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## BQ-AurIPr: a redox-active anticancer Au(I) complex that induces immunogenic cell death†

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Immunogenic Cell Death (ICD) is a unique cell death mechanism that kills cancer cells while rejuvenating the anticancer immunosurveillance, thereby benefiting the clinical outcomes of various immuno-chemotherapeutic regimens. Herein, we report development of a library of benzo[a]quinolizinium-based Au(I) complexes through an intramolecular amino-auration reaction of pyridino-alkynes. We tested 40 candidates and successfully identified BQ-AurIPr as a novel redox-active Au(I) complex with potent anticancer properties. BQ-AurIPr efficiently triggered generation of DAMPs – the hallmarks of ICD – and was superior in terms of efficiency compared to FDA-approved drugs known to induce ICD. BQ-AurIPr significantly increased immunogenicity of cancer cells enhancing their phagocytosis when co-cultured with immune cells. Our investigation reveals that BQ-AurIPr induces oxidative stress inside mitochondria leading to mitophagy, as the mechanism for immunogenic cell death in A549 cells.

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Our immune system is capable of discriminating and eliminating altered self or foreign cells, including cancer cells. However, owing to the high mutation and adaptability rate, cancer cells exhibit numerous mechanisms which aid evasion of the immune system.

Neoplastic cells often overexpress various 'don't eat me' signals on their surface and secrete several factors that create an immunosuppressive microenvironment.<sup>1,2</sup> Recent evidence suggests that some chemotherapeutic drugs actively help reinstate the host immunity against cancer cells, providing long-term protection against cancer and its recurrences.<sup>3,4</sup> The cytotoxic effects of chemotherapeutic agents are primarily manifested through a cell death mechanism called apoptosis, which was until recently considered immunologically quiescent or tolerogenic. A recently identified subset, Immunogenic Cell Death (ICD), is a type of programmed cell death characterized by the release of various Damage-Associated Molecular Patterns (DAMPs) by dying cells that trigger a cascade of innate immune responses leading to long-lasting adaptive immunity. DAMPs are intracellular factors

typically inaccessible to the immune system unless released from the cells at the tumor site. DAMPs help recruit immune cells at the tumor microenvironment and facilitate phagocytosis of cancer cells by antigen-presenting cells (APCs) like macrophages and dendritic cells.<sup>5</sup> Upon phagocytosis, antigens of cancer origin are processed by APCs and presented to T cells, leading to activation and proliferation of effector and memory T cells, thereby providing long-lasting anticancer immunity (Scheme 1). Some of the characteristic DAMPs also referred to as hallmarks of ICD, are translocation of calreticulin (CRT) to the plasma membrane, release of ATP and high mobility group box 1 (HMGB1) protein, and secretion of type-I interferons, IL-1 $\beta$ , IL-17, and CXCL10.<sup>6</sup> Interestingly, cancer cells that underwent ICD induction show protection in immunocompetent mice but not immunodeficient mice that received cancer xenografts, thus highlighting the importance of host immunity against cancer.<sup>7</sup>

Despite the increasing number of clinical studies in immuno-chemotherapy, the number of drugs identified to induce ICD is limited.<sup>8,9</sup> While most of the ICD inducers are non-metallic drugs (doxorubicin,<sup>10–14</sup> epirubicin,<sup>15</sup> mitoxantrone,<sup>16</sup> and cyclophosphamide,<sup>17</sup> etc.<sup>18</sup>), oxaliplatin<sup>19</sup> is the only FDA approved metallic anticancer drugs reported to induce ICD. Owing to the highly immunosuppressive tumor microenvironment, at lower concentrations, many of these ICD inducers hardly produce satisfactory levels of DAMPs to trigger a significant antitumor immune response.<sup>20</sup> As a result, the development of novel, more effective immuno-chemotherapeutics that could generate a robust immune response at comparatively lower concentrations is deemed necessary.

While there have been reports on metal complexes as ICD inducers,<sup>21</sup> recent literature reports disclose some of the most

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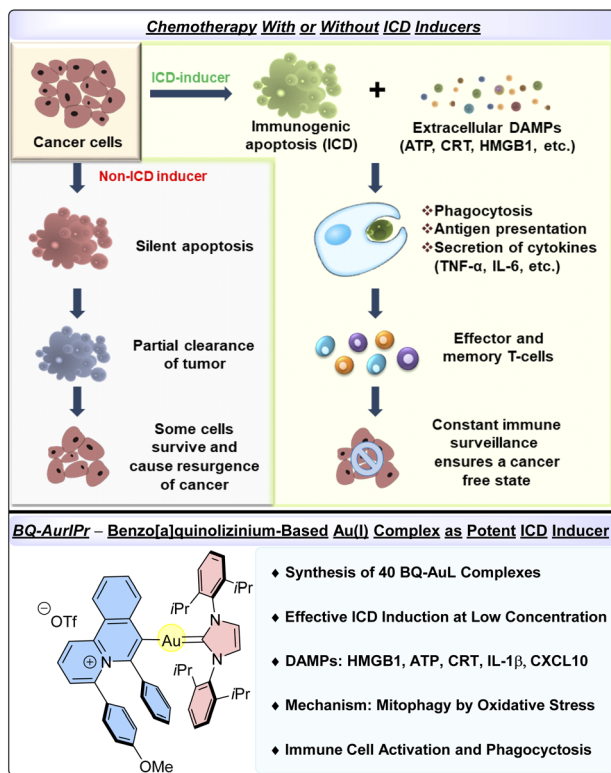
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Scheme 1 Impact of immunogenic cell death inducers on cancer treatment and present work.

potent ICD inducing metal complexes. Ang and co-workers designed a novel Pt-NHC complex which demonstrated excellent ICD and phagocytosis activity.<sup>22</sup> Later, Babak and Ang reported an ER targeting complex: PlatinER, as a modified form of Pt-NHC, which performed superior to its previous version.<sup>23</sup> Likewise, Zhou, Chen, and Liang reported an aminophosphonate ester ligand-containing Pt-complex as a potent ICD inducer.<sup>24</sup> Apart from Pt(II), Pt(IV),<sup>25</sup> Cu(II),<sup>26</sup> and Ru(III)<sup>27</sup> based complexes have also been reported to induce ICD in mice and/or human cancer cell lines. In a recent report by Cui, Sessler, and Arambula, an Au-based complex was reported to induce ICD for the first time.<sup>28</sup> Despite of all these developments, only two metal complexes have been reported to effectively induce ICD in lung cancers, which is the second most commonly diagnosed cancer.<sup>29</sup> Recently, Zhu *et al.* reported an oxaliplatin-based and photocaged Pt(IV) complex: coumaplatin, which induced ICD in cisplatin-resistant non-small cell lung cancer (NSCLC) cells.<sup>30</sup> Later, Chao and co-workers, for the first time, demonstrated the applicability of an Ir-complex in inducing ICD.<sup>31</sup>

Inspired by the growing importance of gold complexes as anticancer agents due to their distinct mode of action<sup>32–35</sup> and banking on our ongoing interest in metal-mediated intramolecular cyclization reactions of pyridino-alkynes,<sup>36–38</sup> we herein disclose a newly designed benzo[a]quinolizinium Au(I)-NHC complex-BQ-AurIPr as a potential ICD inducer in NSCLC A549 cells (Scheme 1). Moreover, we clearly demonstrate its effectiveness in activating immune cells and triggering

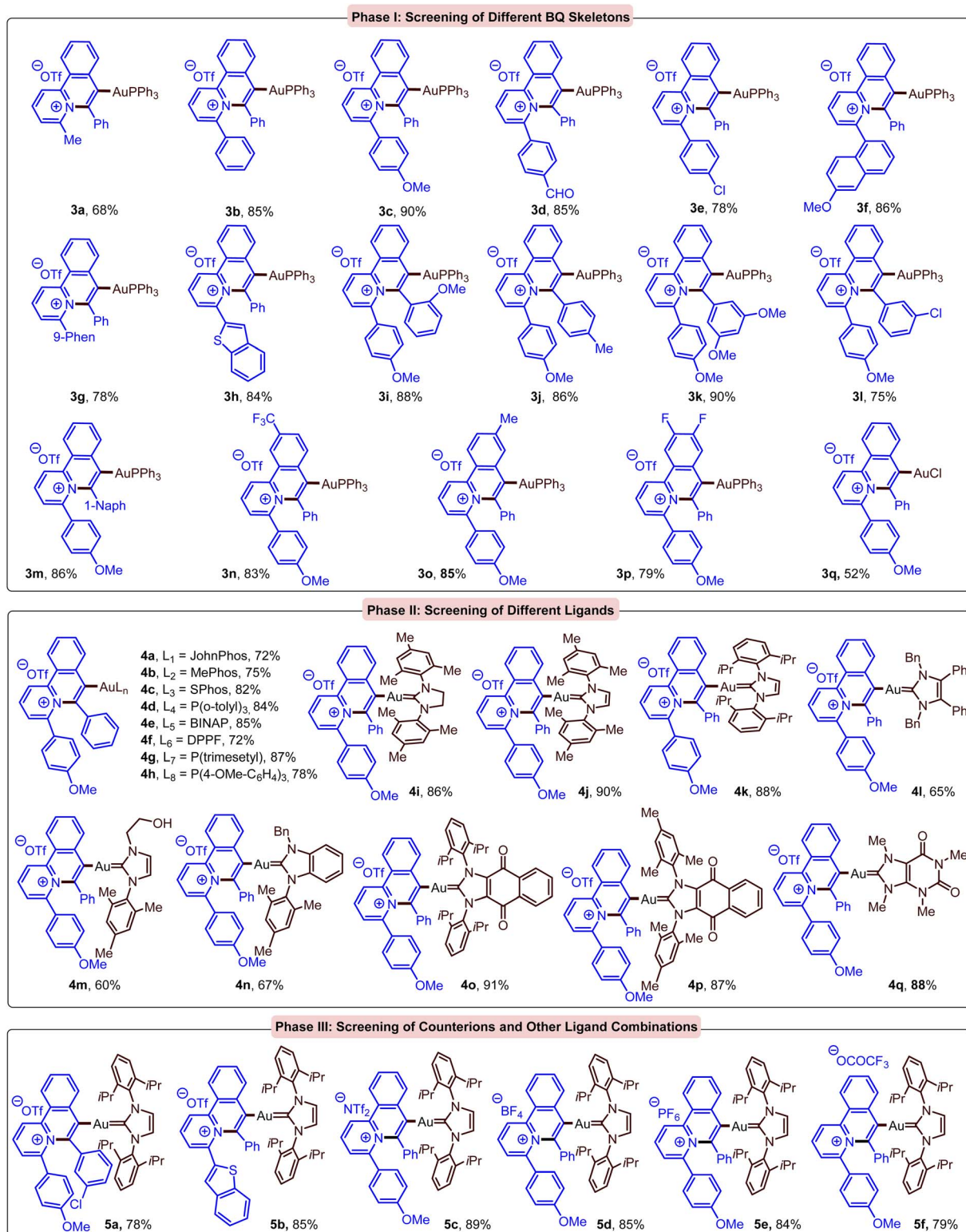
phagocytosis of cancer cells. The mechanism of ICD induction for a gold complex has also been studied for the first time.

A library of 40 BQ-AuL complexes (3a–5f, Scheme 2) was synthesized and evaluated for their anticancer properties through cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and ATP release assays (see ESI† for details). After a systematic structure–activity study and rational designing of BQ core and ligands on Au, we found that BQ-AurIPr (4k) performed exceptionally well in eliminating different kinds of cancer cells (Table 1) and eliciting significant release of ATP, which is a key marker of ICD. To our delight, with a selectivity index (SI) of 5.28 (A549 *vs.* primary human small airway epithelial cells, hSAEC), BQ-AurIPr displayed potent anticancer activity in NSCLC A549 cells through apoptosis (Table 1 and ESI†). Therefore, we focused our study on NSCLC A549 cells. Next, we analysed other aspects of anti-cancer activity by *in vitro* cell mobility and invasion assays and found that 0.25  $\mu$ M BQ-AurIPr doesn't just induce apoptosis in A549 but also decrease their migration and invasiveness (ESI†).

To study the effectiveness of BQ-AurIPr in inducing ICD in A549 cells we analysed the production of DAMPs. In this regard, ATP, HMGB1, IL-1 $\beta$ , and CXCL10 secretion in culture supernatant of BQ-AurIPr treated cells was analysed by luciferin-based assay and ELISA (Fig. 1a–c and ESI†), while translocation of CRT to the plasma membrane was analysed by flow cytometry and confocal microscopy (Fig. 1d and e). Interestingly, we found that BQ-AurIPr produced similar or higher levels of DAMPs in comparison to much higher doses of FDA-approved drugs doxorubicin and oxaliplatin. These findings suggest that cells undergoing apoptosis after BQ-AurIPr treatment are capable of producing DAMPs and might be immunogenic in nature, making BQ-AurIPr a putative ICD inducer.

Redox-active gold(I) complexes have been reported to induce apoptosis and ICD in cancer cells, but the mechanism of action remains elusive.<sup>28</sup> Gold complexes are known to exert anti-cancer activity primarily through oxidative stress.<sup>32</sup> Therefore, to dissect the mechanism of action, we analyzed the level of ROS inside cancer cells upon treatment with BQ-AurIPr using 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) probe and found a marked increase in intracellular ROS levels when cells were cultured in the presence of 0.25  $\mu$ M BQ-AurIPr for 4 hours (ESI†). Interestingly, merged microscopic images of mitotracker red, DAPI and H2DCFDA indicate the accumulation of ROS in mitochondria (mtROS) (Fig. 2a and ESI†). We surmised that mtROS could oxidize mitochondrial DNA (mtDNA)<sup>39</sup> and other contents triggering the degradation of mitochondria by mitophagy leading to DAMP production and ICD.<sup>20,40</sup> To analyze mitophagy, we examined RNA expression of several mitophagy indicator genes by RT-PCR and probed mitochondrial mass by mitotracker red staining. To our delight, BQ-AurIPr treated cells showed higher expression of mitophagy indicator genes (Fig. 2b) and decreased mitotracker red staining in a dose-dependent manner, indicating degradation of mitochondria (Fig. 2c). Moreover, BQ-AurIPr also increased the colocalization of mitochondria and lysosomes (Fig. 2d), confirming that BQ-AurIPr induces mitophagy in cancer cells. Moreover, damaged mitochondria may release mtROS and oxidized mtDNA into





Scheme 2 Synthesized BQ-AuL complexes and their phase-wise screening of IC<sub>50</sub> values and ATP secretion.

cytosol which is sensed by innate immune sensors like NLRP3 and cGAS-STING, respectively.<sup>41,42</sup> This triggers a cascade of innate immune responses through transcription factors IRF3 and NF-κB, leading to secretion of Type-I interferons and pro-inflammatory cytokines like IL-1β, CXCL10, and IL-17 which

are highly potent DAMP signals. To confirm this hypothesis, we tested the activation of interferon stimulation response element (ISRE) and NF-κB by promoter-reporter assay and subsequent secretion of IL-1β by ELISA. Results indicate an increase in ISRE and NF-κB promoter activity (Fig. 2e) correlating to a marked



Table 1 IC<sub>50</sub> values and SI for BQ-AurIPr in different cell lines

Cell line	A549	Caco-2	MDA-MB-231	HeLa	Primary hSAEC	SI in A549
IC <sub>50</sub> (μM)	0.25 ± 0.08	0.45 ± 0.10	0.50 ± 0.06	0.78 ± 0.12	1.32 ± 0.21	5.28

increase in the secretion of IL-1β as shown previously, which was analogous to much higher concentrations of doxorubicin and oxaliplatin.

Noticeably, most ICD studies to date are conducted in cancer cell lines or animal models. However, in order to estimate the true potential of an ICD inducer in humans, it would be equally important to study their effect on activating the human immune system, however, the number of such studies remains fairly small.<sup>23</sup> In this regard, we first analysed the response of human immune cells (peripheral blood mononuclear cells isolated from healthy donors, hPBMCs) to cancer cells treated with BQ-AurIPr. Cancer cells undergoing ICD should be able to activate immune cells like macrophages and dendritic cells, which are the first cells that encounter an antigen or DAMPs. Upon

exposure, these cells become activated and start producing pro-inflammatory cytokines like TNF-α, IL-1β, CXCL10, IL-6, *etc.*<sup>43–45</sup> to activate and invite other more specific types of immune cells at the local site. Hence, we performed indirect co-culture of BQ-AurIPr treated cancer cells with human hPBMCs using Boyden chamber method. Cancer cells were treated for 4 hours with BQ-AurIPr and cultured in the upper chamber, while hPBMCs were cultured in the lower chamber. This method allows soluble factors like DAMPs to cross through the membrane but does not allow direct contact between cancer cells and hPBMCs. Cytokines secreted by immune cells were analysed in culture supernatants after 6 hours of co-culture. Cells treated with 0.25 μM BQ-AurIPr induced markedly higher amounts of IL-6, CXCL10, IL-1β, and TNF-α from hPBMCs, indicating the

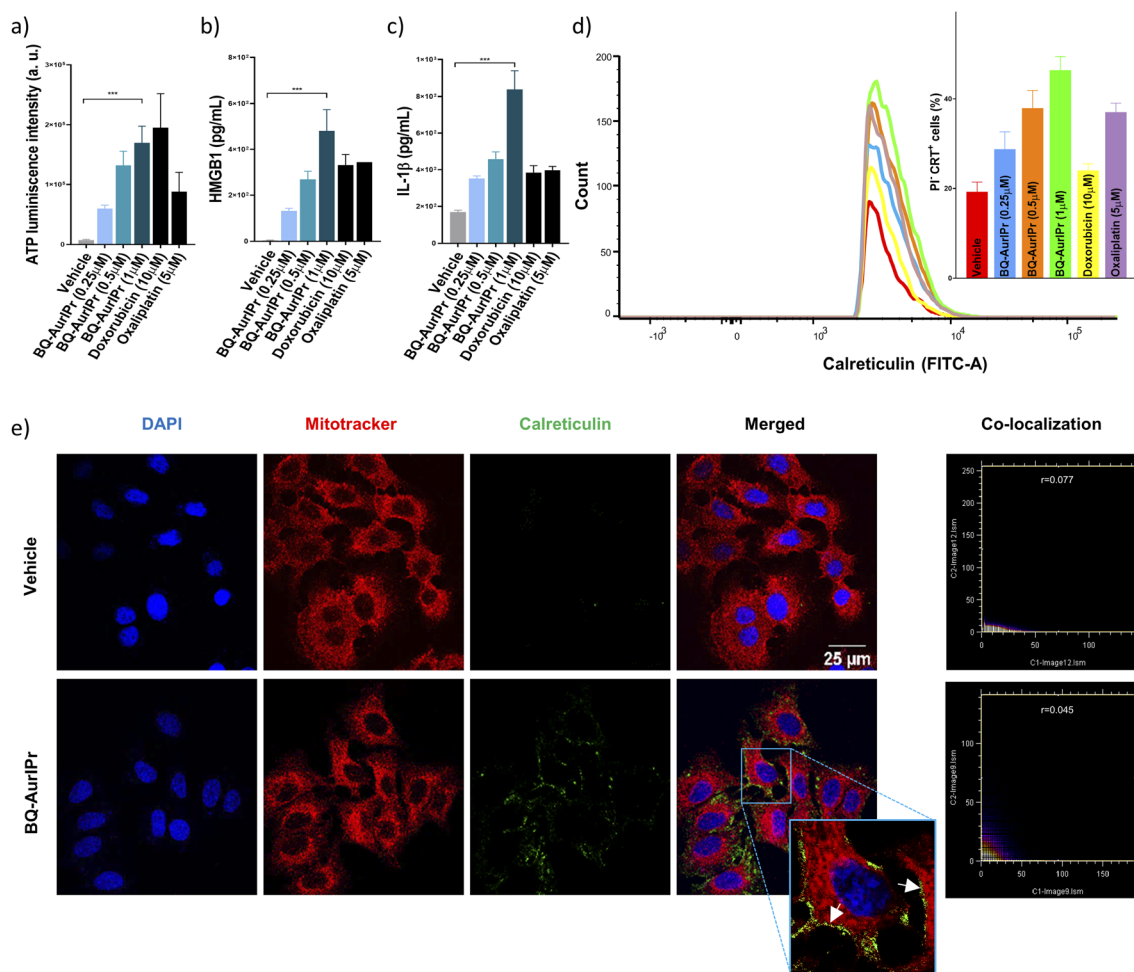
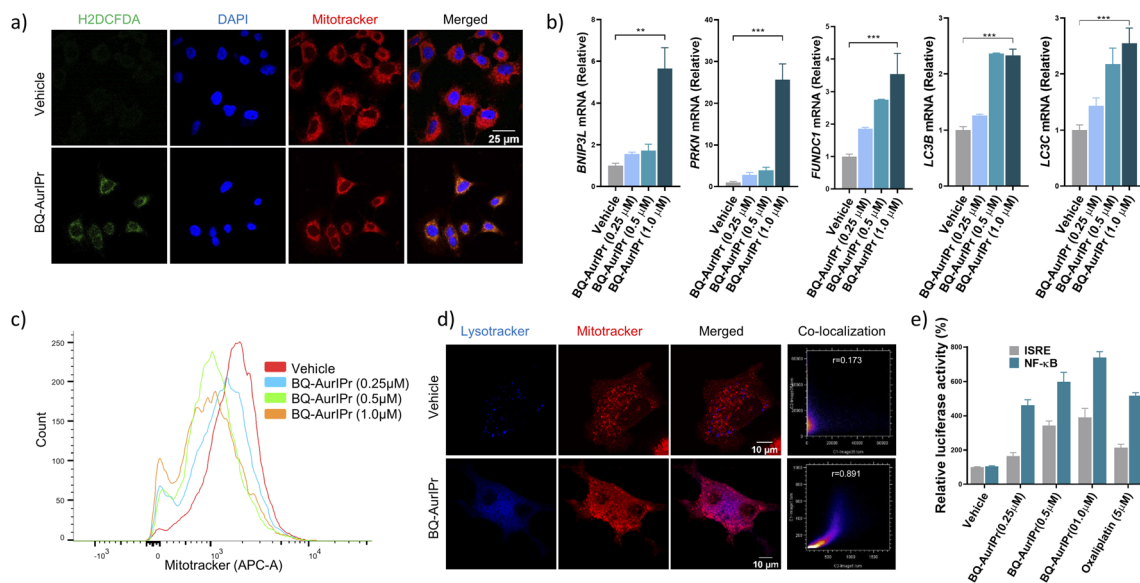


Fig. 1 BQ-AurIPr induces production of DAMPs characteristic of ICD: A549 cells were treated with BQ-AurIPr for 6 hours and supernatant was collected for measurement of DAMPs. (a) Extracellular ATP secretion was measured by luciferase assay, (b) HMGB1, and (c) IL-1β were analysed by ELISA assay and translocation of calreticulin to plasma membrane was analysed by (d) flow cytometry and (e) confocal microscopy. Data are representative of three independent experiments and bar graphs are mean ± SEM.





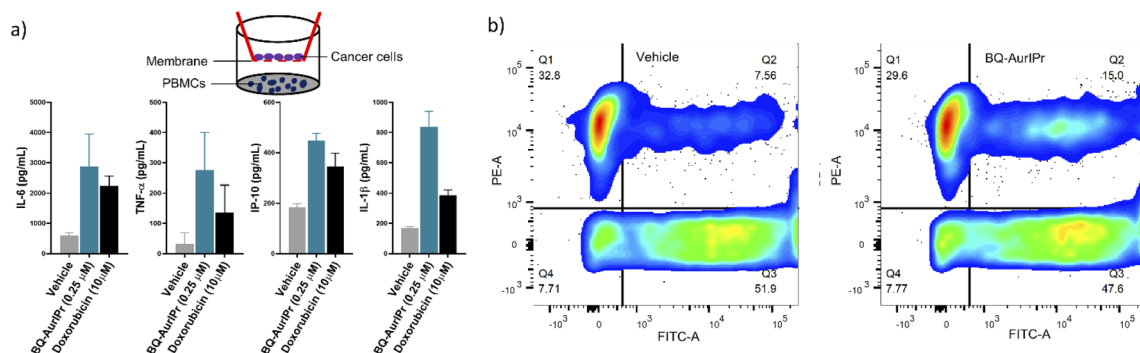
**Fig. 2** BQ-AurIPr induce ROS accumulation inside mitochondria leading to mitophagy: (a) microscopic images indicating production of ROS inside mitochondria. (b) mRNA expression of mitophagy indicator genes by RT-PCR analysis. (c) Flow cytometric analysis of mitochondrial mass by mitotracker red staining. (d) Microscopic images of BQ-AurIPr treated cells probed with mitotracker red and lysotracker blue. Colocalization of both signals indicate mitochondria going through mitophagy. (e) Promoter reporter assay for analysis of ISRE and NF- $\kappa$ B activation. Data are representative of three independent experiment and bar graphs are mean  $\pm$  SEM. Data are representative of three independent experiment and bar graphs are mean  $\pm$  SEM.

DAMPs produced from cancer cells were sufficient for activating human immune cells. The amounts of cytokines produced were found to be analogous to that produced by much higher doses of doxorubicin (Fig. 3a).

Phagocytosis of immunogenic cancer cells by macrophage or dendritic cells initiates the anti-tumor immunity by presenting cancer antigens to adaptive immune cells. Therefore, to see if BQ-AurIPr treatment enhances phagocytosis of cancer cells by human macrophages we performed phagocytosis assay by directly culturing treated cancer cells with differentiated THP1 macrophages. EGFP expressing A549 cells were treated either with vehicle or 0.25  $\mu$ M BQ-AurIPr for 4 hours followed by co-culture with PMA-differentiated THP1 macrophages (labelled red using mitotracker red). After 6 hours of co-culture, cells were

analyzed by flow cytometry for the co-occurrence of green and red fluorescent signals. The rate of phagocytosis of cancer cells by macrophages doubled when cancer cells were treated with BQ-AurIPr compared to vehicle (Fig. 3b). Together these results demonstrate that while killing NSCLC A549 cells directly, BQ-AurIPr also strengthens host's anticancer immunity by instigating immunogenic apoptosis. Such immunotherapeutic agents might have several advantages over traditional chemotherapeutics like faster clearance of tumors, killing of resistant cells by the immune system, fewer side-effects due to high selectivity index, and most importantly fewer chances of relapse because of long-lasting immunological memory.

In summary, we have systematically identified a potential ICD inducer BQ-AurIPr from a rationally synthesized library of



**Fig. 3** BQ-AurIPr treated cancer cells are able to mount successful immune response: (a) A549 tumour cells were treated either with vehicle or BQ-AurIPr followed by co-cultured with hPBMCs. Immune response from hPBMCs was estimated in terms of characteristic cytokines IL-6, TNF- $\alpha$ , IP-10 and IL-1 $\beta$  using ELISA. (b) Phagocytosis assay was performed after labelling BQ-AurIPr treated cancer cells (green) and differentiated THP1 macrophages (red), phagocytosis efficiency was determined using flow cytometric analysis. Data are representative of three independent experiment and bar graphs are mean  $\pm$  SEM.



BQ-based Au(I) complexes through an amino-auration reaction of pyridino-alkynes. BQ-AurIPr, demonstrated excellent anti-cancer properties in various cancer cell lines and was studied to induce ICD in A549 cells. Important biomarkers of ICD studied include: (1) CRT translocation, (2) HMGB1 secretion, (3) ATP release, (4) IL-1b secretion, and (5) CXCL10 secretion. Experimental investigations suggest oxidative stress in mitochondria leading to mitophagy-dependent secretion of various DAMPs as the main mechanism for immunogenic cell death of A549 cells. Furthermore, BQ-AurIPr treatment enhanced the immunogenicity of A549 cells when co-cultured with hPBMCs. In addition to killing cancer cells directly, BQ-AurIPr also substantially enhances the phagocytosis of A549 cells by human macrophages. These findings strongly validate the competence of gold complexes as ICD inducers and are expected to instigate further development in this field of metal-based immuno-chemotherapeutics.

## Data availability

All data are available in the manuscript and in the ESI.†

## Author contributions

Ravindra Mule: conceptualization, investigation, validation, data curation, formal analysis, visualization. Akhilesh Kumar: conceptualization, investigation, validation, data curation, formal analysis, visualization. writing – original draft, Shashank P. Sancheti: investigation, validation, data curation, formal analysis, writing – original draft. B. Senthilkumar: resources. Himanshu Kumar: writing-review and editing, resources, supervision, funding acquisition, project administration. Nitin T. Patil: writing-review and editing, resources, supervision, funding acquisition, project administration.

## Conflicts of interest

There are no conflicts to declare.

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