C and Supplementary Fig. 4A). Vehicle-treated (control) cells expressing NF-κB shRNA did not exhibit altered HEXIM1 expression.

Figure 3. 5-Aza-2′deoxycytidine induced CDK9 recruitment and S2P RNAPII occupancy on the HEXIM1 promoter. C4-2 and LNCaP cells were treated with VE-822 (2 μM) or caffeine (2 μM) for 2 h, followed by 5-AzadC for 2 h. Cells were processed for ChIP analyses of the occupancy of CDK9 on the HEXIM1 promoter and occupancy of serine 2 phosphorylated (S2P) RNAPII on the HEXIM1 coding region. Input DNA was used as normalization control. IgG control was used as a negative control. Figures are representative of at least 3 independent experiments. *P < 0.05 and **P < 0.01 vs. Control.
However, knockdown of NF-κB in LNCaP cells did not result in significant attenuation of 5-AzadC-induced HEXIM1 expression (Fig. 4C), suggesting that there are factors expressed in LNCaP cells that can compensate for downregulation of NF-κB. We further defined the role of NF-κB in the transcriptional regulation of the HEXIM1 gene. Knockdown of NF-κB and the ensuing decrease in promoter occupancy of NF-κB resulted in attenuation

Figure 4. NF-κB is a mediator of 5-Aza-2′deoxycytidine-induced HEXIM1 expression. (A) LNCaP and C4-2 cells were treated with 5-AzadC. Cell lysates were prepared and expression of phospho-NF-κB and NF-κB were analyzed by western blots. Represented are blots cut into strips prior to blotting to minimize the amounts of antibodies required. Figures are representative of at least 3 independent experiments. *P < 0.05 vs. Control. (B) C4-2 and LNCaP cells were treated with 2 μM of VE-822 or caffeine for 2 h, followed by 5-AzadC (5 μM) for 2 h. Cells were processed for ChIP analyses of the occupancy of NF-κB on the HEXIM1 promoter. Input DNA was used as normalization control. IgG control was used as a negative control. *P < 0.05 and **P < 0.01 vs. Control. (C) C4-2 and LNCaP cells were infected with lentiviruses expressing control or NF-κB shRNA, and selected with puromycin. Some cells were treated with 2 μM of VE-822 or caffeine for 2 h, followed by 5-AzadC (5 μM) for 2 h. Cell lysates were prepared and expression of indicated proteins were analyzed by western blot. Represented are blots cut into strips prior to blotting to minimize the amounts of antibodies required. Figures are representative of at least 3 independent experiments. (D) LNCaP and C4-2 cells were infected with control or NF-κB shRNA lentiviruses and selected with puromycin. Some cells were treated with 5-AzadC (5 μM, 2 h). Cells were then processed for ChIP analyses of recruitment of CDK9 and NF-κB to the HEXIM1 promoter. Input DNA was used as normalization control. IgG control was used as a negative control. Figures are representative of at least 3 independent experiments. *P < 0.05 and **P < 0.01 vs. Control.
of 5-AzadC-induced recruitment of CDK9 to the HEXIM1 promoter (Fig. 4D). CDK9 levels were not altered upon knockdown of NF-κB (Supplemental Fig. 4B).

**The relative role of HEXIM1 in the induction of apoptosis and inhibition of cell proliferation by 5-Aza-2′deoxycytidine**. We then examined the functional relevance of 5-AzadC-induced HEXIM1 expression. In particular, we determined the relative role of HEXIM1 in 5-AzadC-induced apoptosis, and if this role involved the reported HEXIM1-induced increases in p53 levels. Treatment with 5-AzadC resulted in increased levels of cleaved caspase-3 in C4-2 cells (Fig. 5A). Downregulation of HEXIM1 resulted in attenuated 5-AzadC-induced increase in cleaved caspase-3 levels, although this could be attributed to the decrease in basal levels of cleaved caspase-3 observed in vehicle-treated/HEXIM1 shRNA-transfected cells (Fig. 5A). Transfection of expression vector for p53 into HEXIM1 shRNA-transfected cells partially restored cleaved caspase-3 levels to that observed in control transfected 5-AzadC-treated cells. Downregulation of HEXIM1 also attenuated 5-AzadC-induced decrease in proliferation of C4-2 cells (Fig. 5B). We examined if 5-AzadC induced other types of cell death, but we did not observe an increase in the levels of a marker for necroptosis, phosphorylated MLKL (Supplementary Fig. 5).

**5-Aza-2′deoxycytidine-induced HEXIM1 expression in triple negative breast cancer (TNBC)** is also mediated by NF-κB and HEXIM1 is critical for 5-Aza-2′deoxycytidine-induced apoptosis. We have also been studying the HEXIM1 as a breast tumor suppressor whose expression is decreased in tamoxifen resistant, triple negative, and metastatic breast cancer. HEXIM1 inhibits metastasis by inhibit-
ing cell invasion, angiogenesis, and the premetastatic niche. Thus, HEXIM1 induction is also a potential therapeutic approach for breast cancer.

5-AzagC induced HEXIM1 expression in three TNBC lines, MDA-MB-231, MDA-MB-468, and MDA-MB-453 (Fig. 6A and Supplemental Fig. 6A). We also observed 5-AzagC-induced HEXIM1 expression in breast cancer cells representative of the luminal subtype, MCF7 and T47D (Supplemental Fig. 6B). A similar mechanism as observed in prostate cancer cells involving induction of DNA damage is supported by the attenuation of the induction of HEXIM1 expression in TNBC cells in the presence of caffeine or NF-κB shRNA (Fig. 6 and Supplemental Fig. 6A). Further support for the aforementioned mechanism is the attenuation of 5-AzagC-induced HEXIM1 expression after downregulating ATM expression (Fig. 6B). The increase in pCHK2 levels in 5-AzagC treated cells was attenuated by transfecting cells with ATM shRNA or by caffeine treatment (Fig. 6B and Supplemental Fig. 6C). Our results are also consistent with an important role for HEXIM1 in 5-AzagC-induced apoptosis (as assessed by levels of cleaved PARP1/PARP1, Fig. 6C). However, the expression of mutant p53 in MDA-MB-231 and MDA-MB-468 suggests this action of HEXIM1 is wild type p53-independent.

Discussion

The biological actions of DNMT1 inhibitors have been attributed to two mechanisms. The first mechanism is reversal of epigenetic aberrations induced by DNMT31,32. The second mechanism is the induction of DNA damage and the formation of irreversible, covalent enzyme–DNA adducts. We determined that 5-AzagC-induced DNA damage resulted in upregulation of the tumor suppressor HEXIM1, and established a role for the DNA damage response pathway in the transcriptional regulation of HEXIM1 in prostate and breast cancer cells. 5-AzagC treatment induced the recruitment of CDK9, a transcription elongation factor, to the HEXIM1 promoter. As a consequence, we observed increased occupancy of S2P RNAPII on the HEXIM1 coding region, a marker for the release of RNAPII pausing. The attenuation of CDK9 recruitment upon inhibition of ATR and ATM, is consistent with the involvement of checkpoint kinases in 5-AzagC-induced HEXIM1 expression.

Our laboratory has invested considerable effort in the identification of agents that can induce HEXIM1 expression. In addition to unique HEXIM1 inducers like the compound 4a17 we have determined that inhibitors of the histone demethylase, KDM5B, also upregulated HEXIM1 expression33. 5-AzagC, as well as other agents that induce release P-TEFb, have been reported to upregulate HEXIM1 gene transcription9,25. While 5-AzagC has also been reported to be a P-TEFb releasing agent that can activate a HEXIM1 promoter luciferase reporter25, the mechanism and consequences were not clearly defined.

DNA damage has been reported to induce the release of P-TEFb from the promoter-bound 7SK snRNP complex10,22 and RNAPII pause release, resulting in transcriptional elongation and activation. NF-κB is a key molecular player that promotes the recruitment of CDK9 and the transcriptional activation of the HEXIM1 gene. The relative role of NF-κB in the activation of HEXIM1 gene transcription was assessed via shRNA-mediated knockdown of NF-κB, which resulted in attenuated 5-AzagC-induced recruitment of CDK9 to the HEXIM1 gene. NF-κB plays critical roles in cellular stress response and modulates transactivation of a large number of genes that participate in various cellular processes involved in DDR34. Along this line, treatment with Tumor necrosis factor (TNF) resulted in the activation of ATM, which was then involved in the proteasomal degradation of IKB and the ensuing release of NF-κB4. NF-κB then promoted the recruitment of PPMiG/PP2Cy phosphatase to their target promoters, resulting in the release of P-TEFb from the inhibitory 7SK snRNP complex and phosphorylation of serine 2 of RNAPII12,26. In our study, 5-AzagC-induced DNA damage promoted recruitment of NF-κB, along with the CDK9 and S2P RNAPII occupancy, on the HEXIM1 promoter to induce HEXIM1 gene transcription. Downregulation of ATM/ATR expression or inhibition of their kinase activity resulted in the attenuation of NF-κB recruitment and/or HEXIM1 gene transcription and expression. NF-κB-mediated induction of the expression of HEXIM1 underscores the divergent role of NF-κB in cancer, with reports supporting not only its canonical role as a tumor promoter but also its role as a tumor suppressor26,26.

Our studies also provide further insight into consequences of 5-AzagC-induced DNA damage by showing that HEXIM1 plays a critical role in 5-AzagC-induced apoptosis and cell proliferation. HEXIM1 upregulates the stability of another tumor suppressor, p53 via an interaction between HEXIM1 and p5344. The implication from this study is that HEXIM1 is another mediator in the upregulation of p53 and p21 resulting from DNA damage and the induction of cell cycle arrest or apoptosis. However, the role of HEXIM1 in 5-AzagC-induced apoptosis appears to be wild type p53-independent in TNBC. Both MDA-MB-231 and MDA-MB-468 express mutant p53. This is critical because p53 is the second most common alteration in CRPC37,38 and 60–80% of TNBC expressing mutated p5339. Wild type p53-independent induction of apoptosis, coupled with downregulation of factors with well-established roles in tumor progression and metastasis by HEXIM117,41, makes re-expression of HEXIM1 via DNMT1 inhibitors an attractive anticancer strategy.

Materials and methods

Cell lines, cell culture and treatment. Prostate cancer cell lines and triple negative breast cancer cell lines were obtained from American Tissue Culture Collection (ATCC) in April 2017. Cells were utilized within 8 passages of the stock obtained from ATCC, and were cultured in RPMI or DMEM media supplemented with 10% fetal bovine serum and antibiotics. Cells were maintained at 37 °C in an incubator (Thermo Fisher Scientific) containing 5% CO2 and 95% humidity. Cells seeded in plates were treated with 5-Aza-2′deoxycytidine (DNMT1 inhibitor), VE-822 (ATR inhibitor), and Caffeine (ATM inhibitor) at different time points and concentrations and then subjected to further experimentation.

Western blotting analyses. Total protein was extracted from cells and subjected to western blot analyses as previously described19. Blots were cut into strips to minimize the amounts of antibodies required. West-
Figure 6. 5-Aza-2′deoxycytidine-induced HEXIM1 expression in triple negative breast cancer is mediated by ATM and NF-κB, and HEXIM1 is critical for 5-Aza-2′deoxycytidine-induced apoptosis. (A) MDA-MB-231 and MDA-MB-468 cells were infected with control or NF-κB shRNA lentiviruses followed by puromycin selection or pre-treated with ATM inhibitor, caffeine (2 μM). Some cells were then treated with 5-AzaC (5 μM) for 48 h. (B) MDA-MB-231 and MDA-MB-468 cells were infected with control or ATM shRNA lentiviruses followed by puromycin selection. Some cells were then treated with 5-AzaC (5 μM) for 48 h. (C) MDA-MB-231 were infected with control or HEXIM1 shRNA lentiviruses followed by puromycin selection. Some cells were treated with 5-AzaC (5 μM, 48 h). For (A),(B), and (C) cell lysates were prepared and expression of indicated proteins were analyzed by western blot. Represented are blots cut into strips prior to blotting to minimize the amounts of antibodies required. *P < 0.01 versus Control. Figures are representative of at least 3 independent experiments.

Cell transduction and transfection. Lentiviral-mediated delivery of shRNAs was used to knockdown NF-κB, ATM1, and ATM expression in cells. Lentiviral particles were generated by transfecting HEK293FT cells with NF-κB shRNA, HEXIM1 shRNA, ATM shRNA, or ATR shRNA expression plasmids (Sigma-Aldrich) along with two other plasmids, envelope expressing plasmid (pMD2.G), and packaging plasmid (psPAX2), using Lipofectamine 3000. Forty to sixty hours after of transfection, medium containing lentiviral particles was collected. C4-2 and LNCaP cells were transduced with lentiviral particles in the presence of polybrene for 18–24 h. Transduced cells were selected using puromycin, harvested, and processed for immunoblotting to confirm knockdown of targeted proteins.

Some cells were transduced with expression vector for p53 (CMV5-p53) using Lipofectamine 3000.
proliferation assays. Media containing DMSO or 5-Aza-2’-deoxycytidine was replaced every other day. After 6 days, cell proliferation was assessed using the WST-1 assay. Absorbance was measured at 450 nm using a Molecular Devices plate reader (San Jose, CA).

**Statistical analyses.** Statistical significance was determined using Student's t test comparison. For some comparisons, probability values for the observed differences between groups were based on one-way ANOVA. A probability (p) value of < 0.05 was accepted as an appropriate level of significance.

**Data availability** The authors declare that all the materials, data and associated protocols related to this manuscript are available upon reasonable request.

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