Nutlins and Ionizing Radiation in Cancer Therapy

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Abstract: Radioresistance stands as a fundamental barrier that limits the effectiveness of radiotherapy in cancer treatment. Recent evidences suggest that radioresistance is due to tumour repopulation and involves several signalling pathways, including p53/MDM2 interaction. Ionizing radiation induces p53-dependent MDM2 gene transcription that, in turn, inhibits p53 transcriptional activity, favouring its nuclear export and stimulating its degradation. In light of the observation that in many human tumours the inadequate function of p53 is the result of MDM2 over-expression, several authors have considered as an attractive therapeutic strategy to activate p53 signalling in tumours by inhibiting MDM2 activities or p53/MDM2 interaction. We retain that, by preventing the interaction p53/MDM2 with Nutlin, a small molecule that binds at the interface between these two proteins, the effectiveness of ionizing radiation treatment could be improved. Promising results have recently emerged from *in vitro* studies performed on laryngeal, prostate and lung cancer cell lines treated with Nutlin in combination with ionizing radiation. Based on these findings, we believe that the combined approach Nutlin/ionizing radiation should be further investigated for efficacy on both solid tumours and lymphoproliferative disorders as well as for side effects on normal cells and tissues. Therefore, the purpose of this review is to report the first results obtained by using Nutlins alone or in combination with other therapeutic agents on primary tumour cells, *in vitro* cell lines or tumour xenografts and to present the most recent advances in the understanding of the molecular mechanisms underlining ionizing radiation cytotoxicity and resistance.

Keywords: p53, MDM2, Nutlins, Ionizing Radiation (IR), Cancer Therapy.

p53: A GENE AT THE CROSSROAD OF SEVERAL STRESS RESPONSE PATHWAYS

The tumour suppressor protein p53 belongs to a small family of related proteins among which p63 and p73 [1-3]. Although structurally and functionally related, p63 and p73 play an important role in morphogenesis and differentiation whereas p53 has a pivotal role in preventing tumour development [4]. The central role of p53 as an oncosuppressor gene is supported by the fact that mutations of p53 in germ line are associated with Li Fraumeni Syndrome characterized by an increased risk of getting tumours at younger age [5]. During the last three decades since its discovery, intensive research on p53 has led to the identification of a complex p53 network involving specific components that are activated under specific circumstances to exert a number of biological responses ranging from cell cycle arrest, apoptosis or cell senescence up to DNA repair, angiogenesis, cell migration and, as recently shown, autophagy [6]. p53 gene is located on the short arm of chromosome 17 at position 17p13 and encodes for a protein consisting of 393 amino acids with an N-terminal trans-activation domain (TAD), a potential conformational element consisting of a proline-rich domain (PRD) adjacent to TAD, a large DNA binding domain (DBD), a tetramerization domain and a basic C terminal domain (CTD) [1]. The primary amino acids sequence of p53 contains many conserved Ser Thre and Lys residues that have probably regulatory functions [3]. Besides canonical p53, up to 10 different protein isoforms produced by alternative splicing, alternative promoter usage and alternative translation initiation have been identified, indicating the existence of complex patterns of regulation at transcriptional, post-transcriptional, translational and post-translational levels [6].

p53 appears to be a major mediator of the genotoxic stress signalling pathway that is triggered by ATM (ataxia teleangiectasia mutated) kinase, proposed to be a sensor of oxidative damage at DNA level [7, 8]. Besides ATM, a number of other stress signals, including oxidative stress and oncogenes activation, converge on p53 through different routes. Downstream ATM or ATR (ataxia teleangiectasia Rad 3 related) kinase, PLK3 (Polo-like kinase-3), a member of the Polo family of protein kinases, may preferentially transduce signals generated by H_2O_2 [9], whereas Chk1 (checkpoint kinase 1) and Chk2 (checkpoint kinase 2) are differentially activated by UV radiation and ionizing radiation (IR), respectively [8]. This leads to a rapid phosphorylation of p53 and the following p53-mediated transcriptional activation or repression of target genes [2, 10]. Indeed, over 4,000 putative p53-DNA binding sites have been identified via bioinformatics and microarray approaches [2].

Several cellular responses to p53 activation have been described, and the choice of response substantially depends on cell type, specific environment and intensity of activation. As already mentioned, once activated the p53 pathway leads to induction of cell cycle arrest, senescence or apoptosis [11, 12]. Cell cycle arrest derives from interaction of p53 with the cyclin-dependent kinase inhibitor (CDKI) p21 and 14-3-3σ, one of the seven isoforms of the 14-3-3 protein family: when p53 activates p21, G1/S or G2/M transitions are prevented, whereas, when p53 interacts with 14-3-3σ, cell cycle is blocked in G2 phase. When cells are arrested during S phase, DNA repair can occur before DNA synthesis, whereas G2/M delay allows transformed cells to repair inflicted damage in DNA preventing them from mitotic catastrophe. When DNA damage is severe or irreparable, cell death or senescence occurs.

DNA damage has been well characterized as one of the many stimuli inducing cell senescence [12-14]. Through this mechanism, which consists in a permanent G2 phase cell cycle arrest, is prevented the proliferation of damaged or stressed cells that are at risk for malignant transformation. On the other hand, cancer cells can be forced into senescence in response to chemotherapeutic agents thus improving therapeutic outcome [15]. In this respect, it has been recently reported that, following the administration of therapeutic IR doses, premature cellular senescence is the principal mode of cell death accounting for the radiosensitivity of human prostate cancer cell lines retaining p53 function [16]. p53 is the pivotal integrator and mediator of these damage signals by means of the recruitment of numerous target genes among which p21 which, in turn, inhibits cyclin-dependent kinase 2 (CDK2), causing maintenance of cell cycle arrest [17]. While the p53/p21 pathway is

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an early response to DNA damage, CDKI $p16^{INK4A}$ represents the player of the slow response to DNA damage that occurs independently from ATM or ATR and involves p38 recruitment [12]. It is worth mentioning that besides p53-dependent senescence other mechanisms of p53–independent senescence related to oncogenes over-expression have been widely documented in both *in vivo* and *in vitro* systems [14, 18], although currently it is not known whether all forms of oncogene-provoked senescence act via damage to DNA.

The pro-apoptotic function of p53 is mediated by a large number of factors and occurs through the activation of target genes including Fas, Puma, Noxa and Bax [19, 20]. Moreover, p53 possesses transcription-independent activities [19]. In fact, it has been widely documented that in response to a stress signal p53 translocates to mitochondria where it interacts with members of the Bcl-2 family. The result of this direct action is the mitochondrial outer membrane permeabilization (MOMP) and the triggering of caspase activity with the following induction of chromatin decondensation [19] Fig. (**1**).

The best-characterized anti-tumour activity of p53 derives from its ability to maintain genome stability and to eliminate cancer cells from the replication pool. In fact, the loss of p53 function results in a decreased ability of the cells to undergo apoptosis and/or cell cycle arrest in response to DNA damage and, consequently, in an increased risk of developing cancer. In cancer cells normal p53 function (^{wt}p53) can be lost through various mechanisms involving genetic deletions (^{del}p53) or mutations (^{mut}p53) inactivating the gene itself or, alternatively, mutations affecting regulators of p53 activity, such as MDM2 (murine double minute 2), and/or downstream effectors of its functions [10, 21, 22]. Mutations of p53 gene occur with a frequency ranging between 10% and 50% of all malignancies. Solid tumours of epithelial origin like ovarian, colorectal, esophageal, head and neck, lung and skin cancers exhibit the highest mutation rates; while other cancer types, such as

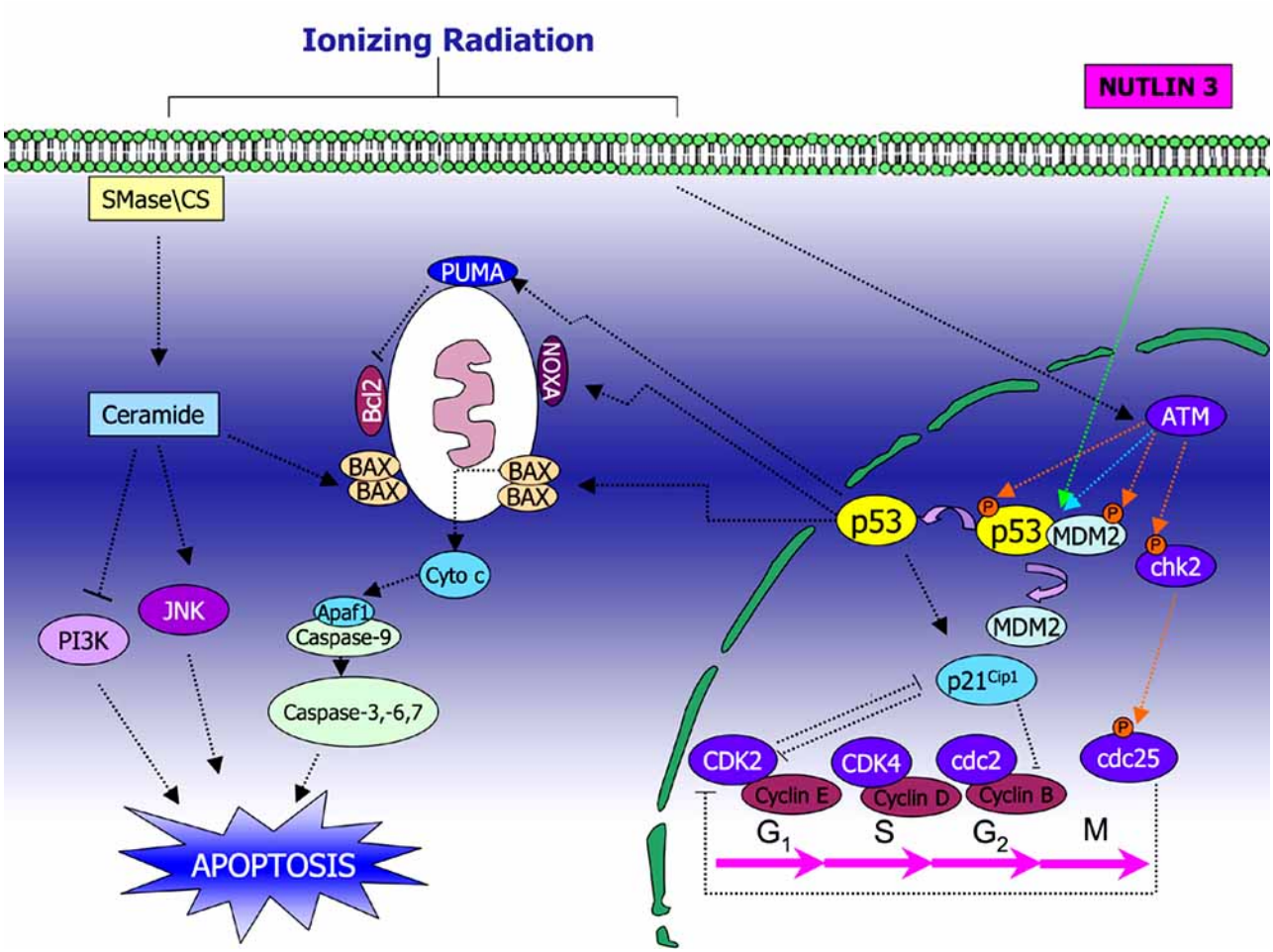

Fig. (1). Schematic representation of the combined approach Ionizing Radiation/Nutlin effects in a ^{wt}p53 hypothetical cell model. See text for details.

Direct stimulatory modification

Direct inhibitory modification

Transcriptional stimulation

Transcriptional inhibition

Separation of subunits

Phosphorylation

Phosphorylation-mediated inhibition of subunits interaction

leukaemia and lymphoma, cervix, bone or prostate neoplasia and sarcomas develop p53 mutation rates of less than 20%. Tumour-associated mutations are predominantly point mutations that occur in 95% of the cases within the DNA binding domain in exons 5 to 8 and lead to the disruption of p53 critical transcriptional activities [23]. Mutations can also occur, although less frequently, in the amino terminal trans-activation and carboxyl-terminal regions interfering with p53 degradation, oligomerization and nuclear trafficking [2].

MDM2: A MASTER REGULATOR OF p53 ACTIVITY

Activity of p53 is regulated at multiple levels, including degradation by H/MDM2 (human/murine double minute 2) protein, stabilization by ARF (alternate reading frame), and phosphorylation by the DNA damage response kinases including ATM [24]. The MDM2 gene was originally identified in Balb/c 3T3 fibroblast cell line [25, 26]. In humans the MDM2 gene is located on chromosome 12q13-14 and encodes a protein consisting of 491 amino acids and several domains: i) N-terminal domain that contains the binding site for p53, p73, and E2F; ii) acidic domain interacting with the tumour suppressor $p14^{ARF}$; iii) putative Zn finger domain and binding site for the retinoblastoma protein Rb; iiii) RING finger domain; iiiii) E3 ligase domain responsible for p53 ubiquitination [26]. MDM2 gene has two promoters (P1 and P2) that direct the synthesis of two mRNA that differ in the presence/absence of exon 1. The P1 promoter drives the synthesis of mRNA that contains exon 1, whereas the P2 promoter directs the synthesis of RNA lacking exon 1 [27, 28]. Two p53 binding sites are positioned in the first intron and p53 stimulates only the downstream P2 promoter [29]. This promoter is practically silent in cells that do not express ^{wt}p53 or express only basal levels of this protein, whereas P1 promoter does not require ^{wt}p53 to become functional. Promoter selection affects the level of MDM2 since mRNA from the P1 promoter appears to be translated less efficiently compared to mRNA from the P2 promoter [30].

A substantial amount of data confirms that MDM2 has a pivotal inhibitory role in p53 pathway since MDM2 and its human homologue HDM2 are the major E3 ligases responsible for p53 ubiquitination [31, 32]. MDM2 and p53 form an auto-regulatory feedback loop through which the two proteins mutually control their cellular levels [31]. p53 binds to the P2 promoter of MDM2 gene and transcriptionally induces MDM2 protein expression; in turn, MDM2 protein binds to p53 and inhibits it through multiple mechanisms [33]. The physiological impact of the p53/MDM2 loop is strongly supported by the observation that embryonic lethality of MDM2-null mice can be rescued only by the simultaneous deletion of p53 gene [34]. Genetic and biochemical studies mapped the p53/MDM2 interaction sites to the 106 amino acid-long N-terminal domain of MDM2 and to the N-terminal part of the trans-activation domain of p53, also called BOX 1 domain [33, 35]. Usually protein-protein interactions involve a large and flat surface. In the case of p53/MDM2 interaction, it has been demonstrated that a limited number of amino acids are involved in the binding of these proteins. In fact, only three amino acids are essential for interaction: Phe19, Trp23, Leu26 [36]; more specifically, upon binding to MDM2, the unstructured p53 trans-activation domain forms an amphiphilic α-helix that projects these hydrophobic residues into a deep hydrophobic binding pocket on the MDM2 surface [37, 38].

MDM2 gene can inhibit p53 not only by blocking its interaction with the basal transcriptional machinery, and consequently interfering with the ability of p53 to induce gene expression, but also in several other ways as recently reviewed [33, 39]. The explanation is provided by the observation, amply documented, that p53 has nuclear activities aside from trans-activation such as base-excision repair and DNA double strand exonuclease activity. For inhibiting these trans-activation independent activities, it is necessary to remove p53 from the nucleus. One way is by promoting the degradation of p53 by adding ubiquitin to it. Ubiquitin is a 76 amino acid residues protein that covalently attaches to substrate proteins at free primary amine groups. The interaction with ubiquitin is critical for proteolysis by 26 S proteasome. In one of the steps that lead to p53 proteolysis an ubiquitine residue is transferred from an MDM2 cysteine residue to p53 [40, 41]. In addition to degradation, on binding to p53 MDM2 favours its export from the nucleus to the cytoplasm, where it cannot function as a transcriptional factor [42, 43]. This mechanism requires that MDM2 constantly shuttles between nucleus and cytoplasm thanks to its nuclear localization (NLS) and nuclear export sequences (NES) [44].

The activity and protein levels of p53 are tightly regulated by MDM2 in normal cells. Deregulation of p53/MDM2 ratio leads to neoplastic transformation. This notion is widely supported by a number of studies performed on mouse models demonstrating a high correlation between MDM2 over-expression, p53 neutralization and tumorigenesis [39, 45, 46]. Up to date a variety of post-translational modification events have been demonstrated to affect p53/MDM2 complex formation: phosphorylation, oligomerization, binding to other proteins, acetylation, methylation, sumoylation and neddylation [24, 39, 40]. p53 phosphorylation at sites within or near the p53/MDM2 interaction domain has been shown to prevent binding of MDM2 [39]. Similarly, phosphorylation of MDM2 might also control p53/MDM2 complex formation [47]. Several reports have identified the importance of phosphorylation of p53 at Ser15 and Ser20 and of MDM2 at Ser17 in modulating p53/MDM2 complex formation [47-50]. Interestingly, it was demonstrated that MDM2 is phosphorylated in response to IR in the presence of ATM gene that is also required for the efficient and rapid activation of p53 after radiation exposure. In response to IR, ATM is activated and activates, in turn, Chk2 that phosphorylates p53 on Ser20, which prevents MDM2 binding and results in p53 stabilization [50]. ATM kinase is also able to phosphorylate p53 on Ser15, which is required for activation of p53 as a transcription factor and may act synergistically with Ser20 phosphorylation [51-53]. This implies that IR triggers ATM to phosphorylate MDM2 and p53, which, in turn, prevents MDM2 from binding [39] Fig. (**1**). Another type of post-translational event that controls p53/MDM2 complex formation is p53 oligomerization that stabilizes the N-terminus into a conformation increasing its affinity for MDM2 [54]. In addition, MDM2 can bind to molecules that modulate the p53 signalling pathway, such as $p19^{ARF}$ in mice and $p14^{ARF}$ in humans, by decreasing the ubiquitin ligase activity of MDM2 [39, 55].

More complex and strictly related to cellular conditions appears the influence of Rb (Retinoblastoma) and CBP (CREB binding protein)/p300 on p53/MDM2 interaction. Indeed, Rb binding to MDM2 blocks the ability of MDM2 to destabilize p53, but at the same time the Rb/MDM2 complex remains bound to p53 and inhibits p53-mediated trans-activation [56]. This suggests how the two MDM2 functions (inhibition and destabilization of p53) can be dissociated. Concerning CBP/p300, it acts as a co-activator of p53 but at the same time appears to be required for MDM2–mediated p53 degradation. MDM2 protein that lacks its p300-binding domain loses its ability to destabilize p53 protein even though the binding between MDM2 and p53 remains unaffected [56].

Finally, the recent identification of new p53 partners has made the scenario even more complicated. Besides MDM2, also MDM4 and its human homolog MDMX bind to p53 with high affinity and effectively inhibit its trans-activation properties but without targeting p53 to degradation [57-59]. At variance to MDM2, MDMX does not possess p53-responsive elements in its promoter and, thus, is not transcriptionally regulated by p53.

Based on these findings that highlight the major role of MDM2 in the regulation of p53 pathway, and based on the observation that in many human tumours the inadequate function of p53 is the result of MDM2 over-expression, several authors have considered as an attractive therapeutic strategy to activate p53 signalling in tumours

by inhibiting MDM2 activities or p53/MDM2 interaction [16, 60-65].

NUTLINS: NON-GENOTOXIC ACTIVATORS OF p53

Nutlins were the first potent and selective small molecules, antagonists of the p53/MDM2 interaction, to be identified 6 years ago [66]. Since then several classes of small-molecule inhibitors with distinct chemical structure have been reported [reviewed in 33]. Nutlins are cis-imidazoline derivatives that displace recombinant p53 protein from its complex with MDM2 with median inhibitor concentration (IC50) in the 100-300 nM range. Up to date many synthetic compounds have been obtained among which Nutlin-1, Nutlin-2, Nutlin-3 (a, b) and the spiro-oxindoles MI-63, MI-219. In spite of this, only Nutlin-3 has been extensively evaluated for its therapeutic potential and mechanism of action in human cancer and represents a promising therapeutic candidate for drug development, together with the even more potent and selective analog MI-219 [33]. The crystal structure of the MDM2/Nutlins complex reveals that Nutlins bind to the hydrophobic p53-binding pocket on MDM2 with high steric complementarity that mimics at a high degree the real interaction of p53 with MDM2 [67]. According to the model proposed for the p53 regulation by MDM2, the treatment of both normal and neoplastic cells with Nutlins should result in: i) stabilization and accumulation of p53, ii) activation of p53 target genes and, consequently, iii) cell cycle arrest in G1 and G2 phases, senescence or apoptosis. Unlike radiation and conventional chemotherapy, MDM2 inhibitors induce accumulation and activation of p53 in cancer and normal cells without inducing DNA damage or post-translational modifications of p53. Nutlins in fact restore p53 function in ^{wt}p53 tumour cells without inducing p53 phosphorylation and with limited effects on primary cells [67, 68]. Interestingly, when used at concentrations higher than 10 μM, Nutlin-3, MI-63 and MI-219 are able to inhibit cell proliferation even in cancer cells lacking ^{wt}p53 [33].

Since their discovery several authors have investigated the effects of Nutlins, used alone or in combination with other therapeutic agents, on primary cells, different cell lines and tumour xenografts [16, 60-65]. In particular, it has been reported that the active enantiomer Nutlin-3a induces i) increased levels of p53, ii) p53- and p21-dependent cell cycle arrest and iii) p53-dependent apoptosis in a number of solid tumours as well as in several types of haematologic malignancies including primary acute myeloid leukaemia (AML) [60, 61], multiple myeloma [69, 70], B-chronic lymphocytic leukaemia (B-CLL) [71-77], Hodgkin lymphomas (HL) [63] and human herpesvirus 8 (HHV8)-related lymphomas [78]. As reported in Table **1**, the most interesting aspect revealed by these studies was the synergistic effect of Nutlins used in combination with several chemotherapeutic agents in terms of apoptosis and cell cycle arrest. It is worth noting that in normal cells p53 activation by MDM2 inhibitors leads to cell cycle arrest but not to cell death [79-81], indicating the absence of toxic effects as a useful requirement for therapeutic purposes.

In spite of the huge number of studies performed, to our knowledge, the identification of the gene set responsible for Nutlin sensitivity is still an open issue [33, 82]. Among several genes related to the p53 pathway, Nutlin-3 up-regulated Notch1 expression [83] and the steady state mRNA levels of PCNA, p21, GDF15, TRAIL-R2, PIG3, and Gadd45 [73]. Recent works have identified other cellular targets of Nutlin including the cell cycle regulator E2F1 [84], the androgen receptor [85], the hypoxia-inducible factor [86], and the NF-κB pathway [87]. Actually, it has been demonstrated that, in p53-deleted or p53–mutated cells, Nutlins are able to activate alternative transcription factors, such as in particular E2F1 [84], capable of inducing either proliferation or apoptosis in dependence on the cell context and, in particular, on the simultaneous activation of the Akt pro-survival pathway [88]. Moreover, a recent study has demonstrated that Nutlins can induce apoptosis in p53-null cells through the activation of p73 [89], providing a rationale for the use of Nutlins also in haematological malignancies with not functional p53.

MECHANISMS OF IONIZING RADIATION CYTOTOXICITY

Ionizing radiation can be defined as any types from electromagnetic (such as X or γ rays) or particle radiation (such as neutron or α particles) with sufficient energy to ionize atoms or molecules. Radiotherapy is the clinical application of IR (γ rays). Indeed, IR is one of the most effective tools in cancer therapy, used to treat localized solid tumours such as skin, tongue, larynx, brain, breast or uterine cervix cancer and blood diseases such as lymphoma and leukaemia. It is worth outlining that a standard course of radiotherapy consists of multiple daily radiation fractions over weeks or months (i.e. total doses in the order of 60-80 Gy), each fraction consisting of a relatively small dose (1.2 to 3.0 Gy). Such fractionated dose schedules appear to amplify the small survival advantage observed between normal organs and tumours when relatively small fraction sizes are used. This important issue can explain some of the difficulties in extrapolating to clinical settings *in vitro* biological data obtained by using large single radiation doses (i.e. 6-20 Gy) rather than more clinically relevant doses (i.e. 1-2 Gy) to assess p53 responses following irradiation.

Ionizing radiation passing throughout living tissues generates reactive free radicals. These free radicals can interact with critical macromolecules, such as DNA and proteins, or membranes, and induce cell damage and, potentially, cell dysfunction and death [90]. The classical theory on the cellular effects of IR identifies DNA as the most important cellular target molecule and the double strand breaks (DSB) as the lesion most closely related to cell death [91]. In addition to DNA damage several data suggest that IR acts directly at the plasma membrane where it activates acidic sphingomyelinase (SMase) that generates ceramide through the enzymatic hydrolysis of sphingomyelin [92]. Ceramide, in turn, acts as a second messenger in initiating an apoptotic response via the mitochondrial system Fig. (**1**).

DSB are generated in dependence of the IR dose [93] and produce a number of cellular responses some of which mutually exclusive. DSB are recognized by proteins exerting both signalling and repair activity; among these proteins the most important are ATM kinases, belonging to the PI3K family, which mediate the early cellular response to DNA damage [94, 95]. ATM exists in the nucleus as a dimer in association with protein phosphatase A (PP2A) that, under physiological conditions, prevents accumulation of trans-phosphorylated active ATM. In response to irradiation, the interaction of ATM with its constitutive inhibitor is abolished; consequently, trans-phosphorylation of ATM can occur leading ATM dimers to dissociate into highly mobile monomers. It is well established that ATM phosphorylation corresponds to autophosphorylation, however the precise mechanism through which ATM switches from its inactive to active form is unclear. Recent studies suggest that the interaction among ATM and DNA-DSB is mediated by MRE11/Rad 50/Nbs1 (MRN) complex, in fact the ability of ATM to phosphorylate various substrates is enhanced in the presence of MRN complex [95]. Another molecule involved in recruitment of ATM to sites of DNA-DSB is 53 BP1, which is considered a sensor of DNA damage. MRN complex and 53 BP1 activate ATM via distinct pathways. Once activated, ATM triggers a series of events involving various downstream substrates important for cell survival after irradiation and including: 1) nibrin (Nbs1), an adaptor molecule for ATM-dependent phosphorylation of Chk2 which inhibits the kinase cell division cycle 25 (Cdc25) and is responsible for S-phase checkpoint control; 2) p53; 3) the E3 ubiquitin ligase MDM2; 4) Chk1 and Chk2; 5) histone 2AX (H2AX) [96].

Radiation-induced damage triggers complex signalling cascades in cells, which result in a variety of responses that include DNA

repair, cell cycle arrest, induction of stress response genes, and cell death [97]. Regarding DNA repair two processes are involved: NHEJ (non homologous end-joining) and HR (homologous recombination) [96]. NHEJ represents a mechanism for the repair of DSB throughout the cell cycle, but particularly active during G0, G1 and early S phase of mitotic cells, and requires the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), Ku 70/Ku 80 heterodimer, XRCC4 (X-ray repair complementing defective repair in Chinese hamster cells), XLF (XRCC like factor), DNA ligase IV and Artemis [96, 98]. The NHEJ process is divided into steps: in the early stage Ku 70/Ku 80 heterodimer binds to both ends of the broken DNA molecule. It is believed that the association of Ku heterodimer with DNA ends creates a scaffold for the assembly of other NHEJ key enzymes, first the DNA-PKcs. In the final step intervenes DNA ligase IV that, with its binding partners XRCC4 and XLF, seals the break. Mammalian cells preferentially repair DSB through HR in the late S and G2 phase of the cell cycle. Yeasts have provided a useful model for HR processes. In yeast, HR is regulated by MRE11/Rad 50/Xrs2, EXO 1 [99-101] and involves Rad family proteins [96, 99]. Although HR proteins are conserved through evolution mammals harbour a more elaborate set of genes, many of which do not have homologs in yeast including BRCA1 and BRCA2. The role of BRCA1 and BRCA2 in HR and the way they receive DNA damage signals are not entirely understood [102].

Regarding cell cycle regulation, exposure to IR arrests the cell cycle at multiple transitions, using multiple mechanisms at each point [101]. Cells that undergo DNA damage during the G1 phase are delayed from entering S phase by the G1/S checkpoint, whereas G2/M checkpoint prevents the onset of mitosis. The arrest before or during S phase requires inhibition of CDK2 activity that can occur in two ways as shown in Fig. (**1**): 1) through the direct link of CDK2 with p21 or 2) through the inhibition of Cdc25 phosphatase activity. p21, activated by p53, binds to and inhibits CDK2/Cyclin E complex which is needed for entering S phase; instead, inhibition of Cdc25 activity can arrest cell cycle progression at various stages. Indeed, Cdc25 activity consists in removing inhibitory tyrosine phosphorylation from CDK2 and CDK1 (cyclin-dependent kinase 1) with the result of promoting entry into and progression through S phase and mitosis [99, 100]. The arrest of G2/M transition can occur in a p53-dependent and -independent manner. The mechanism through which p53 regulates the G2/M transition involves the inhibition of the Cyclin-dependent kinase Cdc2 that is essential for entry into mitosis [101]. Binding of Cdc2 to Cyclin B1 is required for its activity and repression of the Cyclin B1 gene by p53 also contributes to blocking entry into mitosis. Moreover, several of the transcriptional targets of p53 can inhibit Cdc2, including p21, 14-3-3σ, which anchors Cdc2 in the cytoplasm where it cannot induce mitosis, and Gadd45 that dissociates Cdc2 from Cyclin B1 [101]. p53-independent G2 arrest is the result of Cdc2 inhibition exerted by the protein kinases ATR and ATM. ATR and ATM phosphorylate Chk1 and Chk2 respectively, which in turn phosphorylate Cdc25, creating a binding site for proteins of the 14-3-3σ family that sequester Cdc25 in the cytoplasm where it cannot dephosphorylate Cdc2/Cyclin B complex [101, 103].

Finally, another possible outcome of DSB is cell death: indeed, a large body of evidence indicates that un-repaired o mis-repaired DSB in DNA can lead to apoptosis. Apoptosis or programmed cell death is a type of cell death induced by DNA-damaging agents such as IR and chemotherapy [104, 105]. Regulation of apoptosis is delicately balanced by signalling pathways between apoptosis-promoting factors including p53 and caspases, and anti-apoptotic factors such as MDM2 and Bcl-2. p53 is determinant whether cell lives or dies after IR exposure [106]. This occurs via transcription-dependent or transcription-independent mechanisms already described in the p53 paragraph. In particular, it has been reported that, following both UV and IR, MDM2 expression is increased in a p53-dependent manner [28, 107, 108]. This induction ensures that p53 activity returns to low basal levels in surviving cells and prevents uncontrolled cell death after both types of radiation.

Cell choices in response to IR are influenced by many factors: the intensity of DNA damage, the level of p53 expressed, cell type, oncogenic pattern and extracellular stimulus [106]. Massive DNA damage leads to cell death in the form of necrosis and apoptosis, mainly through the depletion of NAD+ [109]. Interestingly, the switch from IR-induced apoptosis to necrosis has been linked to caspase-3 functional activity [110]. Under conditions of low DNA damage (by oxidation, alkylation or IR), cell survival is promoted by PARP-1 as it recruits DNA repair machinery and regulates the functions of p53, NF-κB, and other transcription factors crucial to the stress response. Moreover, recent experiments have demonstrated the existence of pulses of p53 levels [111, 112] whose impact on cell fate is still to be elucidated. Based on these and other authors' findings, Zhang *et al*. [113] proposed an interesting model of the p53-signalling network sustaining a dual transcription-independent role for p53 in DNA repair that would be favoured in the presence of low DNA damage or prevented when damage is severe. The combination of negative and positive feedback loops between p53 and MDM2 would make periodically oscillate p53 and MDM2 levels and the number, more than the amplitude, of p53 pulses would decide the cell fate: at low damage levels, few pulses of p53 would recruit p21 and induce to cell cycle arrest and survival, whereas at high damage levels, sustained p53 pulses would trigger apoptosis by inducing p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1) [113, 114].

As above mentioned, IR also induces activation of the sphingomyelin pathway and several studies report its engagement as a primary event in the pathogenesis of radiation damage [92]. Ceramide is a sphingolipid generated by enzymatic hydrolysis of sphingomyelin via activation of SMase or by *de novo* synthesis involving the enzyme ceramide synthase [115]. There are several SMase isoforms distinguished by pH optimal for their activity in acidic, neutral and alkaline [116]. Acidic and neutral SMase are implicated in the radiation response [117, 118]. More recently it has been reported that IR also activates ceramide synthase [119]. Ceramide, once generated, may be converted into a variety of metabolites and trigger diverse cellular events such as differentiation, senescence, proliferation and cell cycle arrest. Ceramide acts as a second messenger interacting directly with elements of several signalling pathways. Focusing on apoptosis signalling induced by ceramide, there are several mechanistic hypotheses: involvement of JNK system, inactivation of PI3K [92] and, finally, direct action on the outer mitochondrial membrane where ceramide regulates integration of Bax [119] Fig. (**1**). Several studies demonstrate that ceramide is central in the death response to cell irradiation [92]. Indeed, cells deficient for the acidic SMase are resistant to γ radiation. Furthermore, radiations induce ceramide-mediated endothelial cell apoptosis in several organs such as the alveolar septi of the lung [120], the intestinal mucosa [121] and the central nervous system (CNS) [122]. These observations, together with the finding that endothelial apoptosis develops as an early event after irradiation, have led to the concept that the microvascular endothelium serves as the primary target for radiation in the induction of tissue damage [92, 121, 122].

Another mechanism through which IR can induce cell death involves TRAIL (TNF-related apoptosis inducing ligand) signalling. In one of our previous studies we observed a selective up-regulation of TRAIL-R1 in erythroleukaemia cell lines after IR exposure [123]. Since TRAIL-R1 mediates apoptotic signalling [124, 125] its up-regulation results in an increased sensitivity to radiation cytotoxicity.

In addition to these effects derived by its direct action at cellular level, IR induces a series of cellular events known as bystander effect [126]. Radiation bystander effect is defined as "the

induction of biological effects in cells that are not directly traversed by a charged particle but are in close proximity to the cells that are" [127]. Unlike direct irradiation effects, the bystander response is not dose-dependent: instead of an increased response with an increasing radiation dose, the bystander effect becomes saturated at a relatively low dose typically less than 1 Gy. The mechanisms through which the bystander response is mediated involve the release of soluble factors and the presence of gap junction communications [128, 129]. Several signalling pathways, including ERK (extracellular signal-related kinase)/MAPK (mitogen-activated protein kinase) pathway, are responsible for bystander effects [130]. The important role of the MAPK signalling cascade in the bystander process is confirmed by the observation that ERK inhibition suppresses the bystander response [131]. This finding is consistent with the substantial up-regulation of COX-2 signalling which is essential in mediating cellular inflammatory response [130]. Interestingly, an elevation in intracellular levels of reactive oxygen species (ROS) was also observed in bystander cells and postulated to be critical in the transmission of damage [132].

MECHANISMS OF IONIZING RADIATION RESISTANCE

Tumour radioresistance represents a major hurdle in cancer therapy. The term "radioadaptive response" was originally coined to describe a reduced sensitivity to a higher challenging dose of cells previously exposed to a small inducing radiation dose [133]. Although radioresistance stands as a fundamental barrier that limits the effectiveness of radiation therapy, molecular mechanisms underlining the cells radioadaptive response have not been fully addressed [134].

Accumulated evidences suggest that radioresistance is due to tumour repopulation [135]. Cancer can be viewed as an abnormal organ whose growth is driven by a population of cancer stem cells (CSC) [136] that have the ability to self-renew, similarly to normal stem cells. While it is clear that cells responsible for repopulation and consequently for therapeutic failure are CSCs, little is known about factors that induce repopulation reducing tumour control. Several models have been proposed. According to Fowler [137], following radiotherapy well oxygenated cells, proximal to blood vessels, are removed through cell death mechanisms. As a consequence, the nutritional status of the remaining cells population improves, leading to reduction in tumour cell spontaneous death and, as a result, to an increased tumour cell repopulation [137]. This effect is amplified over a course of fractionated radiotherapy. Another model, based on the data derived from normal murine squamous epithelium which shows altered repopulation kinetics after IR [138], establishes that, after fractionated radiotherapy, the relative production of CSCs is increased compared with the production of terminally differentiated cells [139]. To our knowledge, the molecular mechanisms involved in accelerated repopulation during radiotherapy are not well understood yet and need to be further elucidated [134].

A major mechanism through which cancer cells become resistant to IR is through enhanced DNA repair of the lesions [140]. In previous works by our research group we demonstrated ultrastructural and biochemical features of DNA repair in radioresistant murine erythroleukaemia cells [141-144]. Up to 130 genes have been associated with human DNA repair and the corresponding proteins are emerging as important prognostic and predictive markers in solid tumours [145]. Among these proteins, several studies strengthen an important role of DNA-PK in IR resistance, since inhibition of DNA-PK activity is associated with a decrease in DSB repair after irradiation [146].

Multiple lines of evidence indicate a potential role of epidermal growth factor receptor (EGFR) in mediating radioresistance [147, 148]. EGFR is a 170 kDa cell surface receptor frequently expressed at elevated levels in several cancers of epithelial nature [149]. EGFR at the plasma membrane remains localized with caveolin-1 in lipid rafts and caveolae [150] and is activated through phosphorylation at specific amino acid residues in response to ligand binding or after exposure to a variety of unspecific stimuli such as IR, UV radiation, hypoxia and oxidative stresses [151]. Both ligand-dependent and -independent phosphorylation of EGFR results in the receptor internalization and translocation into the nuclear compartment [152, 153]. The mechanism for EGFR activation in response to IR is not fully understood. According to different groups of investigators [154, 155], it is apparent that src kinase activation plays a crucial role during this process. The scenario proposed by Dittman *et al.* [154] can be divided into three phases: 1) IR induces membrane lipid peroxidation and HNE (hydroxyneal) production; 2) HNE activates the redox-sensitive switch of src consisting in a conformational alteration associated with increased kinase activity; 3) activated src phosphorylates EGFR at Y845 and caveolin1 at Y14 leading to internalization of EGFR into caveolae and transport into the nucleus. As a consequence of EGFR phosphorylation, various downstream signalling cascades such as PI3K/Akt, STAT, Ras/MAPK pathways can be stimulated resulting in increased cell proliferation, angiogenesis and decreased apoptosis [156]. Initial data have indicated that two EGFR-dependent pathways, the PI3K/Akt pathway and the Ras/MAPK pathway, are primarily involved in IR resistance, due to their regulatory role in stimulating cell survival by inhibiting apoptosis (PI3K/Akt) and by promoting cell proliferation (Ras/MAPK) [157-159]. It must be underlined that the detailed mechanism through which PI3K/Akt mediates radioresistance has been well characterized, whereas the role of Ras/MAPK cannot be provided so far. In this respect, some authors, based on the observation that the constitutive activity of Ras protein leads to a stimulated production of EGFR-ligands [160], suggest that radioresistance is the result of a constitutive activated autocrine loop of EGFR-ligand production and receptor stimulation [161]. In addition, accumulated evidences have indicated that EGFR induces radioresistance by interfering with DNA repair. In fact, EGFR binds to the catalytic subunit and the regulatory subunit Ku 70 of DNA-PK and controls the disassembly of DNA-PKcs and the physical rejoining of DNA-DSB [156].

The anti-apoptotic signal delivered by PI3K/Akt involves NF-kB recruitment and inactivation of pro-apoptotic proteins [124, 162-164]. Interestingly, the effects of Akt on cell metabolism are also important for radioresistance, since Akt-mediated inhibition of GSK (glycogen synthase kinase) and activation of glycolysis and glucose transport improve the nutritional state of tumour cells resulting in a decreased rate of spontaneous cell death [165]. Accumulating evidences suggest that pro-survival pathways initiated by NF-κB may contribute to tumour radioresistance in a cell type specific manner [reviewed in 134]. The NF-κB/Rel family of transcription factors consists of five members, containing an approximately 300 amino-acid REL homology domain (RHD), which mediates protein dimerization and binding to DNA: 1) RelA also called p65; 2) RelB; 3) c-Rel; 4) p50/p105 also called NF-κB1; 5) p52/p100 also called NF-κB2. Although the heterodimer p50/p65 is shown to be the most abundant form of NF-κB, different combinations of homo- or heterodimers can be formed after irradiation [134]. Under non-stimulated conditions the NF-κB complex, mainly in the form of p50/p65 heterodimer, binds to a member of the IκB family of NF-κB inhibitors, including IκB-α (the most studied), IκB-ß, IκB-γ, IκB-ε, Bcl-3, p105 and p100, and is prevented from nuclear translocation [166]. NF-κB activation can occur in two ways known as classical and alternative. After IR injury, activation of NF-κB typically occurs via the classical pathway involving IKK (IκB kinase)-mediated phosphorylation of IκB proteins. Phosphorylated IκB proteins are then ubiquitinated at Lys residues and, consequently, rapidly degraded by the proteasome [134]. Upon IκB degradation, NF-κB is able to quickly translocate to the nucleus and activate a wide variety of gene promoters, including its inhibitors, suggesting the existence of a

feedback control of NF-κB regulation [163, 167]. Among the NF-κB effector genes cyclin B1, cyclin D1 and HIAP-1 may play a major role in radioresistance. Since NF-κB activation can occur only in the cytoplasm, the mechanism through which IR-induced DNA damage activates NF-κB has remained enigmatic for long. Several evidences [168, 169] suggest that ATM is crucial for NF-κB activation and requires a cytosolic signalling complex containing NEMO (NF-κB essential modulator), ATM, IKK catalytic subunits and ELKS [170].

A large body of evidence supports the role of NF-κB/Rel factors in apoptosis modulation. In fact, it has been demonstrated that the over-expression of p65- or c-Rel-containing dimers can impair apoptosis, while the inhibition of NF-κB/Rel activity can enhance death induced by TNF-alpha, TRAIL, IR or chemo-therapeutic agents in many cell types [164, 167, 171, 172]. As pro-apoptotic stimuli, including TNF-R (TNF-receptor) ligands and stress signals, can result in the activation of an NF-κB/Rel-media-ted anti-apop-totic pathway, cell survival appears to depend on the balance between these opposing inputs [124, 163]. Moreover, by virtue of their pro-survival activity, NF-κB/Rel factors can contribute both to maintain neoplastic clone survival and to impair response to therapy [173]. It is worth noting that in acute lymphoblastic leukaemia, constitutive NF-κB/Rel activation can be caused by the over-expression of MDM2, which can increase p65 promoter activity either directly or through the removal of p53 [174]. In the myeloid compartment, p65 NF-κB is activated by p120BCR-ABL or modulated by the binding with the promyelo-cytic leukaemia protein PML [175].

Moreover, NF-κB binding sites were found to be located in the regulatory regions of the SOD2 gene that encodes MnSOD (mito-chondrial antioxidant manganese–containing superoxide dismutase) [176]. MnSOD is a scavenger enzyme that converts superoxide anions to H_2O_2 that in turn is detoxified by catalase or glutathione peroxidase [177]. Over-expression of MnSOD, maintaining the mitochondrial membrane potential, protects cells from apoptosis and can restore cell resistance to TNF-induced cell death [178]. The connection between NF-κB and MnSOD has been evidenced in several studies on radioresistance reviewed by Guo and Sanders [179] and supporting the concept that MnSOD is a key NF-κB effector gene in radioadaptive resistance [134].

The involvement of PKC isoforms in mediating radioresistance has been object of our recent studies on leukaemia cell lines [180-182]. Among the different PKC isozymes assayed in murine Friend erythroleukaemia cells exposed to 1.5-15 Gy of IR, PKCζ expression was found increased in a dose-dependent manner and localized at nuclear level [181]. These findings are consistent with previous observations of our research group showing an increased percentage of apoptotic cells when wortmannin, a specific inhibitor of PI3K and an upstream regulator of PKC, was added into culture before IR exposure [180]. It is worth noting that other PKC iso-forms, namely PKCδ, were found involved in caspase-3 up-regu-lation and activation in radiosensitive Jurkat and HL60 leukaemia cells exposed to IR alone or in combination with etoposide treat-ment [180, 182].

Finally, since ROS are major mediators of irradiation damage [183], numerous studies have shown that levels of redox-regulating compounds can affect cellular radiation responses. Among these factors, the recently discovered antioxidant Peroxiredoxin (Prx) enzymes appear to be involved in radioprotection [184], so that they have been recently indicated as novel targets of cancer radiotherapy [185]. In particular, the over-expression of Prx-II in head and neck cancer cell lines has been linked to radioresistance [186] and a dose- and time-dependent induction of Prx-I was detected in human HT29 colon cancer and rat C6 glioma cells [187]. Although the irradiation-induced up-regulation of Prx I/II expression has been well documented in both normal and neoplastic cells [186-188], less numerous data are available on Prx gene expression after irradiation of leukaemia cells. IR effects on leukaemia cells are of particular oncologic interest since high doses of whole body γ radiation (typical range 10-12 Gy) may be employed prior to bone marrow (BM) transplantation. In this respect, our recent findings indicate a high stability of Prx-I and II after high dose radiation treatment of radioresistant K562 myeloid leukaemia cells [189], suggesting the use of Prx inhibitors in clinical settings to improve radiotherapy outcome.

THE RATIONALE FOR A COMBINED TREATMENT WITH NUTLINS AND IONIZING RADIATION

Several studies have shown that IR induces MDM2 expression in a p53-dependent manner [107, 108]. The magnitude of MDM2 induction after irradiation differs from that of other p53 target genes such as Gadd45 and p21, in that these genes show a clear dependence on the IR dose rate while MDM2 does not [190]. The key role of p53 in the induction of MDM2 in response to IR is supported by the fact that in mice the activation of MDM2 is higher in tissues in which expression of p53 is much stronger [191]. The activation of MDM2 has a clear role in preventing uncontrolled cell death mediated by p53 in response to ionizing radiation. However, the activation of MDM2, attenuating p53 functions in cell cycle arrest and apoptosis in response to DNA damage, limits the effectiveness of ionizing radiation to treat cancer. As the inhibitory effects of MDM2 on p53 require binding between the two proteins, preventing this interaction may be a major strategy for increasing response to IR as shown in Fig. (**1**). Nutlins, compromising the ability of the two proteins to interact, reduce the MDM2 ability to stimulate p53 degradation, and represent a promising approach for improving radiotherapy effects especially for tumours over-expressing MDM2 such as sarcomas, solid tumours [192] and non-Hodgkin Lymphoma (NHL) [193]. However, two aspects of the use of Nutlins in cancer therapy must be considered: 1) Nutlins may not be effective in cancer with inactivation of p53; 2) Nutlins/IR com-bined treatment may lead to the radiosensitization of normal tissues.

To our knowledge, only a few papers [16, 194-198] have reported the effects of the combined approach Nutlins/IR on tumour cells (Table **1**). According to Arya *et al.* [see Table **1** footnote], among seven cell lines derived from patients affected with laryn-geal carcinoma only those displaying ^{wt}p53 were made significantly more radiosensitive after treatment with Nutlin-3. Lehmann *et al.* [16] investigated the effects of Nutlin-3/IR on different cell lines of prostate cancer. First, they did a systematic comparison of the radiosensitivity of three different cell lines expressing ^{wt}p53 (LNCaP) or ^{mut}p53 at one (22RV1) or both alleles (DU145). In second place, they analyzed the cells surviving fraction after treatment with Nutlin alone and in combination with IR, observing that Nutlin sensitized to irradiation only cell lines expressing ^{wt}p53. These data suggest that p53 function loss, including conditions of p53 heterozigosity, could potentially limit the effectiveness of this therapeutic approach for improving the response to ionizing irradiation. Similar results were obtained by Supiot *et al.* in human prostate cancer cell lines [198] and by Cao *et al.* [194] in ^{wt}p53 lung cancer cells and vascular endothelial cells. Interestingly, these authors [198] demonstrated that Nutlin-3 acts as a radiosensitizer under low O_2 levels via p53-independent mechanisms and proposed this compound as a useful adjunct to improve the therapeutic ratio using precision radiotherapy targeted to hypoxic cells. Moreover, besides anticancer effects, the combination of Nutlin-3 and radiation decreased the ability of endothelial cells to form blood vessels resulting as an effective radiosensitizer of tumour vascu-lature [194]. It is worth mentioning that other groups of investi-gators showed a Nutlin-dependent inhibition of vascular endothelial growth factor (VEGF) production in both *in vivo* and *in vitro* systems [73, 86, 199], highlighting a direct anti-angiogenic effect of

Table 1. Anti Tumour Activity of Nutlin-3 Used Alone or in Combination with Chemotherapeutic Drugs or Ionizing Radiation in Cultured Human Cell Lines, Primary Cells and Murine Xenografts

	Treatment	Tumour Type	Response	Effects	References
Cell lines	Nutlin-3 (2 μM) 30 min + IR (0-8 Gy) 24-72 h	Prostate cancer • LNCaP ($^{wt/wt}$p53) • 22Rv1 ($^{wt/mut}$p53) • DU145 ($^{mut/mut}$p53)	 Radiosensitive Relatively radioresistant Radioresistant	Increased induction of p53-dependent cellular senescence in $^{wt/wt}$p53 cells	[16]
	Nutlin-3 + AraC (1, 2.5, 5, 10 μM each) or + Dox (0, 10, 25, 50, 100 nM) 48 h	AML • OCI-AML-3 (^{wt}p53) • MOLM-13 (^{wt}53) • HL-60 (^{del}p53) • NB4 (^{del}p53)	 Responsive Responsive Less responsive Less responsive	Growth arrest and apoptosis in ^{wt}p53 cells; synergistic increase in apoptosis with chemotherapeutic agents .	[60]
	Nutlin-3a (10 μM) ± Dox (0.1-10 μM) 48 h	HL • MDA-V (^{wt}p53) • KM-H2 (^{wt}p53) • L-428 (^{mut}p53)	 Responsive Responsive Resistant	 39% apoptosis 39% apoptosis 10% apoptosis Enhanced toxicity with Dox	[63]
	Nutlin-3a (30 μM) ± TRAIL (100 ng/mL) 24-48 h	Human osteosarcoma • HOS (^{wt}p53) Human colon cancer • HCT116 (^{wt}p53)	 Responsive Responsive	Sensitization to TRAIL-induced growth inhibition and apoptosis	[64]
	Nutlin-3 (5 μM) + Cisplatin (1 μM) 48 h	Germ cell tumour (GCT)-derived • NT2 (^{wt}p53) • 2102EP (^{wt}p53) • NCCIT (^{mut}p53)	 Responsive Responsive Resistant	Dose-dependent growth inhibition and apoptosis in ^{wt}p53 cells; increased cytottoxicity with Cisplatin	[65]
	Nutlin-3 ± Perifosine (10 μM each) 48 h	B lymphoid • SKW6.4 (^{wt}p53) • BJAB (^{mut}p53) • MAVER (^{mut}p53) Myeloid • OCI (^{wt}p53) • MOLM (^{wt}p53) • HL-60 (^{del}p53)	 Responsive Less responsive Less responsive Responsive Responsive Less responsive	(N) (N+P) 20% *vs* 10% cell viability 80% *vs* 40% cell viability 80% *vs* 40% cell viability 50% *vs* 25% cell viability 25% *vs* 10% cell viability 60% *vs* 5% cell viability	[70]
	Nutlin-3 (10 μM)	B lymphoid • SKW6.4 (^{wt}p53) Control primary cells • HUVEC • PBMC	 Responsive Resistant Resistant	 41% apoptosis 16% apoptosis 15% apoptosis	[73]

(Table 1) Contd….

	Treatment	Tumour Type	Response	Effects	References
Cell lines	Nutlin-3a (7 μM) 12-120 h	NHL PEL (KSHV^{+}, ^{wt}p53) • BC-1 (HIV positive) • BC-3 (HIV negative) • BCBL-1 (HIV negative) EBV-transformed LCL • CZE (^{wt}p53) • IHE (^{wt}p53) Bukitta lymphoma • DG-75 (^{mut}p53) Promyelocytic leukaemia • HL-60 (^{mut}p53)	 Responsive Responsive Responsive Resistant Resistant Resistant Resistant	at 96 h 96% apoptosis 89% apoptosis 95% apoptosis 20.7% apoptosis 18.8% apoptosis 5.8% apoptosis 6.5% apoptosis	[78]
	Nutlin-3 (1 μM) 48 h ± IR (0-6 Gy)	Lung carcinoma • H460 (^{wt}p53) • Val138 (^{mut}p53)	 Responsive Resistant	Radiosensitization of ^{wt}p53 cells; increased apoptosis and cell cycle arrest after combined treatment	[194]
	Nutlin-3a (5-10 μM) 5 days	Retinoblastoma • Y79 (^{wt}p53) • WERI-RB-1 (^{wt}p53) • MDA-MB-435 (^{mut}p53)	 Responsive Responsive Resistant	Dose-dependent reduction in cell viability in ^{wt}p53 cells	[195]
	Nutlin-3 (10 μM) ± RITA (10 μM) ± IR (0.02-8 Gy) 3-6 h	Human lung cancer • H1299 (^{wt}p53) • H1299 (^{mut}p53)	 Responsive Resistant	Accumulation of p53, HDM2 and iNOS after low-dose irradiation	[196]
	Nutlin-3 (10 μM) 24 h ± IR (10 Gy) ± Cisplatin (15 μM) 72 h	Human colon cancer • HCT116 (^{wt}p53) Human osteosarcoma • U20S (^{wt}p53)	 Resistant Resistant	Tetraploid cells formation after Nutlin-3a treatment. Tetraploid cells were more resistant to IR- e C-induced apoptosis than diploid counterparts	[197]
	Nutlin-3 (5 μM) 48 h + IR (2 Gy) 2 h	Prostate cancer • 22RV1 (^{wt}p53) • DU145 (^{mut}p53) • PC-3 (^{del}p53)	 Responsive Resistant Resistant	Nutlin-3-increased apoptosis and decreased clonogenic survival in ^{wt}p53 cells; radiosensitization not dependent on p53 status	[198]
	Nutlin-3 ± IR	Laryngeal carcinoma • ^{wt}p53 • $^{mut/mut}$p53 p53 heterozygous and homozygous non-sense mutation	 Radioresistant Radiosensitive Radioresistant	Radiosensitization of ^{wt}p53 cells	Arya *et al.* 2008 see footnote
Primary Cells	Nutlin-3 (1-10 μM) + Dox (0, 10, 25, 50, 100 nM) 48-96 h	AML • ^{wt}p53 • ^{mut}p53	 Responsive Less responsive	Dose- and time-dependent apoptosis; synergistic increase in cytotoxicity of ^{wt}p53 cells after combined treatment	[60]

(Table 1) Contd….

	Treatment	Tumour Type	Response	Effects	References
Primary Cells	Nutlin-3a ± Fludarabine (1-10 μM each) 24-72 h	B-CLL • ^{wt}p53 • ^{mut}p53	Responsive Resistant	Dose- and time-dependent apoptosis; synergistic increase in cytotoxicity of ^{wt}p53 cells after combined treatment	[61]
	Nutlin-3a (0.5-10 μM) ± Dox or Chlorambucil or Fludarabine 24-72 h	B-CLL • ^{wt}p53 • ^{mut}p53 Normal B cells Normal T cells	Responsive Resistant Less responsive Resistant	Apoptosis in ^{wt}p53 B-CLL; synergistic increase in cytotoxicity after combined treatments	[71]
	Nutlin-3a (0.01-30 μM) ± Fludarabine (0.1-10 μM) 24-48 h	B-CLL (CD19$^+$ ^{wt}p53) Normal B cells (CD19$^+$) PBMC Normal CD34$^+$ cells	Responsive Resistant Resistant Resistant	Dose-dependent increase in apoptosis; synergistic increase in cytotoxicity after combined treatment	[62]
	Nutlin-3 (10 μM) ± CpG-ODN 48-96 h	B-CLL (^{wt}p53) Co-treatment Pre-treatment	Responsive Responsive	(N) (N+CpG-ODN) 60% *vs* 50% cell viability 60% *vs* 40% cell viability	[75]
	Nutlin-3 (10 μM) 48 h	• B-CLL (^{wt}p53) • B-CLL ($^{del/mut}$p53)	Responsive Less responsive	62% cell viability 69% cell viability	[76]
	Nutlin-3 (10 μM) 24-48 h	B-CLL (^{wt}p53)	Responsive	GEP signature correlation with cytotoxic potential	[77]
	Nutlin-3 (10 μM) 24 h	B-CLL (^{wt}p53)	Responsive	Transcriptional activation of Notch1	[83]
Xenografts	Nutlin-3a 20mg/Kg every other day for 2 weeks	BC-3 cells implanted subcutaneously into Balb/c nude female mice and allowed to grow untill the tumours were palpable	Responsive	Marked regression of all tumours in in treated animals; no weight loss or signs of toxicity	[78]

abbreviations:
N: Nutlin; P: Perifosine; IR: Ionizing Radiation; RITA: 5,5'-(2.5–Furanidyl)-bis-2-thiophenemethanol; C: cisplatin; GEP: gene expression profile
Footnote:
Arya AK, Devlin T, El-Fert A, Banfield M, Rubbi C, Lloyd B, *et al.* The use of Nutlin-3 as a radiosensitiser in laryngeal carcinoma cells harbouring wild-type p53. Clin Otolaryngol 2008; 33: 641.

Nutlins. This effect together with the increased induction of p53-mediated cellular senescence in cancer cells after irradiation [194, 198] suggests broader therapeutic implications of these molecules in cancer therapy and warrants the need of further *in vitro* and *in vivo* investigations on solid tumours and haematological malignancies. In this respect, promising results have been obtained with the use of Nutlin in combination with chemotherapeutic drugs as already mentioned and shown in Table **1**. The molecular basis of this combined therapeutic activity appears to be due to the exertion of both p53-dependent and p53-independent mechanisms by chemotherapeutic drugs that synergize with p53-dependent effects exerted by Nutlins [199]. In light of these findings, it would be worthwhile to evaluate the potential to target the p53/MDM2 interaction in combination with IR in models of HL, NHL and B-CLL. Indeed, radiotherapy is the election therapy for human lymphomas, since these pathologies start as neoplasms localized to single group of lymph nodes, then spread to contiguous lymph nodes, and, eventually, may invade blood vessels and spread to organs by haematogenous dissemination. Ongoing trials for patients affected with HL in early stages are investigating lower radiation doses, smaller radiation fields and possible reductions in the doses or number of cycles of chemotherapy given. We expect that the clinical application of Nutlins/IR treatment might improve survival rates or reduce therapeutic doses in sensitive patients. With this aim, in light of the TRAIL-induced radiosensitization [123] and Nutlins-mediated up-regulation of TRAIL-R2 in haematological malignancies [199], the impact of the triple sequential combination TRAIL/Nutlins/IR might be investigated on TRAIL-sensitive tumours.

Another important issue regarding the combined approach Nutlins/IR is the potential toxicity to normal cells and tissues. As far as we are concerned, no data are available on this topic. In light of the therapeutic perspectives of this combined treatment, it is essential to determine whether Nutlins increase the side effects of radiotherapy on normal cells and tissues. According to the original

study of Vassilev *et al*. [66, 67], the p53 activation resulting from the use of Nutlins as a single treatment is not associated to severe cytotoxicity and is well tolerated *in vivo* in nude mice at doses that determine tumour growth inhibition. A strong support in this regard comes from studies performed on mice where the genetically-induced reduction of MDM2 expression and the consequent p53 up-regulation in all tissues did not compromise mice normal development and lifespan and resulted in a mild toxicity to the haematopoietic system and small intestine [200, 201]. As well, *in vitro* findings have shown that only high concentrations of Nutlins are able to induce apoptosis on $CD19^+$ B lymphocytes, peripheral-blood mononuclear cells and bone marrow haematopoietic progenitors [62], or cell cycle arrest and cell senescence on normal human fibroblasts and endothelial cells [73]. Based on these and other observations (see Table **1**), it appears that leukaemia cells display a higher sensitivity than normal counterparts to Nutlins cytotoxic action and, thus, require a lower Nutlins dose for therapeutic effects [62]. Unlike non-genotoxic activation of p53 by Nutlins, DNA damaging agents, such as chemo/radiotherapy, activate p53 by inducing its post-translational modifications, such as extensive phosphorylation, therefore altering the protein stability [81]. In addition, chemo/radiotherapy induce robust accumulation of p53 in normal tissues, leading to tissue damage in p53-sensitive tissues [80]. In comparison with IR and chemotherapy, Nutlins compounds like MI-219 activate p53 in normal tissues with minimal p53 accumulation [81].

IR toxicity remains an important issue that limits the success of therapy and adversely affects patients' quality of life. It is well known that therapeutic exposure to IR can induce side effects at the haematopoietic, gastrointestinal (GI) and CNS level [90]. The nature and degree of such unwanted side effects depend upon the IR dose administered and the sensitivity of the irradiated organs, and, moreover, differ among individuals, suggesting a possible genetic control [202]. Although these complications are well documented at the clinical level, their patho-physiology and molecular mechanisms are poorly understood. Since the haematopoietic system has a high level of cell turnover, it is among the most radiosensitive tissues in the body. Many cancer patients receiving IR develop acute and residual BM injury [203]. IR impairs haematopoiesis, with the result of an acute myelo-suppression, through a variety of mechanisms: i) inducing apoptosis of haematopoietic stem cells (HSC), ii) altering the capacity of BM stromal elements to support haematopoiesis *in vivo* and *in vitro* and, finally, iii) inducing redistribution and apoptosis of mature formed elements of the blood [204]. While many patients recover rapidly from acute myelo-suppression with HGF (haematopoietic growth factor) treatment, a large proportion of patients develop residual long-term injury that is characterized by a decrease in HSC and an impaired HSC self-renewal. The molecular mechanisms of BM long-term injury are not clearly defined although many *in vitro* observations suggest that it is the result of chronic oxidative stress that induce HSC senescence [203, 205]. Regarding GI toxicity, it is induced by higher irradiation doses compared to haematopoietic syndrome and represents a major limiting factor in abdominal and pelvic radio-therapy [206]. The molecular determinants of intestinal radio-sensitivity are not clearly elucidated: some authors advocate a possible role of p53 and its effectors in mediating apoptosis of the crypt cells [206, 207], others believe that endothelial apoptosis, independent of p53, is involved in the pathogenesis of GI syndrome [208]. CNS toxicity induced by IR remains a major cause of morbidity in patients with cancer. It is possible to distinguish acute (during irradiation), early delayed (up to 6 months after irradiation) and late delayed (from more than 6 months to several years post irradiation) neurological side effects [209]. Although the precise mechanisms underlying different types of radiation-induced CNS lesions are not well understood, experimental data suggest that vascular endothelial cells and oligodendrocytes are direct radiation targets in the CNS [210].

Based on these findings it is mandatory to assess if the combined approach Nutlins/IR results in an enhancement of side effects on normal cells and tissues.

CONCLUDING REMARKS

Since its discovery in 1979 p53 gene and protein have been widely characterized in normal and neoplastic cells. Although p53 is not a typical cancer-specific antigen, its central role in the control of cell growth and apoptosis and frequent mutations in tumours indicate p53 as an attractive target for cancer therapy. Besides restoring transcriptional activation to mutant p53 proteins in tumours, considerable interest has been given to modulating p53 activity in normal cells to protect them from toxic side effects of chemo/radiotherapy. Several new compounds targeting p53 pathway are entering clinical trials, indicating that p53-oriented therapy will be one of the hottest topics in the coming years even if its effectiveness will regard only tumours with p53 malfunction. As an alternative strategy, Nutlin-3 used alone or in combination with chemo/radiotherapy appears to be a valid approach for the treatment of tumours that harbour wild type as well as null or mutant p53. In fact, in the absence of functional p53, Nutlin-3 would promote cell cycle arrest in normal cells and tissues that surround the p53-null or -mutant tumour, without affecting tumour cells themselves that would continue to proliferate to be selectively killed by anti-proliferative drugs [211]. In spite of the great number of scientific reports on Nutlins, only a few clinical trials employing Nutlins alone or in combined regimens are ongoing. Although further studies must be performed to test the *in vivo* cytotoxicity and to determine the effective radiation dose in the eradication of tumours, with this review we hope to have given a useful contribution to the scientific community with the perspective to move rapidly from bench to bedside.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

ATM	=	Ataxia teleangiectasia mutated
ATR	=	Ataxia teleangiectasia Rad 3 related
Chk1	=	Checkpoint kinase 1
Chk2	=	Checkpoint kinase 2
UV	=	Ultraviolet
IR	=	Ionizing radiation
CDKI	=	Cyclin-dependent kinase inhibitor
CDK2	=	Cyclin-dependent kinase 2
MDM2	=	Murine double minute 2
HDM2	=	Human double minute 2
ARF	=	Alternate reading frame
Rb	=	Retinoblastoma
CBP	=	CREB binding protein
AML	=	Acute myeloid leukaemia
B-CLL	=	B-chronic lymphocytic leukaemia
HL	=	Hodgkin lymphoma
DSB	=	Double strand breaks
SMase	=	Sphingomyelinase
Cdc25	=	Kinase cell division cycle 25

NHEJ	=	Non homologous end-joining
HR	=	Homologous recombination
DNA-PK	=	DNA-dependent protein kinase
CDK1	=	Cyclin-dependent kinase 1
PI3K	=	Phosphoinositide3 kinase
CNS	=	Central nervous system
TRAIL	=	TNF-related apoptosis inducing ligand
ERK	=	Extracellular signal-related kinase
MAPK	=	Mitogen-activated protein kinase
COX-2	=	Cyclooxygenase 2
ROS	=	Reactive oxygen species
CSC	=	Cancer stem cells
EGFR	=	Epidermal growth factor receptor
STAT	=	Signal transducers and activators of transcription
IKK	=	IκB kinase
MnSOD	=	Mitochondrial antioxidant manganese–containing superoxide dismutase
PKC	=	Protein kinase C
Prx	=	Peroxiredoxin
NHL	=	Non Hodgkin Lymphoma
GI	=	Gastrointestinal
BM	=	Bone marrow
HSC	=	Haematopoietic stem cells

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