Safety and efficacy of quadrapeutics versus chemoradiation in head and neck carcinoma xenograft model

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Abstract: Chemoradiation is the strongest anti-tumor therapy but in resistant unresectable cancers it often lacks safety and efficacy. We compared our recently developed cell-level combination approach, quadrapeutics, to chemoradiation to establish pre-clinical data for its biodistribution, safety and efficacy in head and neck squamous cell carcinoma (HNSCC), as a clinically challenging aggressive and resistant cancer. In vitro and in vivo models of four carcinomas were treated with standard chemoradiation and quadrapeutics using identical drug and radiation doses. We applied liposomal cisplatin or doxorubicin, colloidal gold, near-infrared laser pulses and radiation, all at low safe doses. The final evaluation used a xenograft model of HNSCC. Quadrapeutics enhanced standard chemoradiation in vitro by reducing head and neck cancer cell proliferation by 1000-fold, inhibiting tumor growth in vivo by 34-fold and improving animal survival by 5-fold, and reducing the side effects to a negligible level. In quadrapeutics, we observed an “inversion” of the drug efficacy of two standard drugs: doxorubicin, a low efficacy drug for the cancers studied, was two times more efficient than cisplatin, the first choice drug in clinic for HNSCC. The radical therapeutic gain of quadrapeutics resulted from the intracellular synergy of the four components employed which we administered in a specific sequence, while the reduction in the toxicity was due to the low doses of all four components. The biodistribution, safety and efficacy data for quadrapeutics in HNSCC ensure its high translational potential and justify the possibility of clinical trials.

Keywords: Carcinoma, drug resistance, laser, plasmonic nanobubble, quadrapeutics, chemoradiation, nanomedicine

Introduction

Chemoradiation is generally considered as the strongest therapy for unresectable tumors but even so, it often fails to safely and efficiently treat resistant and aggressive cancers [1-3]. To overcome this challenge, we recently converted current standard macro treatments-surgery, chemo- and radiation therapies-into one cancer cell-specific intracellular micro-modality named quadrapeutics [4]. To achieve such a macro-to-micro conversion of standard therapies, we employed non-stationary mechanical intracellular nanoevents, plasmonic nanobubbles (PNBs) [4, 5] in a four-component treatment (Figure 1). Firstly, systemically administered antibody-targeted gold colloid and a liposomal drug are aggregated by the cancer cell into an intracellular mixed cluster [4, 6]. Secondly, a low-energy near-infrared (NIR) laser pulse is applied locally and is instantly converted by the gold cluster into an expanding and collapsing vapor nanobubble named a PNB [7]. A PNB is not a particle, but a nano-explosion, with a mechanical, non-thermal, intracellular impact. The high cancer cell specificity of a PNB is due to its
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In order to determine the translational potential of quadrapeutics and to prepare for future clinical trials, firstly for head and neck squamous cell carcinoma (HNSCC), we continue our previous work here and report the in-depth study of this novel technology focusing on the biodistribution, safety and efficacy of quadrapeutics and its components and on its applicability to other cancers. We also optimize the quadrapeutics protocol (because the time sequence of the component administration is the key to quadrapeutics), and compare it to standard chemoradiation in several resistant carcinomas, with an in-depth evaluation in HNSCC. Compared to our previous works, the focus here is on the key preclinical data for HNSCC: the biodistribution and toxicity of the employed gold and drugs, the influence of the drug type on the therapeutic efficacy, and the analysis of the key therapeutic metric—the overall animal survival rate for chemoradiation versus quadrapeutics.

Materials and methods

Cancer models

In vitro, we used cell lines of four epithelial carcinomas associated with high aggressiveness and drug resistance, and poor therapeutic outcomes for chemo- and chemoradiation treatments: head and neck squamous cell carcinoma, HN31, triple-negative breast adenocarcinoma, MDA-MB-468, ovarian adenocarcinoma, SKOV3 IP1, and colon adenocarcinoma, SW48. The cells were obtained from UT MD Anderson Cancer Center (Houston, TX). All these carcinomas overexpress Epidermal Growth Factor Receptor (EGFR) which was targeted in our study to deliver the gold and drugs by using two clinical antibodies, cetuximab (C225, ImClone Systems Inc., Branchburg, NJ) and panitumumab (Vectibix, Amgen Inc., Thousand Oaks, CA). Epithelial cancers with a tumor depth up to several millimeters can be reliably and safely (for adjacent normal tissues) accessed with laser radiation in the NIR spectral range and therefore are a good translational model. To evaluate specificity, we used normal epithelial cell line NOM9 (from J. Myers's Lab, UT MD Anderson Cancer Center, Houston, TX).

In vivo, a HNSCC xenograft tumor was raised on a flank of mice (athymic nude, strain CRL-490) to 5 mm diameter by s.c. injection of HN31 cells. We chose HNSCC as a model due to its

threshold generation mechanism: the threshold energy (fluence) of the laser pulse decreases with the cluster size [7] and the cluster size is the largest in cancer cells [4, 8]. This cluster-threshold mechanism results in PNB generation only in cancer cells even despite the non-specific uptake of some gold nanoparticles by adjacent normal cells (which cannot produce a PNB at the same laser fluence [4, 9]). An intracellular gold cluster and a PNB deliver three therapeutic mechanisms by using four components: a mechanical, non-thermal impact destroys a cancer cell [4, 5, 10-12], or disrupts the co-localized drug-bearing liposomes thus ejecting the drug into the cytoplasm [4, 6, 13], and radiosensitizing the cell [4]. The intracellular synergy of these three mechanisms is the foundation of quadrapeutics and significantly amplifies chemoradiation in resistant cancer cells and tumors [4].

Figure 1. Principle and protocol of quadrapeutics as the combination of four therapeutic components: ①, ② the systemic concurrent administration of the conjugates of encapsulated drug and colloidal gold creates mixed gold-drug intracellular clusters in cancer cells via receptor-mediated endocytosis during the delay 1; ③ the local administration of a single near-infrared laser pulse after delay 1 generates plasmonic nanobubbles only in cancer cells resulting in the intracellular mechanical impact, drug ejection and radiosensitization, which achieves the maximum after the time-delay 2; ④ during the maximal radiosensitization of a cancer cell, the local administration of radiation completes the procedure. The intracellular co-localization of the mechanical, chemotherapeutic and radiotherapeutic effects synergistically enhances standard chemoradiation resulting in a multi-fold therapeutic gain.

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high aggressiveness and drug resistance which has not allowed an improvement in patient survival time over the last 30 to 35 years [2, 3, 15]. Two standard metrics of therapeutic efficacy were obtained: tumor volume (within 2 to 3 weeks after the treatment) and the animal overall survival time (measured until the animal reaches the moribund condition which was defined as a tumor size of 10 mm). Six animals were treated in each group. The animals were treated according to the IACUC protocols approved by the Rice University and the Methodist Hospital Research Institute.

Drug and gold targeting and analysis in vivo

Gold spheres of 60 nm diameter (VanPelt Biosciences LLC (Ijamsville, MD) and nanoComposix Inc (San Diego, CA) were covalently conjugated to the anti-EGFR antibody clinically-approved for HNSCC, Panitumumab, by using a proprietary conjugation method (performed by VanPelt Biosciences LLC (Ijamsville, MD)). In vitro, gold conjugates were incubated with cells for 24 hours under physiological conditions at the concentration of gold conjugate suspension corresponding to the optical density of 0.08 at the wavelength of 540 nm, the maximum of the optical absorption spectrum. This corresponds approximately to a dose of 0.7 μg/ml. In vivo, gold conjugates were administered intravenously in the low dose of 4 mg/kg of body weight and a certain time-delay was allowed in order to form intracellular clusters of gold and drug nanoparticles [4-6, 13].

Two standard liposomal drugs were used: Doxil (Ben Venue Laboratories Inc, Bedford, OH) for doxorubicin and Lipoplatin (Regulon Inc, Alimos, Greece) for cisplatin (the first drug of choice in chemoradiation therapy of HNSCC and other studied carcinomas since the platinum in this drug is known to radio-sensitize the cells). For targeting, these 100 nm drug liposomes were covalently conjugated to the same antibodies as GNPs by using our previously established and validated methods that show high stability, low toxicity and the long shelf life (> 6 months) of such liposomal conjugates [4, 6, 13].

In vitro, GNPs and drug liposomes were mixed and incubated with the cells under physiological conditions for 24 hours at specific concentrations of gold and drugs. This incubation time was previously shown to provide the maximal efficacy and specificity of cluster formation in cancer cells via receptor-mediated endocytosis [10, 13]. In vivo, GNPs and drug liposome conjugates were administered systemically and concurrently. We previously verified the high efficacy and tumor specificity of the formation of gold conjugate clusters in HNSCC tumors in vivo under low doses of colloidal gold conjugate through the mechanism of receptor-mediated endocytosis [4, 6]. The influence of cancer aggressiveness on the cluster size was also observed previously [4]. Therefore, the described protocol provides the safe and reliable formation of mixed gold-drug clusters in vitro and in vivo.

The efficacy of gold and drug conjugate targeting in vivo was analyzed by measuring the level of gold and platinum in the tumor and other organs which were harvested 24 hours and 72 hours after the systemic administration of the conjugates. Three animals were studied for each time-point. The level of gold and platinum was measured with the mass-spectroscopy method (Perkin Elmer Nexion 300 ICP-MS, Perkin Elmer, Inc., Waltham, MA). The toxicity of the gold conjugates in vivo was measured short-term (24 and 72 hours after administration) and long-term (over 1 month). To determine the short-term toxicity, the harvested liver, kidney, spleen, and lung were analyzed with a pathological method for necrosis, apoptosis and other standard signs of toxicity. The long-term toxicity was assessed by monitoring the animal weight for one month and longer.

Histology

The harvested organs (kidney, lung, liver, heart,) and the tumor were placed in 10% neutral buffered formalin and fixed for 48 hours. The organs were then processed routinely and sections were stained with hematoxylin and eosin (H&E). Sections were examined by a board certified veterinary pathologist (BCVP). Regions of tumor and necrosis were delineated with the assistance of the BCVP. For the histological study of the therapeutic effect of standard chemoradiation and PNB-enhanced chemoradiation, animals were sacrificed on Day 12, where Day 0 was the day of treatment initiation. This corresponds to 72 hours following the end of the dual treatment. Following sacrifice, the tumors were collected along with the underlying muscle and ribcage, and fixed in 10% neu-
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central buffered formalin for at least 48 hours. Samples were then processed to HE slides as outlined above.

**PNB generation and detection**

On-demand intracellular PNBs were generated around clusters of gold colloids with single NIR laser pulses (782 nm, 30 ps, Ekspla PG 500, Ekspla UAB, Lithuania) which were absorbed by the gold spheres and converted into heat. While the stationary optical excitation of gold colloids at 782 nm is not efficient due to their poor optical absorbance (just 6% relative to that in their visible spectral peak of 500-600 nm), our non-stationary excitation method [9] provides an efficient generation of PNBs with a 30 ps laser pulse at the NIR wavelength of 782 nm. The *in vivo* experiments used our photothermal microscope described previously [7]. In the *in vivo* experiments, the laser pulse was delivered to the tissue via a custom-made endoscope (Figure 2A). To detect PNBs optically in individual cells *in vitro*, we used our established optical scattering method [7]. *In vivo*, PNBs were detected with ultrasound sensors installed in the tip of an endoscope via a PNB-specific acoustic signal (Figure 2B).

**Radiation treatment**

Cells and animals were irradiated with a RS 2000 machine (Rad Source Technologies, Inc., Suwanee, GA). *In vitro*, single treatment was used. *In vivo*, two fractions were administered locally with a one day interval. The radiation was administered with a specific time delay after the laser treatment.

**Statistical considerations**

We used two-tailed t-tests to compare the cell and animal group metrics. We performed statistical analyses with Origin software (Origin 9.1, OriginLab Corporation, Northampton, MA). *P* values of < 0.05 were considered statistically significant.

**Results and discussion**

This study was aimed at the optimization of the quadrapeutics protocol in several resistant carcinomas to achieve maximal safety and efficacy in comparison with the standard of care, chemoradiation therapy.

**Optimization of the gold and drug targeting *in vivo***

Since the therapeutic efficacy of the quadrapeutics mechanisms depends upon the clustering of gold and drugs in the tumor, we first analyzed the safety and efficacy of the systemic targeting of gold and liposomal conjugates in a xenograft model of head and neck squamous cell carcinoma (HNSCC) induced with HN31 cell line. According to our previous observations [4, 9], this is a very resistant and aggressive form of HNSCC. Both conjugates were concurrently i.v. injected with doses of 4 mg/kg (gold) and 12 mg/kg (cisplatin). The systemic administration followed the standard approach in chemo-

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**Figure 2.** A. The laser pulsed treatment has been applied locally through the custom-designed endoscope with a ring-shape acoustic sensor around the optical exit window (the endoscope was coupled to the wound optically and acoustically through transparent ultrasound gel); B. Acoustic time-responses to a single laser pulse (782 nm, 30 ps, 65 mJ/cm²) of the primary tumor of the animal pretreated with gold conjugates (red) and the tumor without such pretreatment (black).
The concurrent systemic administration of gold colloids and liposomal drug (Lipoplatin) was used to maximize the endocytosis-based intracellular formation of the mixed drug-gold clusters which requires the synchronous internalization of gold and drug liposomes by cancer cells [13]. To reduce the treatment toxicity, mainly associated with the drug, the drug dose was administered at the level within 15-20% of the human clinical dose equivalent. The dynamics of the biodistribution of the liposomal drug was measured for several organs harvested at 6, 24 and 72 hours in animals with relatively small tumors (within 3 mm). The maximum level of drug in the tumor was achieved in 24 hours (Figure 3A), which coincided with the optimal time for the formation of the maximal gold-drug clusters in cells as observed previously in vitro [4, 13]. The efficient retention of the drug in a tumor was observed up to 72 h. This relatively long retention time (compared to that for small molecule-based free drugs) should further improve the...
radiation treatment by amplifying the radiosensitization effect of the intracellular drug clusters.

The influence of the drug targeting antibody combination for drug was measured for the drug levels in relatively small (3 mm) tumors harvested 24 hours after the systemic injection of bare gold and drug liposomes versus those conjugated to identical or different antibodies (C225 and Panitumumab). The combination gold-Panitumumab and Lipoplatin-C225 provided the maximal levels of the drug in the tumor (Figure 3B). After optimizing the timing and the antibody combination, we analyzed the influence of the tumor size (early vs mature tumor, Figure 3C). By comparing the drug biodistribution achieved in 24 hours in animals with early (< 3 mm) and well-developed (> 5 mm) tumors, we concluded that 5 mm tumors provide a much better accumulation of the drug when it is conjugated to the optimal antibody (Figure 3C). The better-developed vasculature in larger tumors improved the systemic delivery of the gold and drug. Nevertheless, even small early tumors can be successfully targeted systemically with low doses of the drug and gold. After optimizing all targeting conditions, we achieved the relatively high tumor specificity of accumulation of the drug (Figure 3D). In the therapeutic experiments, we used animals with 5 mm tumors. Note that despite using the anti-EGFR antibody, its drug liposome conjugates escaped the so-called “liver sink” effect [14] and the liver levels of the drug was relatively low (Figure 3D). Among normal organs, a relatively high amount of the drug was found in the lungs and liver (Figure 3D), although it should not induce any PNB treatment-related toxicity because the laser pulse (which activates the gold and drug) cannot reach either the lungs or the liver.

Coupled with our previous observation of gold biodistribution and gold clusters in tumor cells for a similar targeting of the HNSCC xenograft in mice [6], and a comparison of the intratumoral versus the systemic administration of gold conjugates [6], we chose to use the concurrent systemic administration of 60 nm gold-Panitumumab and 100 nm drug liposome-C225 conjugates. An advantage of the concurrent administration is an ability to personalize and independently tune the doses of GNPs and drugs in order to optimize the cluster formation and the therapeutic effect. The advantage of systemic administration over local intra-tumoral injection is in using the standard chemotherapy route for drug delivery, whereas the local intratumoral injection of drug liposomes or gold nanoparticles will have limited delivery and selectivity due to the strong limiting effect of the tissue upon the nanoparticle diffusion. This was directly observed by us previously by comparing the local versus the systemic delivery of the same gold nanoparticles in similar tumor models [6].

Toxicity of systemically administered gold and drug conjugates

Although colloidal gold, drug and antibodies are clinically validated, we studied their short- and long-term toxicity in mice. A histological evaluation of organs harvested at 24 hours and 72 hours from intact (Figure 4A-C) and gold/drug-treated mice (Figure 4D-F) revealed no short-term gold- and drug-related toxicity at all (Table 1). This result is in line with (1) the generally safe nature of gold nanoparticles of this relatively large size because they remain chemically and biologically inert (this was also verified in clinic for similar colloidal gold nanoparticles [16-20]), (2) the low doses of gold we used in our experiments which were 5-100 times lower than those reported in the diagnostic and therapeutic use of gold nanoparticles in vivo [21-29], and (3) the significantly reduced dose (25% of the clinical equivalent) of the chemotherapy drug, cisplatin, which is associated with major clinical adverse effects of cisplatin-based chemotherapy and chemoradiation therapy. In addition, we observed the relative changes in the body weight in two groups of intact and quadrup-deals-treated mice for a relatively long term of over one month. We found no toxicity expressed in the weight loss and/or abnormal behavior of the treated animals (Figure 4G). These results reveal that quadrup-deals and its components under the doses employed were safe in vivo and their optimized administration protocol provided a relatively high accumulation of both the gold and drug in the tumor. This systemic pre-treatment was designed to support the next stage of local treatment with a laser and radiation.

Quadrup-deals versus standard therapy efficacy in vitro in various carcinomas

We first compared standard chemoradiation, chemoradiation with targeted liposomal nano-
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therapeutics and quadrapeutics in cultures of resistant and aggressive head and neck squamous cell carcinoma (HNSCC, HN31) and normal epithelial (NOM9) cells by using free and liposomal forms of cisplatin (Lipoplatin), the main clinical choice in HNSCC chemo- and chemoradiation therapies. After optimizing the drug dose to the safest level for normal cells by using the clonogenic test (Figure 5A), both standard and targeted chemoradiation therapies revealed the high therapeutic resistance of HNSCC (Figure 6A), which is in line with clinical experience [1, 3, 15]. In contrast, quadrapeutics showed a 1000-fold therapeutic gain compared with standard chemoradiation (Figure 6A). At the same time, identically treated normal cells were not damaged (Figure 6A) because they were unable to generate PNBs unlike cancer cells (Figure 6B).

This 1000-fold therapeutic gain (which exceeded a similar previous result by almost ten-fold [4]) was achieved by: -optimizing the timing of the radiation administration after the laser treatment (Figure 5B)-24-hour delay was found optimal to radiosensitize the cell and to apply a single fraction of the radiation of 4 Gy; -optimizing the antibody conjugation of drug and gold (Figure 5C)-the combination of Lipoplatin-Panitumumab and gold-C225 provided both high efficacy and selectivity of quadrapeutics; -optimizing the laser pulse administration (Figure 5D). Firstly, a single laser pulse (782 nm, 30 ps, 30 mJ/cm²) was applied 24 hours after administering the drug and gold to ensure their intracellular clustering and co-localization. This pulse induced PNBs only in cancer cells (Figure 6B) and therefore all quadrapeutics mechanisms were triggered only in cancer cells, while the identical treatment of normal cells did not trigger the quadrapeutics mechanisms.

After optimizing the quadrapeutics sequence timing, targeting antibodies and the number of laser pulses, we studied the influence of the drug type on the therapeutic efficacy in quadrapeutics and chemoradiation modes. Cisplatin (the first drug of choice in clinic for the cancer studied) was replaced with a similar clinically equivalent dose of liposomal doxorubicin (Doxil) (whose clinical efficacy against HNSCC is considered to be low). The chemoradiation mode revealed the predictably better efficacy of cisplatin (Figure 6C). In contrast, in the quad-
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In vitro response of HNSCC HN31 (red dots and bars) and normal epithelial NOM9 (grey dots and bars) cells to the treatments measured as the surviving fraction with a clonogenic test: A. Chemotherapy (24 hour incubation) with liposomal cisplatin (Lipoplatin) in plain (solid circle) and targeted forms (hollow circle-with C225 antibody, hollow triangle-with Panitumumab antibody); B. PNB-enhanced chemoradiation with a variable time-delay between the laser and radiation treatments (Lipoplatin-Panitumumab: 200 ng/ml, gold-C225: $2.4 \times 10^{10}$ NPs per ml, laser 782 nm, 30 ps, 45 mJ/cm$^2$, single pulse, radiation: 4 Gy, single fraction), red arrow indicates zero colonies; C. Influence of the intracellular co-localization of gold and drug on the efficacy and selectivity of PNB-enhanced chemoradiation (NSP-plain gold colloids, L-plain Lipoplatin, NSP-C225-conjugate of gold colloids with C225, L-P-Lipoplatin conjugated with Panitumumab); D. Influence of the number of laser pulses applied on the efficacy and selectivity of PNB-enhanced chemoradiation.

Quadrapeutics mode, doxorubicin, a “poor” drug against HNSCC, improved the therapeutic efficacy by four-fold (Figure 6C) and, most unexpectedly, outperformed cisplatin by two-fold (Figure 6C). This inversion of drug efficacy (never observed previously) is associated with the PNB-induced mechanical impact and the intracellular drug ejection and radiosensitization it provided.

Such an inversion may significantly improve current chemoradiation and therefore it was further studied in three additional drug-resistant and aggressive cancers which are currently also treated preferably with cisplatin (triple-negative breast adenocarcinoma, ovarian adenocarcinoma and colon adenocarcinoma). To generate similar PNBs in these different cancer cells, we optimized the level of laser pulse fluence for each cell line (Table 2). After identical targeting with gold conjugates, the laser pulse fluence was adjusted to provide the generation of sub-lethal PNBs of a 60-70 ns lifetime as was previously done for HNSCC cells (Table 2). The difference found in the laser fluences is associated with the different efficacy of gold clustering in these cells which was apparently the highest (lowest fluence required to generate similar PNBs) in SW48 cells and the lowest in SKOV3 cells (the highest fluence required to generate similar PNBs). In this experiment, we used the same drug and radiation doses as in
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In addition to the cell viability measurements, the clonogenic test resulted in zero colonies in the samples of all the additional cancer cell lines treated with quadrapeutics (not shown). We therefore used an additional metric-cell viability 24-48 hours after the treatment. The treatment of all three additional cancer lines with standard chemoradiation and quadrapeutics revealed a similar inversion effect for doxorubicin vs cisplatin (Figure 6C): doxorubicin was more efficient than cisplatin.

Interestingly, in all four carcinoma cell lines, the therapeutic gain for both drugs (quantified through the ratio of cell survival for standard chemoradiation/quadrapeutics) correlated with the PNB generation efficacy of these cell lines (quantified through the PNB lifetime in individual cells under identical exposure to gold conjugates and laser pulses) (Figure 6D). Therefore, the intracellular mechanical impact of PNBs plays a key role in enhancing chemoradiation in cancer cells in the quadrapeutics.

Figure 6. In vitro study of quadrapeutics in cancer and normal cells. A. Surviving fractions of HN31 cancer (red) and NOM9 normal (grey) cells in a clonogenic test after identical treatment with: Chemotherapy (Chem) with liposomal cisplatin (Lipoplatin)-Panitumumab conjugated (200 ng/m), chemoradiation therapy (ChemoRad) with Lipoplatin-Panitumumab and a single radiation dose (4 Gy), quadrapeutics (ChemoRad+PNB): NSP-C225 conjugates (2.4 × 10^10 NPs per ml) + Lipoplatin-Panitumumab (200 ng/ml), single laser pulse (782 nm, 30 ps, 30 mJ/cm^2) and a single dose of the radiation (4 Gy) 6 hours after the laser treatment (data: mean ± SE, n = 3); B. Optically-detected typical time-responses of individual cells to a single laser pulse (782 nm, 30 ps, 30 mJ/cm^2): cancer HN31 (red) and normal NOM9 (black), only cancer cells show the PNB-specific optical time-response; C. The ratio of the therapeutic efficacy of liposomal cisplatin (Lipoplatin), 200 ng/ml versus liposomal doxorubicin (Doxil) in standard chemoradiation (PNB lifetime = 0) and quadrapeutics (PNB lifetime > 0) in four different carcinoma cell lines (delay between laser treatment and X-ray treatment was 6 hours for Doxil and 24 hours for Lipoplatin): head and neck-black, breast-white, colon-grey and ovarian (shaded) cancer. The ratio < 1 means the higher efficacy of cisplatin, the ratio > 1 means the higher efficacy of doxorubicin; D. Therapeutic gain delivered by the quadrapeutics (as the ratio of the cell survival for chemoradiation divided by that for quadrapeutics) as the function of the PNB generation efficacy in cancer cells (measured for individual cells as PNB lifetime under identical optical excitation and gold pre-treatment) for Lipoplatin (black) and Doxil (red) for carcinoma cells of the following cancers: head and neck (star), breast (hollow circle), ovarian (square), colon (solid circle), solid lines show the best polynomial fit, the correlation (Pearson) coefficient is 0.99 for both drugs (data: mean ± SE).

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Quadrapeutics versus standard chemoradiation in vivo

We further compared standard chemoradiation, targeted chemoradiation and quadrapeutics in vivo in a xenograft mouse model of HNSCC. All three treatments used identical doses of the drug at the level of 18% of its clinical equivalent: free cisplatin in the standard mode (12 mg/kg) and Lipoplatin conjugate in targeted chemoradiation and in PNB-enhanced chemoradiation (12 mg/kg). After optimizing targeting antibodies for the drug (C225, Figure 3B), gold (Panitumumab) and their administration timing (Figure 3A), we achieved the tumor-specific bio-distribution of the gold and drug (Figure 3C). A single NIR laser pulse (782 nm, 30 ps, 65 ml/cm²) was applied locally via an endoscope (Figure 2A) 24 hours after systemically injecting the drug and gold. The PNB generation in the tumor was confirmed in real time by detecting PNB-specific acoustic signals (Figure 2B). The radiation (two fractions, 6 Gy (day 3) and 3 Gy (day 4)) was administered locally. Each treatment was administered twice with a one-week interval. Compared to the standard mode, chemoradiation with targeted Lipoplatin did not significantly inhibit tumor growth (Figure 7A), although the improvement in the animal survival time was 1.9-fold (Figure 7B). In contrast, the Lipoplatin-based quadrapeutics provided a 5-fold inhibition of the tumor growth and 3.1-fold improvement in the animal survival time (Figure 6A, 6B) compared to standard chemoradiation.

Next, after replacing liposomal cisplatin with a similar clinically equivalent dose (18%) of liposomal doxorubicin (Doxil), we observed a further increase in the therapeutic gain in the quadrapeutics mode versus standard chemo-

radiation: 34-fold for tumor growth inhibition (Figure 7C, the histological data are shown in Figure 8) and 5.4-fold for the animal survival time (Figure 7D). No short-term (Figure 4A-F; Table 1) or long-term (Figure 4G) toxicities were observed after the treatment with quadrapeutics, a problem associated with full clinical doses of standard chemoradiation.

Thus, our in vitro and in vivo results showed that a drug with a relatively poor clinical efficacy in HNSCC, doxorubicin, outperformed the first choice drug, cisplatin, in the quadrapeutics mode and provided a more than 5-fold improvement in the overall animal survival rate, compared to that for standard chemoradiation. Such a radical therapeutic improvement was achieved with a combination of low, safe doses of liposomal doxorubicin and radiation with the cell-level mechanical impact of PNBs (applied locally).

Quadrapeutics mechanisms versus other investigational therapies

Such a radical improvement both in the safety and efficacy of standard chemoradiation was achieved through the intracellular synergy of several PNB-based mechanisms which are specific to quadrapeutics:

The high intracellular concentration of the drug due to its instantaneous ejection by a PNB [4, 6, 7, 13] efficiently sensitizes the target cancer cell. While this mechanism is well-known, PNBs further enhance it by enabling better radiosensitization with doxorubicin than with cisplatin (Figure 6C, 6D). This “drug efficacy inversion” is probably the most promising part of radio-sensitization because it broadens the drug choice for chemoradiation, while at the same time reducing the efficient therapeutic dose of the drug to a safe level. The intracellular mechanical impact of PNBs [4, 5, 10-12] (similar to the mechanical macro-sensitization demonstrated earlier [30-35]. The re-emission of secondary electrons by gold clusters [36-38] both achieve maximal strength in cancer cells with the largest gold clusters and PNBs, while sparing adjacent normal cells (which do not generate PNBs and have much lower levels of non-specifically accumulated gold).

All these mechanisms were not triggered in normal tissue because no PNBs were generated

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<th>Cell line</th>
<th>Laser pulse fluence, mJ/cm²</th>
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<tr>
<td>MDA-MB-468</td>
<td>30</td>
<td>70±6</td>
</tr>
<tr>
<td>SW48</td>
<td>22</td>
<td>63±4</td>
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<td>SKOV3 IP1</td>
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<tr>
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<td>NOM9</td>
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there under identical laser exposure due to the threshold mechanism of PNB generation. The high cancer-cell specificity of PNB-enhanced chemoradiation stems from the localized mechanical impact of a PNB and its threshold nature, not only from using antibodies to target cancer cells. The PNB generation threshold fluence decreases with the gold cluster size, with the lowest PNB threshold in large clusters in cancer cells and the highest PNB threshold in single gold nanoparticles or their small clusters in normal cells [4, 12, 13].

The mechanical non-stationary mechanism employed in quadrapeutics principally differs from gold nanoparticle-based drug delivery, hyperthermia and radiation therapies which all employ the stationary effects of drugs, heat or secondary radiation (Table 3) [21-29, 36-38]. Such “stationary” nano-therapies suffer from poor cancer cell specificity due to the unavoidable uptake of nanoparticles by normal cells and their gain is relatively incremental compared to that of the quadrapeutics.

Conclusions

Our study investigated and optimized the quadrapeutics protocol for the delivery, safety and efficacy:

1. The delivery of the gold and drugs was verified via a systemic route: no non-specific toxicity was observed due to the reduction of the drug dose to 25% of the clinical one and because of using relatively low doses of clinically-safe colloidal gold and clinically-approved antibodies (also at low, sub-clinical doses). A
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Liver “sink effect” did not prevent the efficient targeting of the tumor via EGFR antibodies and the levels of non-specific uptake of drugs and gold were relatively low.

2. After optimizing the quadrupleutics protocol, we achieved a more than 5-fold increase in overall survival (compared to that after chemoradiation therapy), without increasing either the drug or radiation doses. This gain was achieved mainly due to the conversion of the standard macro-treatment into an intracellular and cancer cell-specific quadrupleutics micro-treatment which activates the therapeutics mechanisms only in cancer cells.

3. Unlike standard cisplatin-based chemoradiation therapy the quadrupleutics mechanisms revealed the higher efficacy of another drug, doxorubicin, which does not provide high efficacy under the standard treatment.

The reported results establish a foundation for translating quadrupleutics in HNSCC to clinical trials with an unprecedented combination of efficacy and safety compared to standard chemoradiation: a more than five-fold improvement in overall animal survival and a significant reduction in side-effects. They also establish the potential of quadrupleutics in other aggressive and resistant carcinomas, including triple-negative breast cancer. The high translation potential of quadrupleutics relies upon using only clinically validated components in low, safe doses: 25-30% for the drugs and 15% for radiation (of clinical doses). This broadens patient eligibility, especially among those who already failed standard therapies, ensures easy integration with standard protocols and flexibility in choosing the drug type. Quadrupleutics is a non-stationary nanomedicine which can be administered as a stand-alone or adjuvant treatment after standard practices fail. While quadrupleutics is a local treatment by definition, by efficiently destroying a primary or residual epithelial tumor it effectively reduces the probability of both local recurrence and metastases.

Table 3. Therapeutic mechanisms and performance of PNB-enhanced chemoradiation vs current practices

<table>
<thead>
<tr>
<th>Therapeutic Feature</th>
<th>Current Practices (Drugs, NPs, radiation)</th>
<th>PNB-enhanced chemoradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanisms</td>
<td>Biological and chemical</td>
<td>Mechanical and physical</td>
</tr>
<tr>
<td>Agent</td>
<td>Materials with permanent properties</td>
<td>Non-stationary events with transient, on-demand properties</td>
</tr>
<tr>
<td>Efficacy</td>
<td>Low for resistant cancers and decreases with cancer’s aggressiveness</td>
<td>High for resistant cancers and increases with cancer’s aggressiveness</td>
</tr>
<tr>
<td>Specificity and safety</td>
<td>Low due to macro-nature and non-specific uptake of therapeutic agents by normal tissue</td>
<td>High due to cluster-threshold intracellular therapeutic mechanisms</td>
</tr>
<tr>
<td>Treatment time</td>
<td>Long</td>
<td>Short</td>
</tr>
<tr>
<td>Therapeutic doses</td>
<td>High and unsafe</td>
<td>Low and safe</td>
</tr>
</tbody>
</table>

Figure 8. Histological images of the primary HNSCC tumor samples harvested from (A) untreated, (B) chemoradiation treated, and (C) quadrupleutics-treated mice (drug: liposomal doxorubicin (Doxil)-Panitumumab, 3 mg/kg, gold colloid-C225: 4 mg/kg, laser: 782 nm, 30 ps, 65 mJ/cm², radiation: 9 Gy total). All tumors were harvested 12 days following start of the treatment. Scale bar is 2 mm; inset is 10 × magnification of the specified location. Green-border of viable tumor, yellow-border of necrotic tumor or edematous tissue associated with the tumor necrosis). All tumors had similar size in the beginning of the treatments.
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Disclosure of conflict of interest

None.

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References

Comparison of quadrapeutics versus chemoradiation


