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New hopes from old drugs: revisiting DNA-binding small molecules as anticancer agents

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Abstract

Most of the anticancer chemotherapeutic drugs that are broadly and successfully used today are DNA-damaging agents. Targeting of DNA has been proven to cause relatively potent and selective destruction of tumor cells. However, the clinical potential of DNA-damaging agents is limited by the adverse side effects and increased risk of secondary cancers that are consequences of the agents' genotoxicity. In this review, we present evidence that those agents capable of targeting DNA without inducing DNA damage would not be limited in these ways, and may be as potent as DNA-damaging agents in the killing of tumor cells. We use as an example literature data and our own research of the well-known antimalarial drug quinacrine, which binds to DNA without inducing DNA damage, yet modulates a number of cellular pathways that impact tumor cell survival.

Keywords

9-aminoacridine; cancer; cancer therapy; DNA; DNA-binding molecule; FACT; HMG domain protein; NF-κB; p53; quinacrine

DNA as a target of anticancer therapy: advantages & disadvantages

DNA was a target of anticancer drug discovery before its structure was even discovered. Empirically identified compounds with anti-cancer activity were later shown to target DNA either directly or through inhibition of enzymes that control DNA integrity or provide building blocks for DNA. By the time the structure of DNA was revealed by Watson and Crick in 1953 [1], there were several established therapeutic modalities targeting DNA: antimetabolites, which deplete nucleotides, including folic acid antagonists such as methotrexate; alkylation agents, which cause direct DNA damage, such as nitrogen mustard and its derivatives; and intercalators such as actinomycins, which bind DNA and inhibit the activity of many enzymes that use DNA as a substrate. Among the most widely and successfully used anticancer agents today are nonspecific DNA-damaging chemicals, including inhibitors of topoisomerases (TOPO) I and II, antimetabolites, alkylating agents and agents causing covalent modification of DNA (mitomycin C and platinum compounds), as well as γ-irradiation, for which the main target is also DNA.

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As the mechanisms underlying development of cancer were uncovered, it became clear that DNA is the critical entity driving tumorigenesis. The rationale for targeting DNA to treat cancer is based on three facts: DNA is the substance that is 'broken' in tumor cells (gene mutations); the life cycle of DNA is different in tumor cells from normal cells; and tumor cells (as compared with normal cells) are more likely to acquire additional DNA damage, owing to their higher rate of DNA replication, deficits in checkpoint control and DNA repair machinery. The latter point suggests that alteration of cellular DNA can actually be considered a targeted therapy, since rapidly proliferating tumor cells depend upon DNA integrity more than normal quiescent cells.

Although targeting DNA as a cancer therapy is well justified and, at least to some extent, successful, there are a number of disadvantages to this approach. First, it is not as effective as would be required to cure cancer. Second, it produces significant adverse side effects. Third, it can cause secondary cancers (cancers not related to the primary cancer and appearing 10–15 years after successful elimination of the primary disease). All three of these problems stem from the DNA damage caused by the therapeutic agent. If DNA was not damaged, most of the side effects would be minimized, and with no severe side effects, patients could be treated more intensively in order to achieve complete cure. In addition, the risk of secondary cancer developing would be reduced if the drugs targeting DNA did not create additional mutations in cellular genes. Therefore, we are interested in the possibility of modifying the strategy of targeting DNA as an anticancer therapy to achieve two improvements: increased specificity towards tumor cells as compared with normal cells by taking advantage of the greater dependence of tumor cells on certain DNA-related processes, and elimination of adverse side effects including secondary cancer, by using compounds that bind to DNA, but do not damage it.

Types of small molecule: DNA interaction

An understanding of the types of chemical interactions that occur between small molecules and DNA is important for predicting the potential physiological and/or therapeutic consequences of such interactions. There are three general modes of binding that characterize small-molecule interactions with double-stranded DNA: intercalation, groove binding and covalent binding (for review, see [2–4]). Pure representatives of the first two categories do not directly damage DNA, but may cause indirect damage by altering the activity of topoisomerases or other enzymes involved in maintaining DNA integrity or, alternatively, by forming particularly strong hydrogen bonds with residues of the DNA helix [4–6]. It should be noted that a single compound may utilize more than one mode of DNA binding (e.g., intercalation and covalent binding).

Despite detailed chemical/structural knowledge of the modes of DNA binding described above, there remain deficits in our understanding of small molecule–DNA interactions. First, structural studies of DNA–small molecule interactions typically consider the consequences of all such interactions as cytotoxic without discrimination of the type or mechanism of toxicity or the targeted cell population. Second, the indirect DNA-damaging consequences of small molecule–DNA binding have only been thoroughly studied for topoisomerases, and not for the many other enzymes, DNA-related factors and processes critical for the cellular functions of DNA. Furthermore, the general tendency in the development of DNA binders as anticancer drugs has been to identify stronger and more specific DNA binders and provide them with DNA-damaging substituents [7–10]. This narrow focus has left a relative deficit in our understanding of what types of effects, other than DNA damage, are elicited by different DNA-binding compounds. Nevertheless, there is evidence that the consequences of DNA–small molecule binding can vary significantly depending upon the nature of the small molecule, the mode of DNA binding, the cell type, processes ongoing in the cell and so on. Enzymes other

than topoisomerases, as well as nonenzymatic regulatory factors with activities that involve DNA recognition and/or binding, may be sensitive to small molecules bound to DNA. The literature provides many examples that demonstrate that DNA-binding compounds are not always genotoxic (for review see [6]) and that, therefore, their cytotoxicity is not due to induction of DNA damage.

Among the best-studied DNA-binding small molecules are acridine compounds [11], which interact with DNA via intercalation. Depending on the nature of a particular compound and its substituents, the results and cellular consequences of acridine–DNA interaction may vary significantly [5,8,11]. In this review, we present the literature and our own results on a class of DNA-binding small molecules with 9-aminoacridine-based structures. Our work on these molecules indicates that they do not induce DNA damage, but act through a mechanism that has not been previously described to modulate multiple stress-related transcriptional pathways. The net result of specific tumor-cell killing presents the possibility of using such molecules as anticancer therapies.

9-aminoacridine & quinacrine

9-aminoacridine (9AA) is well known in pharmacology. This scaffold has been used in many drugs for the treatment of many different diseases since the beginning of the 20th century [11]. 9AA was first used as an antiprotozoa and antibacterial agent [12]. In the 1930s, Mellanby discovered that a close analog of 9AA, acriflavine, prevented growth of transplanted tumors [13], and in 1948 9AA was shown to block fibroblast mitosis [14]. While this activity was correctly assigned to the ability of 9AA to interact with nucleic acids, 9AA derivatives were not studied further as potential anticancer agents.

The most well-known and widely used drug based on the 9AA structure is quinacrine (QC), a synthetic compound discovered in the 1920s and used for decades worldwide for a number of different indications. One of the best reviews of the pharmacological use of QC is provided by Wallace [15]. Briefly, QC was first discovered as an antimalarial compound, effective not only as a treatment, but also for prophylaxis of this disease. QC was administered as a protective measure to millions of US soldiers in the Pacific region during World War II [16–18]. The safety and generally benign nature of the compound was first demonstrated during this period [19,20]. Besides malaria, QC was also used to treat a variety of other parasitic infections, including amoeba, liamblia, giardia and so on [15,21–23]. Early in its use, an important feature of QC was noted: anti-inflammatory activity in patients with systemic chronic inflammation or, as they are now known, autoimmune disorders (for review, see [15]). QC was used to treat lupus erythematosus, rheumatoid arthritis, bronchial asthma and other inflammatory diseases [15,24–26]. Patients with these diseases frequently used QC for months at a time to control their symptoms [15]. QC was only pushed out of clinical use for such indications by the appearance of cortisone on the market in the 1950s [15].

Although the wide use of QC demonstrated that it was a generally safe compound, some side effects were identified. This led to preferential use of chloroquine over QC for treatment of malaria. The observed side effects of QC included ‘yellow discoloration of the skin’ [27,28], which we now know is actually not indicative of a pathological effect, but simply due to accumulation of the bright yellow compound in the skin; and psychosis, characterized by temporary delirium-like hyperactivity [29,30]. Development of psychosis was only seen at high doses (greater than that typically used for treatment of malaria), resulting in QC penetration of the blood–brain barrier [31,32]. Psychosis was found to be curable by cessation of QC administration and/or by sedative therapy [32]. Severe side effects of chloroquine, such as macular degeneration leading to blindness, were not described for QC [33]. Other side effects of QC that were documented (e.g., dermatitis and anemia) were idiosyncratic, meaning that

they occurred without strict dependence on the dose or duration of QC treatment, and typically in patients predisposed to the particular type of reaction. These side effects developed gradually, starting from minor lesions for dermatitis or a slight decrease in blood counts for anemia, and were completely reversible if QC use was discontinued at this early stage [15, 27,34–36]. Side effects such as these are not typical for DNA-damaging chemotherapeutic agents, which, unlike QC, cannot be administered chronically. Importantly, the anti-inflammatory activity of QC was not accompanied by immunosuppression [15]. This characteristic makes QC more similar to non-steroidal anti-inflammatory agents than to DNA-damaging chemotherapeutics, which are also used to treat autoimmune disorders based on the importance of proliferation of autoreactive lymphocytes in such disorders.

A search for the mechanism of action of QC was undertaken soon after QC implementation in the clinic, and since that time, many biological activities of QC have been described. Interestingly, the molecular mechanism underlying QC's effect against plasmodia still remains unknown. However, a number of reports have documented that the anti-inflammatory effect of QC is a result of the compound's ability to inhibit the activity of phospholipase A2 (PLA2) and other enzymes of phospholipid metabolism (Box 1 and references therein). In the course of mechanistic studies of QC, it was noted that the compound binds directly to DNA, and QC was even used in chromosome-staining techniques [37,38]; however, no immediate connection between this property and the therapeutic capacities of QC was established.

Rediscovery of quinacrine as an anticancer agent

Quinacrine was recently rediscovered in a blind search (chemical library screening) for small molecules able to activate p53 in tumor cells without causing genotoxicity (DNA damage) [39]. The goal of the library screen was to identify safe (nongenotoxic) p53 activators for potential use as anticancer therapeutics based upon the critical role that p53 plays in tumorigenesis. p53 is a transcription factor that responds to DNA damage by inducing pathways leading to either growth suppression or programmed cell death. By preventing growth of cells containing damaged DNA, p53 acts as a tumor suppressor. The importance of p53 activity for tumor suppression is illustrated by the fact that *p53* is inactivated by mutations in more than 50% of human tumors. Moreover, even in tumors with wild-type *p53*, the activity of the protein is frequently suppressed by other mechanisms involving altered expression of negative or positive regulators [40–43].

We noted that renal cell carcinoma (RCC) tumors rarely have *p53* mutations and do not appear to utilize any other obvious mechanism of p53 suppression. Nevertheless, in RCC cells, p53 is not fully activated in response to DNA damage [41]. Since p53 is not activated by DNA damage in RCC cell lines, we proposed that use of such cell lines would allow identification of p53 activators that work through mechanisms not involving DNA damage. An RCC cell line containing an integrated p53-dependent luciferase reporter was used to screen a library of small molecules for p53 activators. This resulted in selection of the 9AA-based compound (Z)-3-(acridin-9-ylamino)-2-(5-chloro-1, 3-benzoxazol-2-yl)prop-2-enal [39], and we subsequently found that the 9AA scaffold of the compound was responsible for p53 activation in the RCC cells (Figure 1). Initially, this result was somewhat disappointing, since 9AA is a well-studied compound, known to be a DNA intercalator and commonly believed (according to the literature) to be a DNA-damaging agent [44–46]. The question raised was why 9AA was able to activate the p53-dependent reporter in the RCC cells when other DNA-damaging agents could not. Even a compound structurally similar to 9AA, the topoisomerase II inhibitor amsacrine (m-amsa, Figure 1), did not activate p53 in this system [47]. The critical difference between QC and m-amsa, from a structural standpoint is that m-amsa has an alkylating substituent (methanesulfonyl), while QC does not (Figure 1). Therefore, the 9AA scaffold gives m-amsa the ability to bind DNA, and methanesulfonyl provides DNA-damaging capacity. QC

interacts with DNA through the same 9AA scaffold, but, lacking an alkylating substituent, does not (contrary to some previous reports) induce significant DNA damage (discussed later). The answer to the question of how 9AA activates p53 if not via DNA damage was provided by the finding that 9AA inhibits NF-κB activity in tumor cells, thereby 'unleashing' p53 from NF-κB-dependent inhibition [39]. By contrast, DNA-damaging agents stimulate NF-κB activation, leading to further suppression of p53 [48–50]. The effect of 9AA on NF-κB-dependent p53 inhibition validated our expectation that p53 activators identified in RCC cells would not function through induction of DNA damage.

Box 1

Consequences of quinacrine interactions with phospholipid bilayers

Primary effect

- Quinacrine avidly binds to several types of phospholipids in lipid layers (primarily phosphatidylethanolamine), thereby penetrating membranes and perturbing their physicochemical properties, stability, charge, viscosity, and so on [134–136].

Major secondary effect

- PLA2, PLC binding to membranes impregnated with QC is reduced; this leads to reduced enzymatic activity. PLA2 is more affected than PLC due to its greater dependence on phosphatidylethanolamine [137–139,140].
- Inhibition of membrane enzymes, channels and receptors that require the presence and correct architecture of phospholipids [137,139,141].

Consequences (first line)

- Synthesis of arachidonic acid (aa) is decreased [142]
- Inhibition of Ca^{2+}, Na^{+} and K^{+} channels [143], nicotinic acetylcholine receptor [144], histamine receptor [145] and ABC transporters [146].

Consequences (second line)

- Reduced COX activity (production of prostanoids) [140], LOX activity (synthesis of leukotrienes) [147] and MOX activity (production of eicosanoids) [81].
- Inhibition of Na^{+}/K^{+} exchange [148–150] and Ca^{2+} efflux [143].
- Membrane stabilization [148].

End results (combination of several consequences)

- Inhibition of:
  - Platelet aggregation [151]
  - Smooth muscle contraction [152]
  - Renal vasodilatation [153]
  - Chemotaxis [154]
  - Adhesion of some cells [155,156]
  - CYP450 MOX, enzymes of liver [147,157–161]
  - Bradykinin release [152]
  - Lipid peroxidation [147]

- Hemolysis [162]
- Kallikrein–kinin system, angiotensin and histamine signaling [142,145, 153]

- Decrease of oxidative burst of macrophages and neutrophils [145]
- Modulation of hormone release [163–166]
- Increase of water absorption in small intestine [167,168].

Pharmacological effects (combination of several end results)

- Reduction of local and general inflammation
- Decrease in coagulation
- Protective effects in several models:
  - Ischemic injury to kidney [77]and heart [78–83]
  - Toxin-induced death of liver hepatocytes [84–86]
  - Astrocyte cell death after intracerebroventricular carrageenan injection [87]
  - Kainate-induced neuronal injury in the rat hippocampus [88]
  - Reduction of local and general inflammation
  - Decrease in coagulation
  - Ischemic injury to kidney [79] and heart [80–85]
  - Toxin-induced death of liver hepatocytes [86–88]
  - Astrocyte cell death after intracerebroventricular carrageenan injection [89]
  - Kainate-induced neuronal injury in the rat hippocampus [90]
  - Neurotoxicity in an experimental parkinsonism model [91]
  - Muscle cells in an experimental model of cachexia [92]
  - Oxalate-induced renal injury [93]
  - Oxidative stress-induced kidney cell death [79]
- Reduction of gastric ulcer [169]
- Decrease in gastric motility [170,171]
- Decrease in amylase secretion by pancreatic cells [172]
- Local anesthetic [134,150]
- Anti-arrythmogenic effect [32,84,141]

Based on our results with 9AA, we tested the p53 activation and antitumor efficacy of several other 9AA-containing molecules, including the antimalarial drug QC [39]. QC and 9AA both displayed a set of very favorable antitumor properties: they induced p53 and downstream p53-dependent target genes, leading to death of tumor cells; they were more toxic to tumor cells than normal cells; and QC had antitumor effects *in vivo* in several mouse models of cancer [39]. While the *in vivo* antitumor effects of QC were not very strong and it was only able to suppress tumor growth but not cure mice [39], the data were promising enough to warrant

further investigation of the anticancer potential of QC. This involved two different strategies: improve upon QC biologically by identifying a related molecule that shows greater efficacy at lower concentrations, and/or improve upon QC pharmacologically by optimizing the delivery or biodistribution of QC or a close analog of QC. To our surprise, very few 9AA-based compounds, while all being DNA intercalators, were able to activate *p53* in the RCC reporter cells [39]. A new generation of nongenotoxic 9AA-based compounds with 9AA/QC-like effects on p53 and tumor cell viability (named curaxins) has been identified, and is currently being evaluated for future pharmacological optimization and potential use as anticancer therapeutics.

Genotoxicity of quinacrine

9-aminoacridine and QC are known DNA intercalators [11], and, in the past, were largely assumed to be DNA-damaging agents (data described below). However, the nature of our screen that identified 9AA as a p53 activator (use of an RCC cell line in which p53 is not activated by DNA damage) and differences in the effects of 9AA/QC versus known DNA-damaging agents in patients suggested that 9AA-based compounds actually do not induce DNA damage. Indeed, we found that *in vitro* treatment of mammalian cells with either 9AA or QC did not result in any signs of DNA damage [39]. We used a number of standard assays for detection of DNA damage and the results of all were clearly negative: no detection of phopshorylation of histone H2AX, no indication of ATM activation and no standard DNA-damage-associated pattern of p53 phosphorylation [39]. In addition, direct measurement of DNA breaks by Comet assay or by detection of TopoII–DNA cleavable complexes did not show any difference between QC-treated and control groups [39]. Moreover, both 9AA and QC were able to protect eukaryotic cells from the toxicity of the known DNA-damaging agents doxorubicin and bleomycin in a ‘bleomycin protection assay’ [51]. This finding demonstrates that in the presence of the DNA intercalators 9AA and QC, bleomycin and topoisomerase-inhibiting drugs cannot cause double-strand breaks in DNA [Canhui Guo, Katerina Gurova, Clevel and Clinic, Cleveland, OH, USA, and Roswell Park Cancer Institute, Buffalo, NY, USA. Unpublished observation;51,52]. Finally, QC did not promote tumor formation *in vivo*, as would be expected for a genotoxic compound. This experiment was performed in $p53^{+/-}$ mice, which demonstrate a high frequency of radiation-induced tumor formation, and thus are widely used to assess *in vivo* tumor-promotion/repression [53].

A summary of data from others regarding the potential genotoxicity and mutagenicity of QC is provided in Table 1. These data show that QC was weakly positive in some *in vitro* studies – predominantly those using prokaryotic cells [54–58]. However, no mutagenic effect was observed in most of the *in vivo* studies. The only indication of potential mutagenicity was in micronuclei assays, the results of which are complicated by the pro-apoptotic effect of QC [54,59,60] and the fact that all of these studies were carried out in the ‘preapoptosis’ era.

A major deficit of the studies that used prokaryotic cells to assess the potential mutagenicity of QC is that the doses at which QC was claimed to be mutagenic are toxic to the cells [54–56]. The strong antibacterial activity of QC [54,55] means that, in seeking to identify QC-induced mutants or revertants, one cannot distinguish between selection of pre-existing mutants and induction of mutants by the compound. Moreover, the ‘mutagenic’ effect of QC was incomparably lower than that of the positive controls used in these studies [54,55]. A truly positive mutagenic effect was only observed in frameshift-sensitive strains of bacteria [54, 55].

QC was reported to be a clastogen (inducer of chromosome breaks leading to gain, loss or rearrangement of chromosome segments) in Chinese hamster ovary (CHO) cells [54,60]. However, polyploidy was not seen in these experiments, suggesting that the observed DNA

breaks might have actually been due to what is now known as apoptosis rather than a direct effect of QC on DNA integrity. Using the 'micronucleus assay', Snyder and Arnone did not detect chromosome aberrations or any direct interactions between QC and topoisomerases [61]. In one study, treatment with QC led to an increase in antinucleoside immunofluorescence. This type of staining reveals regions of single DNA strand, what was interpreted as degradation of other strand as a result of DNA damage [62]. However, inhibited replication and stalled replication forks, rather than actual DNA damage, could explain the observed increase in fluorescence [62].

Several studies have come to the opposite conclusion that QC reduces the mutagenic effects of genotoxic compounds or the basal level of genetic instability. QC prevented the emergence of antibiotic-resistant bacteria [63,64], had apparent antimutagenic activity in Detroit-98 human sternal marrow cells grown in culture [65], reduced the frequency of appearance of cytarabine-resistant clones in L1210 murine leukemic cells [66], and suppressed the mutagenic activity of an *Escherichia coli* mutator gene and of 2-aminopurine [67].

Taken together, these past studies suggest, but do not unequivocally demonstrate, that QC is not genotoxic or mutagenic. However, this position is further supported by our recent experiments (discussed earlier), as well as the history of QC use in humans. Widespread administration of QC to hundreds of thousands of young people for prophylaxis against malaria [16,68], and to women in many different countries for sterilization, had no frequent obvious adverse consequences, including development of cancer [69,70]. Moreover, studies assessing the potential carcinogenic effect of QC (expected to be a direct consequence of a mutagenic effect) showed that QC had no carcinogenic effect on its own (see Table 2 and references therein). In various studies, QC either promoted or reduced the effects of known carcinogens, but in no case was QC found to be carcinogenic itself [71–74].

Finally, in searching for QC-like molecules with improved anticancer activity, we identified a number of compounds that were many times more active than QC against mouse models of cancer and completely lacked DNA-damaging properties in all assays run, including mutagenicity in bacteria (Ames test). Nevertheless, the DNA-binding activity of these optimized compounds is proportional to their toxicity to tumor cells [Gasparian A, Burkhart C, Sviridov S *et al.*: Multitargeting of cancer pathways by curaxins is mediated through the CK2/FACT complex. Manuscript submitted].

The preponderance of the evidence described above led us to conclude that QC does not cause DNA damage. Thus, the general assumption that the cytotoxicity of DNA-binding compounds is exclusively due to their DNA-damaging properties or genotoxicity needs to be revised. However, this conclusion also raises additional questions, most importantly, if the DNA-damaging activity of QC is negligible, then what is the mechanism responsible for the antitumor cytotoxic effect of the compound?

Why QC has antitumor activity

In our investigation of the cellular consequences of QC treatment, we found that QC and 9AA not only modulate NF-κB and p53 activity in the directions desired for limiting tumor cell growth (activation of p53 and inhibition of NF-κB), but also have effects on several other cellular signaling pathways that could play an important role in cancer treatment. First, 9AA and QC inhibit heat shock factor (HSF)-1 [75], a major transcriptional regulator of the 'unfolded protein response', without which tumor cells cannot handle the massive translational load that accompanies uncontrolled proliferation. Second, these compounds also inhibit hypoxia inducible factor (HIF)1-α [Gasparian A, Burkhart C, Sviridov S *et al.*: Manuscript submitted], a transcription regulator that both promotes tumor cell survival under the conditions of limited oxygen supply that frequently exist in solid tumors, and induces angiogenesis. These findings

suggested that 9AA and QC have a profound effect on the cellular transcription machinery, particularly targeting cell stress-related transcription. Additional work demonstrated that the mechanism of inhibition of all of the transcription factors known to be affected by the compounds was similar and quite unusual. Previously identified inhibitors of NF-κB and HSF1 anchor these factors in the cytoplasm and prevent their translocation to the nucleus where they function. By contrast, 9AA and QC do not interfere with the nuclear translocation and DNA binding of the transcription factors, but convert them from transactivators into transrepressors [39,75]. At the same time, general transcription was not significantly inhibited by 9AA or QC and some transcriptional responses (such as those involving activity of Xbp1, CRE, ARE, Oct, VDRE, C/EBP, RARE and Sp1) were actually stimulated many-fold [Gasparian A, Burkhart C, Sviridov S *et al.*: Manuscript submitted]. Thus, the impact of 9AA and QC on transcription is relatively specific.

The importance of the multiple pathways affected by 9AA and QC for their toxicity was illustrated by the finding that cells lacking *p53* are also killed by these drugs, just at higher doses than *p53* wild-type cells [39; Gasparian A, Burkhart C, Sviridov S *et al.*: Manuscript submitted]. Thus, it is clear that activation of p53 by the compounds is not in itself sufficient to produce toxicity. Moreover, modulation of any of the identified QC-targeted transcriptional pathways independently (activation of p53, or inhibition of NF-κB, HSF1 or HIF1-α) is not likely to determine the antitumor effects of the compounds. This is illustrated, for example, by data showing that pure inhibitors of NF-κB or HSF1 are not as toxic to tumor cells as QC [76–78]. Therefore, the toxicity of QC is most likely owing to the combined effects of simultaneous modulation of several tumor-related signaling pathways.

In summary, 9AA-based nongenotoxic compounds modulate transcriptional responses in tumor cells in a way that includes repression of several inducible stress-related and tumor-promoting pathways and induction of the p53 tumor-suppressor pathway, as well as the activity of some basic housekeeping factors (Xbp1, CRE, Oct1, C/EBP, Sp1 and so on). In contrast, the DNA damage caused by many standard chemotherapeutic agents (e.g., dactinomycin antibiotics, cisplatin compounds and antimetabolites) frequently results in suppression of basal transcription and induction of stress responses. The latter effect is because of the fact that DNA damage is one type of stress recognized by cellular stress sensor pathways that lead to activation of cytoprotective mechanisms (e.g., activation of the antiapoptotic NF-κB pathway in tumor cells in response to chemotherapy-induced DNA damage [50]).

The observed effects of 9AA and QC on a number of transcriptional pathways relevant to cancer clearly contribute to, if not completely account for, the antitumor efficacy of the compounds. However, the next step in our work was to identify the direct target(s) of 9AA/QC in cells and determine how interaction of QC with those targets is translated into modulation of transcription.

Direct targets of quinacrine in cells

It is well established that the physicochemical properties of QC determine its ability to interact with at least two types of biomolecules: phospholipid bilayers, and double helices of nucleic acids (DNA and RNA). We set out to determine whether targeting of either of these types of biomolecules underlies the ability of QC to alter cellular transcription and, ultimately, survival. Both of these types of interactions are characterized by a relative degree of specificity, meaning that QC preferentially interacts with certain phospholipids under certain conditions (e.g., pH and so on), and the same is true for nucleic acids. Both types of interactions have been demonstrated in cell-free and protein-free assays on purified biochemical moieties, and the physicochemical parameters of these interactions are well established.

The consequences of QC interactions with phospholipids are shown in Box 1. QC inhibits many enzymes of lipid metabolism in cells, including phospholipase (PL) A2 and C, cyclooxygenase and so on. Inhibition of these enzymes leads to decreased production of arachidonic acid and, consequently, reduced levels of tissue mediators of inflammation, such as prostanoids, leukotrienes and so on. QC was also shown to inhibit many membrane-spanning channels and receptors that require the presence and correct architecture of phospholipids. These include the acetylcholine and histamine receptors and ABC transporters. According to the literature, QC avidly binds to several types of phospholipids in lipid layers (primarily phosphatidylethanolamine), allowing the compound to penetrate and perturb the physicochemical properties of the layer. This leads to reduced binding of PLA2 to the substrate and subsequent inhibition of PLA2 function and arachidonic acid release. Among enzymes involved in lipid processing, PLA2 and PLC are primarily involved in cell signaling. Among these, QC affects the activity of PLA2 to a greater extent because of its greater dependence on phosphatidylethanolamine as compared with PLC.

It is unlikely that any of these consequences of QC interactions with phospholipids would be independently sufficient to cause death of a wide spectrum of tumor cell types. Moreover, some of the effects of QC interaction with phospholipids (e.g., stabilization of membranes, inhibition of lipid peroxidation) would be expected to actually enhance cell survival under stress conditions. Indeed, the protective effect of QC on different normal tissues was shown in many studies [79–93]. Therefore, we hypothesized that the mechanism underlying the antitumor effects of QC primarily involves interaction of QC with DNA. This possibility is supported by our finding that the cytotoxicity of QC-related compounds is well correlated with their DNA-binding potency.

How quinacrine interacts with DNA

The ability of QC to intercalate into double-stranded DNA has been established by a number of experiments, including calorimetric [94], fluorescence resonance energy transfer [95] and different photophysical and physico–chemical studies [96,97]. The parameters of QC intercalation are well established and are described in the studies mentioned earlier. True intercalators are defined as compounds that can insert between base pairs of a nucleic acid double helix owing to their small chromophore size and cross-sectional width [5]. If an intercalator does not have strong hydrogen bond donor or acceptor groups, or other substituents that can interact with DNA, it will not cause any direct modifications in gene coding and is expected to be relatively inert and safe [5]. Thus, the commonly-held view that all intercalators are mutagenic is erroneous. Nevertheless, intercalation of rather rigid planar polycyclic molecules between base pairs can significantly change the physico–chemical properties of DNA, even in the absence of chemical interaction between the DNA molecule and the intercalator. For example, both 9AA and QC increase DNA length and thermostability (resistance to temperature-induced loss of appropriate 3-dimensional double-stranded structure) [98,99]. A recent study showed that binding of QC to DNA resulted in strong stabilization of DNA against thermal strand separation in both optical melting and differential scanning calorimetry assays [94]. This could predispose DNA containing an intercalator to introduction of frame shift mutations in the process of replication, since enzymes involved in DNA metabolism are adjusted to specific degrees of spatial and dynamic architecture of the DNA molecule. However, this type of mutagenic effect is basically not observed in QC-treated eukaryotes, and is only weakly apparent in prokaryotes [54,55]. This is likely owing to the relatively weak effect of QC on the physico–chemical properties of DNA and the fact that QC suppresses replication through inhibition of DNA polymerase I [40]. Moreover, in eukaryotes, mutations that arise are probably efficiently handled by DNA-repair machinery.

QC does not interact with DNA solely by intercalation, since in addition to its tricyclic planar body, it also has a 'tail', which, based on molecular modeling, extends into the minor groove of the DNA double helix (Figure 2). This provides additional QC–DNA binding contacts, and thereby increases the affinity of binding [94]. The minor groove of DNA is narrow and less accessible to proteins compared with the major groove. Furthermore, the relative deficiency of chemical features presented in the minor groove is typically considered to be insufficient for specific recognition. However, there are several groups of proteins that bind to DNA through the minor groove with high affinity and varying degrees of sequence specificity (for review, see [100]). There are also groups of small molecules that bind to DNA in the minor groove and thereby interfere to varying degrees with minor groove protein binding [101–103]. Thus, the presence of a portion of the QC molecule in the minor-groove of DNA could be important for its cellular effects. Interestingly, QC and related small molecules bind preferentially to AT-rich sequences [94,101], a characteristic shared with the minor groove-binding DNA-bending high mobility group (HMG) domain proteins.

Quinacrine has two optical isomers (enantiomers) with a chiral center in the middle section of its 'tail' [104]. This results in two different positions of the tail in the minor groove: the *S*-enantiomer tail is directed against the direction of the minor groove, while the *R*-enantiomer tail is positioned along the minor groove (Figure 2A). Interestingly, the *R*-enantiomer, which has the better fit in the minor groove, is a slightly better inducer of p53 and inhibitor of NF-κB, and has proportionally greater cytotoxicity than the *S*-enantiomer (Figure 2B). Moreover, direct measurement of QC enantiomers binding to DNA demonstrated that, in fact, the *R*-enantiomer has a higher binding constant than the *S*-enantiomer (Figure 2C). Correlation of the strength of DNA binding with the biochemical (e.g., activation of p53 and so on) and cytotoxic consequences of QC treatment suggests that these consequences are due to a direct effect of QC on DNA, albeit an effect that does not involve DNA damage.

Structure–activity relationship (SAR) studies aimed at identifying QC-related molecules more active than QC itself demonstrated the importance of two features of the molecule for the QC activities of interest (p53 activation, NF-κB/HSF1/HIF1α inhibition and toxicity to tumor cells): a planar tricyclic body of the appropriate size to intercalate between base pairs and fit between the oligosaccharide walls of the DNA double helix, and a tail of sufficient length and structure to extend into the minor groove. In general, the QC-like properties of different compounds correlated well with their strength of DNA binding. However, among the many compounds assessed in our SAR study, there were some that bound to DNA and were cytotoxic, but showed only very weak induction of p53 and no inhibition of NF-κB. These were likely intercalators with DNA-damaging activity. We also identified some 9AA-based molecules that bound to DNA, but did not cause p53 induction, NF-κB inhibition or cytotoxicity.

How quinacrine interferes with DNA-related processes

Focusing on those QC-related molecules for which p53 activation, NF-κB inhibition and cytotoxicity all correlated with DNA binding (without the induction of DNA damage), we set out to determine what the consequences of the molecule binding to DNA were and how they are translated into the observed downstream effects. As mentioned above, intercalation can alter the physico–chemical properties of DNA, and thereby affect the activity of enzymes for which DNA is a substrate, such as TOPOs. Although it has not been clearly demonstrated in cell-free systems for other enzymes [6], the link between intercalation and inhibition of TOPOII is well-established [6,105–107]. Interestingly, the inhibition of TOPOII by QC that we observe in cell-free assays is quite different from that caused by some other intercalators, such as amsacrine and doxorubicin [Gasparian A, Burkhart C, Sviridov S *et al.*: Manuscript submitted]. These latter agents inhibit TOPOII-mediated religation, resulting in the appearance of DNA breaks. By contrast, although QC and 9AA alter the electrophoretic mobility of DNA, no DNA breaks

are detected. This finding suggests that the compounds bind directly to supercoiled DNA, and that TOPOII is not able to cut the intercalator-impregnated DNA. The finding that intercalation of QC or 9AA prevents DNA from being a substrate for TOPOII-mediated cleavage is consistent with the ability of the compounds to protect cells from the cytotoxic effects of several TOPOII inhibitors [Canhui Guo, Katerina Gurova, Cleveland Clinic, Cleveland, OH, USA, and Roswell Park Cancer Institute, Buffalo, NY, USA. Unpublished data;6,52,61]. Regardless, inhibition of TOPOII activity by QC is not likely to play an important role in its cytotoxicity since it is known that QC also inhibits DNA-polymerase 1 activity (shown in a cell-free system [108]) and TOPOII activity is not needed in cells with suppressed replication.

In addition to intercalation, minor-groove binding by the 'tail' of QC-like molecules appears to be important for their biological activity. While some minor-groove-binding small molecules act by interfering with protein binding to DNA [3,101–103], there are also minor-groove binders whose effect is autonomous. Some compounds of this type that can bind to DNA with sequence specificity and thereby deliver a functional molecule to specific sites are of interest for therapeutic uses [103]. Other compounds like netropsin binding to DNA increases the twist per base. Therefore, it removes supercoils when interacting with positively supercoiled DNA and introduces (additional) negative supercoils when binding to relaxed or negatively supercoiled DNA [109]. Among chemists and structural biologists, such compounds are named cytotoxics, although it is clear that not all of them are truly toxic [103–105]. The classic minor groove binder distamycin A inhibits helicase activity, which can lead to introduction of mutations if DNA replication is ongoing [110]. Distamycin A also inhibits binding of high mobility group (HMG) domain-containing proteins to DNA [111,112].

HMG domain proteins comprise a family of extremely abundant nuclear proteins that bind to DNA in the minor groove and have a variety of functions. A number of HMG domain proteins are so-called 'DNA architectural proteins' [100], and others are directly involved in processes such as chromatin remodeling, transcription elongation and so on [100]. The HMG proteins that are 'architectural' factors organize chromatin by appropriately bending and plasticizing DNA [113]. HMG domain proteins have a typical wedge shape, the concave surface of which can delve into the minor groove of DNA in AT-rich regions and cause a small degree of bending [114]. Some HMG domain proteins cause more extensive DNA deformation due to the presence of amino acids with aromatic side chains that intercalate between bases and separate them [101,115].

Many DNA-binding proteins involved in disparate biological functions require different degrees of DNA bending in order to recognize DNA [116–118]. Formation of fully functional transcriptosomes and enhanceosomes requires DNA bending, which can be achieved by some transcription factors on their own or through the assistance of HMG domain proteins. Thus, while HMG domain proteins do not activate transcription directly, they play a critical role in the assembly of the required transcriptosome and enhanceosome complexes [119,120]. In addition, some specific transcription factor binding sites (e.g., the NF-κB binding site) have an inherent bending angle (for the NF-κB site, 0–20°) that must be adjusted to bind different transcription factors (for the NF-κB site, up to ~14° for p65 binding and up to 18° for c-Rel binding) [121]. In these cases, again either the transcription factor itself or an interacting HMG domain protein can adjust DNA bending.

Based upon the importance of HMG domain protein activity for transcription and the fact that QC treatment impacts a number of transcriptional pathways (p53, NF-κB, HSF1 and HIF1-α), we hypothesized that HMG domain proteins might play a role in the cellular effects of QC. This possibility was supported by data showing that QC does not affect *in vitro* binding of transcription factors that it inhibits (e.g., NF-κB) to their isolated cognate DNA elements in electrophoretic mobility shift assays [39,75]. Moreover, QC does not inhibit RNA-polymerase

II activity in *in vitro* transcription assays and is not a general inhibitor of transcription [Gasparian A, Burkhart C, Sviridov S *et al.*: Manuscript submitted]. Therefore, it appears that QC may affect transcription factor recognition of DNA sequence elements only in intact cells (chromatin) or at the level of assembly of an active multicomponent transcriptosome, which again suggests involvement of HMG domain proteins.

The strong and specific transcriptional induction of genes *in vivo* by stress-activated transcription factors such as NF-κB and HSF1 relies upon 'dynamic DNA architecture', including remodeling and modification of chromatin and changes in the actual length of DNA, the degree of DNA winding and the flexibility of DNA, which affects its capacity to be bent (for review, see [100,122]). In addition to the direct impact of QC binding to DNA on DNA length and flexibility, we propose that minor groove-binding DNA-bending proteins such as HMG domain proteins also play a role in the mechanism of action of QC. There are several pieces of evidence supporting this.

First, we found that QC inhibits the activity of a particular minor-groove-binding HMG domain protein, structure specific recognition protein (SSRP)-1, and that this plays a critical role in the signal leading to p53 activation in cells treated with QC. This was clearly demonstrated by the finding that genetic knockdown of SSRP1 prevents p53 activation by QC [Gasparian A, Burkhart C, Sviridov S *et al.*: Manuscript submitted]. SSRP1 is a component of the FACT complex, which plays a critical role in transcription elongation by disassembling nucleosomes in front of RNA polymerase II [123]. Second, QC inhibits the transcriptional activity of nuclear NF-κB, and it has been shown that NF-κB-dependent transcription from the interferon-β promoter requires the HMGA1 protein for transcriptosome assembly [124]. It is likely that the HMGA1 protein participates in a similar manner in NF-κB-dependent positive regulation of many additional genes [125], including those encoding interferon-γ, E-selectin, Kit ligand, IL-2 receptor α, the inducible form of nitric oxide synthase (iNOS) and the insulin receptor [126–131]. Third, QC inhibits the activity of nuclear glucocorticoid receptor (GR), and GR has been shown to interact with the HMGB1 protein [Gasparian A, Burkhart C, Sviridov S *et al.*: Manuscript submitted;132].

Thus, for NF-κB and GR, the effect of QC on the ultimate activity of the transcription factor could be explained by a negative impact of QC on HMGA1 or HMGB1, respectively (such as reduced presence of the HMG domain protein in the nucleus or inhibition of its DNA-binding activity). The effects of QC on several other specific transcriptional responses that involve HMG domain proteins support this potential scenario. For example, QC is a potent inhibitor of HSF1-dependent transcriptional responses, and HSF1 has been shown to require the HMG domain protein SSRP1 for transcription in *Drosophila* [133]. In addition, QC inhibits the activity of interferon-inducible factors (IRFs), and IRFs interact with HMGA1 to form an enhanceosome for several interferon genes [125,126,128].

Proposed model of quinacrine mechanism of action leading to effective killing of tumor cells

Based on all of the available data, we propose the following scheme of events in cells treated with QC and QC-like molecules. QC intercalates into DNA and its 'tail' fills the minor groove in AT-rich regions of cellular DNA. This causes a disturbance in DNA architecture at these sites, making it less flexible and interfering with the architectural efforts of HMG domain proteins. The activity of transcription factors dependent on correct DNA architecture and/or interaction with HMG proteins is blocked. Such architecture-sensitive factors include a number of inducible stress-responsive factors, such as NF-κB, HSF1, HIF1α, GR, IRF and so on, many of which are important for tumor cell survival but are used by normal cells only under specific conditions. The presence of QC bound to DNA is also sensed by the FACT complex (of which

the HMG protein SSRP1 is one component), which activates p53. Since QC-induced p53 activation occurs in a cellular environment of inhibited NF-κB (also due to QC), it is not attenuated as it is in the case of DNA-damaging treatments. Together all of these QC-induced events overwhelm the prosurvival mechanisms activated in tumor cells and result in cell death. By contrast, normal cells do not constitutively depend upon many of the transcriptional pathways that are fully employed in tumors and affected by QC. This could explain why QC and QC-related compounds display relatively specific toxicity *in vivo* against tumor tissue as compared with normal tissue. Importantly, similarly selective cytotoxicity is not observed when tumor and normal cells are treated with QC *in vitro*. This may be explained by the fact that cultured normal cells are exposed to multiple stresses owing to their *in vitro* growth conditions and, thus, may be more dependent upon QC-targeted stress-related pathways than normal cells *in vivo*.

While this scheme is still hypothetical in many parts, it provides a rational scaffold for additional studies aimed at gaining a complete understanding of the mechanism of action of QC-like DNA-binding molecules that do not damage DNA, yet display significant specific toxicity against tumor cells. Such molecules represent a novel and highly promising strategy for safe and effective treatment of cancer.

Conclusion

DNA remains a promising target for anticancer drug development, but DNA damage is not a prerequisite for anti-tumor activity by DNA-binding molecules. Different modes of binding of compounds to DNA may produce different cellular outcomes, which may lead to selectivity of toxic effects against different cells, depending on tissue and cellular conditions (context) and transformed status. The effects of small DNA-binding molecules on numerous DNA-related processes in cells are not well-studied and require additional investigation, since they may still be a valid approach for cancer treatment.

Future perspective

I would expect that in the near future the focus of the drug discovery field will shift away from selective inhibitors of single enzymes towards agents with broader activities based on the efficacy data of such drugs in the clinic. In addition, we will begin to understand how currently used anticancer agents actually cause their effects and what their molecular targets are. Since most of these compounds are DNA-binding agents, this may lead to an improved understanding of how small molecules affect different functions of DNA, and what consequences this may have for different categories of cells.

Targeting of DNA without induction of DNA damage presents an interesting, and, thus far, poorly explored approach for anticancer treatment. By changing the properties of DNA, DNA-binding small molecules have the potential to modulate many processes and signaling pathways simultaneously. While such a multipronged approach might provide improved anti-tumor efficacy and limit development of drug resistance, the question is whether we would be able to achieve the desired level of specificity and avoid adversely reshuffling cellular physiology.

Quinacrine and more potent next-generation compounds (curaxins, from ‘cure’) may become prototype molecules for this approach. We are aiming for curaxins to enter clinical trials in 2010, and hope that this will provide additional support for use of nongenotoxic DNA-binding small molecules as anticancer therapies. In the meantime, these new groups of molecules are ‘seeking efforts’ of molecular biologists to further investigate the effects that they cause in cells and determine what combination of DNA-binding properties in small molecules will be the most valuable for cancer treatment.

Executive summary

DNA is the target of many successful anticancer agents

- Tumor cells depend more than normal cells on DNA integrity.
- DNA is sensitive to a wide variety of physical effects and chemical substances.
- Significant variation in the level of DNA damage induced by a drug is possible; therefore, a range of doses lethal for tumors yet not lethal for an organism may be identified.

Induction of DNA damage is a major problem with many anticancer therapies targeting DNA

- Normal cells die from DNA damage in highly proliferative organs and tissues such as the blood, intestine and immune system; the potential lethality of such adverse side effects limits the dose (and, therefore, the effectiveness) of the therapy.
- Irreversible changes in DNA integrity cause short- and long-term side effects.
- DNA damage significantly increases the risk of developing secondary cancers.

Induction of DNA damage is not a prerequisite for antitumor activity by DNA-binding small molecules

- Binding of small molecules to DNA causes significant changes in the physico–chemical properties and plasticity of DNA.
- Changes in the physico–chemical properties and plasticity of DNA may impact many DNA-related processes and, therefore, have more consequences for cellular physiology than DNA damage.
- DNA damage induces stress responses that may actually promote survival of tumor cells.
- Cisplatin, currently the most widely-used anticancer agent, causes significant changes in the physico–chemical properties and plasticity of DNA; this, rather than mutations associated with cisplatin treatment, may be responsible for cisplatin efficacy.

Quinacrine (QC) is a prototype DNA-binding molecule that demonstrates anticancer activity without inducing DNA damage

- Quinacrine has antitumor activity *in vitro* and *in vivo*.
- QC has no mutagenic activity in eukaryotes.
- QC has no carcinogenic activity.
- Side effects of QC treatment are milder and very different than chemotherapy-related side effects.
- By using QC as a prototype, we have identified a new class drugs, curaxins, with improved DNA binding and anticancer activity, yet no DNA-damaging properties.

Physico–chemical properties of DNA may be especially critical for stress-related transcriptional responses

- Induced transcription depends more on DNA architecture than basal or constitutive transcription.

- DNA architecture is impacted by changes in the three-dimensional structure of DNA caused by small-molecule binding.
- In cases of changed DNA architecture, the activity of stress-inducible transcription factors may be affected more than that of basal transcription machinery, since inducible factors need to find and recognize *de novo* their binding sites.

Tumor cells depend on stress-inducible transcriptional pathways much more than normal cells

- Tumor cells utilizes many stress-related pathways on a constant basis for survival.
- Tumor cells do not have a reserve of additional inducible stress-related transcription, while normal cells have substantial reserves.
- Inhibition of stress-related transcription (especially several particular transcriptional pathways) has detrimental consequences for tumor cells, but not for normal cells *in vivo*.

Conclusion

- Design of new DNA-binding molecules that have the ability to change DNA architecture and block different types of transcription without inducing DNA damage is needed for experimental and clinical oncology.
- Simultaneous inhibition of multiple stress-related pathways in cells at the level of transcription may present a new safe and effective approach to cancer therapy.

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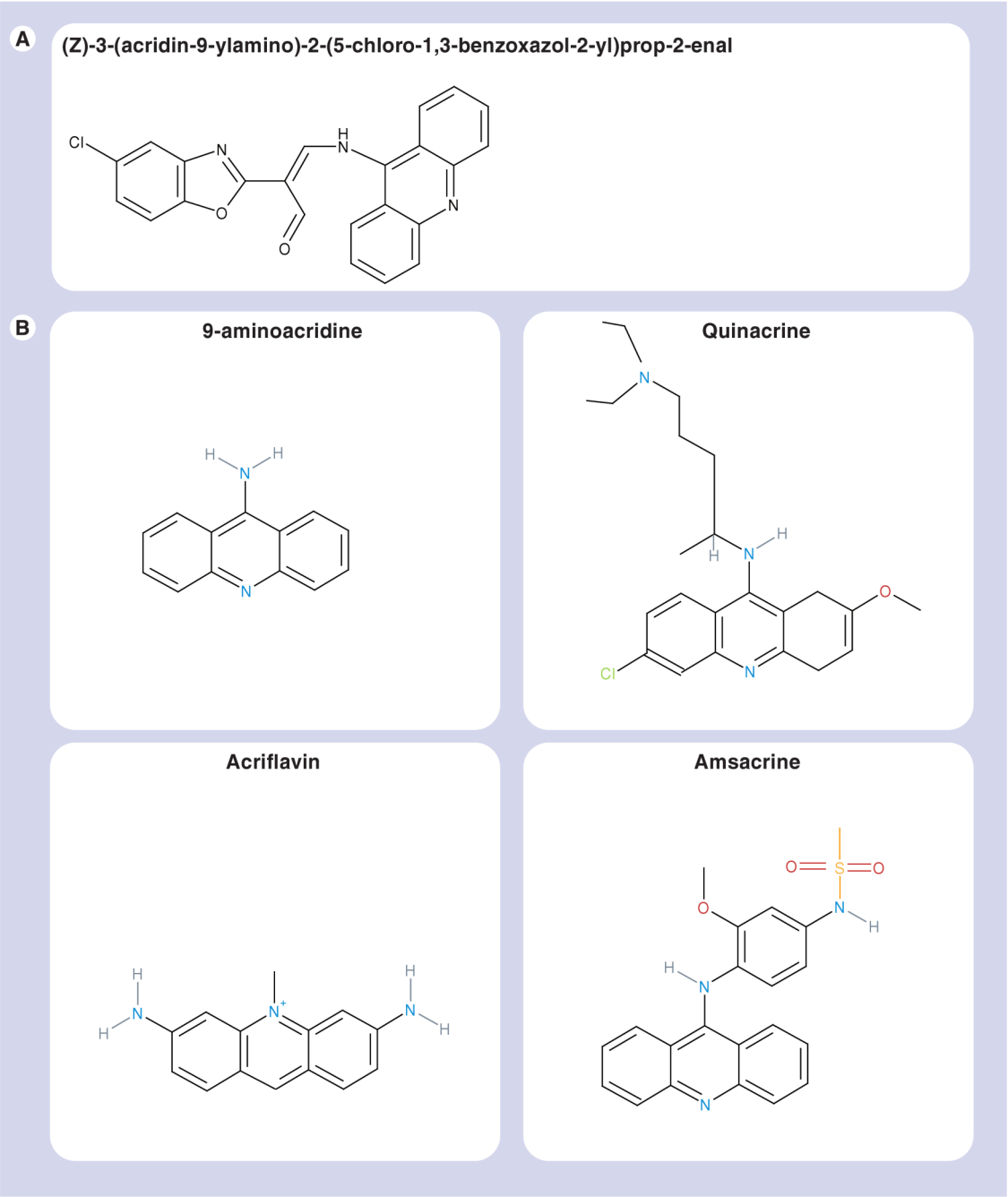

Figure 1. Compounds discussed in the text

(A) Structure of the compound identified in the screening of p53 activators. This compound was in Diversity Set Small Molecule Library (Chembridge, San Diego, CA, USA) ID# 561611. Treatment of renal cell carcinoma cells with this compound induced p53-dependent reporter activity. **(B)** Known compounds with 9-aminoacridine scaffold used in medicine for different purposes. 9-aminoacridine – antiseptic, acriflavin – experimental anticancer compound, amsacrine – anticancer drug, inhibitor of topoisomerase II and quinacrine – antimalarial and anti-inflammatory agent.

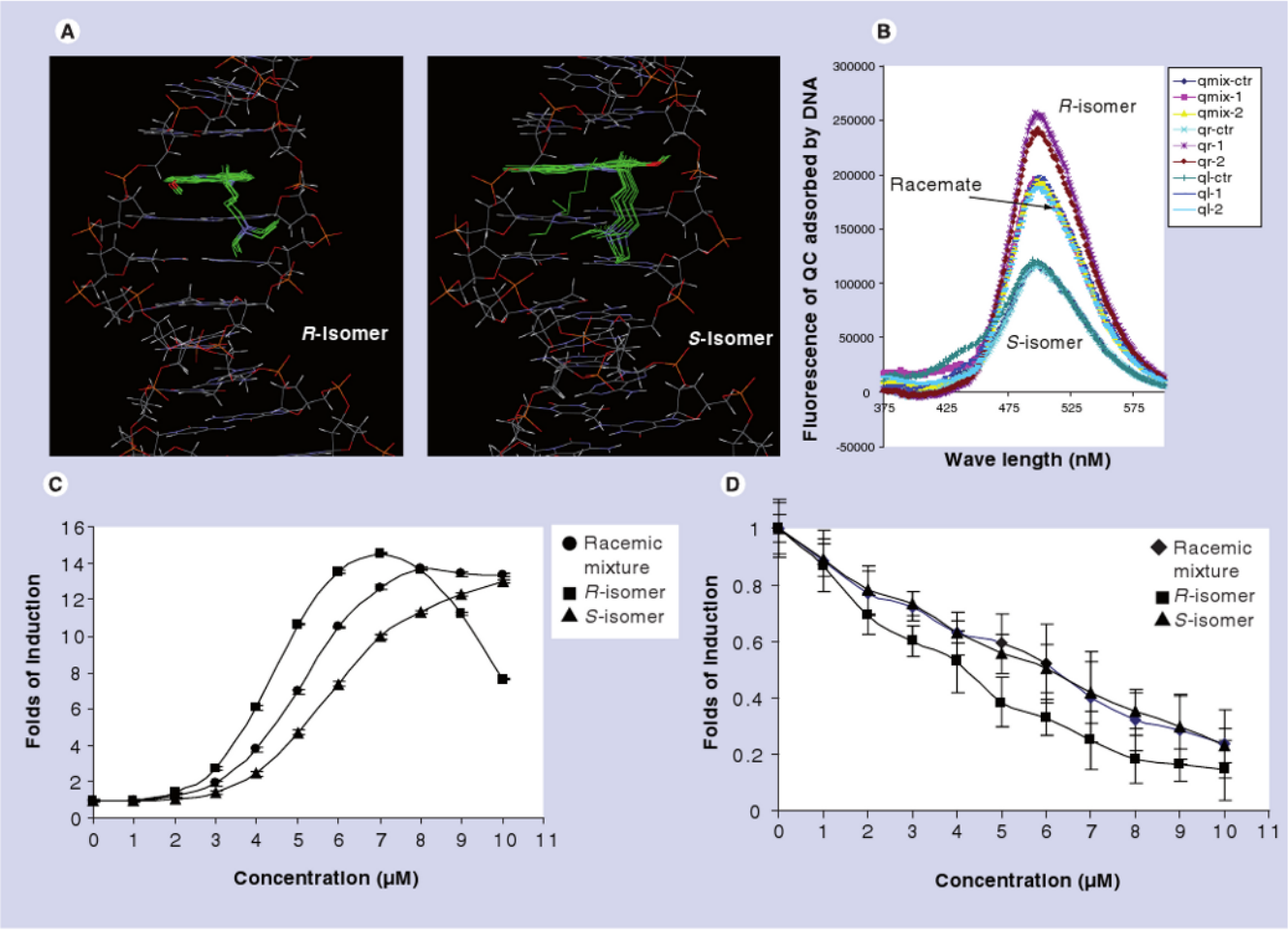

Figure 2. DNA-binding capacity of quinacrine enantiomers correlates with p53 activation and NF-κB inhibition

(A) Computer modeling of the fitness for *R*- and *S*-isomer binding to double-stranded DNA. The average fitness of *R* conformers is 7% better than that of *S* conformers, ($p < 0.00001$). The better fitness of R conformers is primarily due to one of the fitness score components; van der Waals potential energy between ligand and DNA atoms. It is also likely that the *S* isomer disrupts DNA Watson–Crick pairing through hydrogen bonding by the nitrogen in quinacrine (QC)'s tail. Modeling was carried out by L Brodsky (Cleveland BioLabs, NY, USA). **(B)** Binding of QC isomers and racemic mixture to plasmid DNA in a competitive dialysis experiment. 30 μM double stranded plasmid DNA were placed in a mini-dialysis unit (Pierce, #69560) and dialyzed for 24 h against 200 ml of 1 mM QC solution. DNA-bound QC was measured by fluorimetry. The multiple curves represent replicates of the experiment. The fluorescence on Y-axis reflects the amount of QC bound by DNA. Experiment was carried out by A Gasparian (Cleveland BioLabs, NY, USA). **(C)** Activation of p53-dependent luciferase reporter in RCC45 cells by QC isomers. **(D)** Inhibition of NF-κB reporter in H1299 cells by QC isomers. Error bars in **(C)** and **(D)** show the standard error between eight replicates of experiment. Reporter activity was measured 24 h after chemicals were added by Promega (WI, USA) Bright-Glo™ assay. Experiments **(C)** and **(D)** were performed by D Bosykh (Cleveland BioLabs, NY, USA).

Table 1
Studies of quinacrine mutagenic properties

Type of assay	Indicator cells and/or species/ strain	Results (in at least one strain)	Comments	Ref.
Bacterial reversion assay	*Salmonella typhimurium* (TA100, TA98, TA1535, TA1537) and *Escherichia coli* WP2 *uvrA*	Positive	Some positive results are obtained on toxic concentrations. Only results on frameshift strains were truly positive	[53]
Mouse lymphoma	L5178Y cells $TK^{+/-}$ (clone 3.7.2C)	Negative	–	[53]
Chromosome aberration	Chinese hamster ovary	Positive	No polyploidy	[53]
Micronucleus assay	Mice	Negative	–	[53]
Bacterial reversion assay	*Salmonella typhimurium*	Positive	–	[54]
Bacterial reversion assay	*Salmonella typhimurium*	Positive	–	[55]
Bacterial reversion assay	*Streptococcus pneumoniae*	Positive	Mainly frameshift	[56]
DNA synthesis/repair	*Escherichia coli*	Positive	–	[57]
DNA synthesis/repair	Opossum lymphocyte	Positive	–	[173]
Micronucleus assay	Chinese hamster lung V79	Negative	–	[104]
Chromosome aberration	Human/lymphocytes	Positive	Although positive type of aberration were not similar to other mutagens or ionizing radiation	[59]
DNA strand separation	BALB/c3T3 1-13 mouse cells	Positive	Staining was positive at doses inhibiting replication (inhibiting replication forks presents zone of unwound DNA and these are stained positive)	[61]
Micronucleus assay	Mouse/CBA	Positive (?)	Maier and Schmid refuted the conclusion of this work by showing that the inclusion bodies observed by Jenssen *et al*. were artifacts; the quinacrine-induced lysis of the nucleus resulted in micronucleus-like structures	[58]
Micronucleus assay	Mouse/NMRI	Negative	–	[174]
Sex-linked recessive lethal and sex-chromosome loss	*Drosophila melanogaster*	Negative	–	[175]

Table 2

Studies of quinacrine carcinogenicity

Type of study	Description	Species	Results	Ref
1-year neonatal mouse carcinogenicity	Neonatal CD-1 mice were given injections of QC at dosages of 0, 10, 50 and 100 mg/kg on postnatal days 8 and 15. This study was conducted according to US FDA GLP regulations, 21CFR58.	Mouse (neonatal)	Slight increases in the incidence of endometrial hyperplasia; benign uterine stromal polyps, statistically significant by trend test but not by pairwise comparison. The authors pointed out that 'uterine stomal polyps are not considered to be precancerous lesions in rodents. Both endometrial hyperplasia and endometrial stromal polyps are common findings in older mice'. There were no increases in liver or lung tumors in either sex.	[70]
Induction of preneoplastic liver cell foci	Male F344 rats were injected i.p. with DEN and, starting 2 weeks later, rats were given QC at dietary levels of 20, 100 and 500 ppm for 6 weeks. At week 3 following DEN administration, all animals were subjected to two-thirds partial hepatectomy, and after killing the animals at week 8, development of preneoplastic liver cell foci was investigated using the glutathione S-transferase placental form as a marker.	Rat	No lesions being induced by quinacrine alone (500 ppm) Increase in foci number and area in DEN/QC (500 ppm) group as compared with DEN alone, partial increase at 100 ppm, but not the 20 ppm treated group.	[72]
Transplacental CNS tumorigenicity	QC was administered in combination with ethynitrosourea to pregnant Wistar rats. The offspring (30 in the quinacrine group and 57 in the control group) were followed for up to 400 days post-partum.	Rat	No increase in the incidence of spinal ependymomas. The latency of these tumors was decreased	[71]
Post-initiation phase of carcinogenesis	30 female Syrian hamsters were given three weekly subcutaneous injections of BOP at a dose of 10 mg/kg followed by 100 or 300 ppm QC in the diet for 37 weeks. Additional groups of animals received the BOP injection alone, or only the 300 ppm QC treatment. At week 40, all surviving animals were killed and development of proliferative lesions was assessed histopathologically.	Hamster	Pancreatic adenocarcinomas and dysplastic lesions per hamster were higher in the BOP/QC100 group (1.92 and 1.78), but not in the BOP/QC300 group compared with the BOP-alone group (1.07 and 0.79).Hepatocellular adenomas plus carcinomas were also increased in the BOP/QC300 and BOP/QC100 groups. Lung adenomas plus adenocarcinomas were decreased by the QC300 treatment. Renal cell tumors (adenomas and carcinomas) or nephroblastomas were not different between the BOP-treated groups. Therefore, QC enhances pancreatic and hepatic carcinogenesis while inhibiting lung tumor development in hamsters initiated by BOP. No tumors or preneoplastic lesions were found in the QC-only group.	[73]

BOP: N-nitrosobis(2-oxopropyl)amine; DEN: N-nitrosodiethylamine; GLP: Good Laboratory Practice i.p.: Intraperitoneal; QC: Quinacrine.