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Biological Pathways as Substantiation of the Use of Copper Radioisotopes in Cancer Theranostics

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Copper, a cofactor for many enzymes, is a bioelement that is involved in many main biochemical processes; although high levels of copper promote the proliferation of cancer cells. Further development of radiopharmaceuticals based on copper radioisotopes depend on understanding and taking advantage of its biochemical pathways in oncogenesis. As with other radiometals used in molecular imaging and/or targeted therapy, biological vectors are employed to transport copper radioisotopes to a target, aiming for high specific uptake at tumor sites and precise delivery of ionizing radiation. Evidence of the clinical utility of copper radioisotopes in the ionic form $CuCl_2$ were also proven in an *in vivo* study of the copper metabolism, guiding personalized copper-chelating treatment in cancer patients and in imaging pathological sites associated with copper imbalance. Five of the copper radioisotopes have gained interest for nuclear medicine applications, based on their emissions, energies, and half-lives, as they can be produced with pharmaceutical-grade quality. The uptake mechanism, kinetics, and metabolic parameters are important findings in molecular imaging, which are decisive when designing individualized targeted radiotherapy for dose calculations of high linear energy transfer Auger electrons and β^- emissions of ^{64}Cu and ^{67}Cu. As radiation deposits a high amount of energy within the intra-cellular space, the biochemical involvement of copper determines targets in drug design and validation. The biochemical pathways depict copper metabolism in normal cells and highlight its increased activity in tumor progression and angiogenesis. The avid uptake of copper into inter- and intra-mitochondrial spaces, as constituents of cytochrome C oxidase, substantiate the selection of $^{64/67}CuCl_2$ as theranostic agents.

Keywords: molecular imaging, theranostics (combined therapeutic and diagnostic technology), copper, emergent radioisotopes, cancer, Cu-64/67

INTRODUCTION

The natural occurrence of copper (69.17% ^{63}Cu, 30.83% ^{65}Cu [1]), either in metallic form or as a mineral, confers a wide exposure to humans. Its inorganic salts are highly toxic but its varied coordination complexes have gained a lot of interest in drug design, as they are selective and also exhibit convenient pharmacokinetics and pharmacodynamics.

Copper is an essential microelement involved in important biochemical processes, such as: homeostasis, iron transport, respiration, and metabolism, as a result of its redox abilities in the biological environment: reversible translation between oxidized form (cupric ion, Cu^{2+}) and the reduced form (cuprous ion, Cu^{+}). It is a transition metal with 29 isotopes, out of which 27 are radioactive [2].

Along with the progress of nuclear medicine practices and technology, approaching molecular imaging and personalized treatment, five of the copper radioisotopes have gained interest for medical applications, considering their emissions, energies, production route, and availability, with half-lives ranging from 9.7 min (^{62}Cu) to 2.6 days (^{67}Cu) [2, 3]. Especially, ^{64}Cu and ^{67}Cu were intensively investigated as medically emergent radioisotopes for theranostic applications and therapy, respectively. Still, there is a need for more data regarding the production of ^{60}Cu, ^{62}Cu, and particularly ^{67}Cu in medical small cyclotrons.

Recent studies demonstrated the usefulness of $^{64/67}$Cu agents, containing biological vectors to carry radioisotopes to target, aiming for high specific uptake at tumor sites, and precise delivery of ionizing radiation, such as peptides, antibodies, or other biologically active small molecules [2, 3]. Besides using such carriers, the clinical utility of copper radioisotopes in their most simple chemical form, copper chloride, was also proven, either for an *in vivo* study into the copper metabolism, guiding personalized copper-chelating treatment in cancer patients, or to image pathological sites associated with copper imbalance in inflammation, tumor angiogenesis, and metastasis.

As many of these findings are evidence-based and sourced directly from clinical practice (e.g., the significantly higher copper levels measured in serum and tumor cells of patients with cancer compared to normal subjects [4]), there is a need for an in-depth biological evaluation of the involved mechanisms and quantification. Therefore, we reviewed the relevant literature regarding the biological and biochemical pathways of copper, to substantiate the use of copper radioisotopes in oncology and promoting its further development.

BIOLOGICAL PATHWAYS OF COPPER IN HUMANS

Copper Bioavailability and Dietary Interactions

Humans are exposed to environmental copper from water, food, and tools or household goods, therefore the World Health Organization (WHO) defined a safe range for copper intake and acknowledged its effects, either positive or negative, on human health [1]. In an adult organism there is approximately 1.5 copper mg/kg bw, still up to 2.2 mg/kg bw is considered acceptable in the physiological range. Foods most abundant in copper are seafood, dry nuts and seeds, dark chocolate, and mushrooms [5]. A high nutritional intake does not represent any risk considering copper toxicity, as the human organism has a dynamic mechanism of homeostasis.

Copper bioavailability is fairly affected by dietary factors, such as carbohydrate, iron, zinc, molybdenum, and ascorbic acid co-ingestion. Large quantities of dietary zinc can decrease copper absorption and induce the symptoms of systemic copper deficiency. Also, an increased molybdenum intake drives the organism toward secondary copper deficiency, which can be rapidly corrected by copper supplementation. On the other hand, iron-copper interactions in the intestines conduct the regulation of copper transport modulation by the iron levels. Reduced levels of copper lead to a series of physiological changes, inducing pathological conditions, while high intake of copper, found as chronic or acute exposure, can result in liver damage [1].

Copper Metabolism and Physiological Role

The intestines are the main absorption site, the process being conducted by the enterocytes, with the participation of copper permease and human copper transporter-1 (hCTR1) [1–7]. Dietary Cu^{2+} is reduced to Cu^{+} by reductases, prior to being transported through the brush border membrane of the enterocytes by hCTR1 [1, 5, 8], yet the mechanisms for selective permeation of Cu^{+} ions across cell membranes are unknown [9]. After absorption, copper, bound to metallochaperone proteins, is delivered to the mitochondrion [10, 11] by the SLC25A3 inner membrane transporter [11, 12], which is required for the metalation of enzymes within the mitochondrial inter-membrane space [13]. The exceeding amount of copper can be deposited in an inert form in metallothionein, the main intracellular copper storage protein. Subsequently, it is released under the influence of ATP7A. At the end of the process, Cu^{+} is effluxed from enterocytes, chemically reconverted to Cu^{2+}, and is thus able to bind to the transport proteins, albumin and alpha-2-macroglobulin. The carrier proteins deliver copper to the hepatic tissue, from where it is subsequently redirected to the target sites; therefore liver is the main organ that controls copper homeostasis mechanisms [1, 5]. Copper is distributed mostly in the bone and muscle tissues (up to 67%), but also in the liver, brain, and heart [1, 8].

Copper is further transferred to the cytoplasm, in inter-mitochondrial and intra-mitochondrial spaces, where it becomes a constituent of cytochrome c oxidase (CcO) and superoxide dismutase-1 (SOD1) [10–12]. Under normal circumstances, copper is transferred into the trans-Golgi network, where it is used for the synthesis of other cuproenzymes (ceruloplasmin, lysis oxidase, peptidylglycine alpha-amidating monooxygenase, and dopamine beta-hydroxylase) [1]. In the case of high intracellular copper influx, the same transporters will move to the cell surface, where they will mediate the efflux of excessed copper to the plasma (ATP7A) or bile (ATP7B) [1, 8]. Copper excretion is mainly achieved through bile, in the form of bile salts; the urinary excretion is rather insignificant [1, 8]. The ubiquitarian role of copper derives from its structural importance in a wide array of

functional and modulatory proteins that are deeply involved in physiological and pathological mechanisms [13–15] (**Supplementary Table S1**). Copper is an enzymatic cofactor, an essential component of Cu-dependent enzymes: ceruloplasmin, cytochrome C oxidase, metallothionein, Cu/Zn superoxide dismutase-1, amine oxidases, lysyl oxidase, tyrosinase, zyklopten, and mono-oxygenases, and also represents an up-regulating trigger for a series of redox status modulatory enzymes: catalase, glutathione peroxidase, hepaestin, cartilage matrix glycoprotein, and Protein-6-lysine oxidase [1, 13]. Reduced activity of these enzymatic proteins is found in copper deficiency states [13, 16].

Copper Deficiency and Pathological Implications

Reduced or minimal activity of copper-dependent enzymes results in symptoms that may include hypochromic anemia, neutropenia, thrombocytopenia, and hypopigmentation, bone, cardiovascular, and neurological abnormalities, as well as immune system depression [1, 8]. Copper-related genetic diseases include Menkes syndrome (a mutation of ATP7A gene) which is expressed by reduced intestinal copper absorption and Wilson's disease (a mutation of the ATP7B gene), when copper accumulates in excess in different organs (liver, brain, cornea) [1, 5, 8]. Wilson's disease is caused by the cerebral and hepatic tissue accumulation of copper, leading to neurologic and psychiatric symptoms, and liver impairment [16, 17].

Children can develop potentially fatal idiopathic copper toxicosis when drinking contaminated water or food [8, 18, 19]. Correlations with Alzheimer's disease have also been observed; elevated levels of free (unbound) copper in the blood were present, as well as high copper levels in amyloid senile plaque deposits [18, 20]. Diabetic patients exhibit elevated plasma copper levels [19, 21]. Copper deficiency is also associated with cardiovascular diseases [22].

The proliferation of cancer cells is promoted by high levels of copper [8, 23]. Elevated copper levels were found in different types of tumors while cancer growth was minimized when copper was chelated [5, 24]. Considering its redox properties, copper is a source for reactive oxygen species [1].

MEDICAL RADIOISOTOPES OF COPPER

Molecular Imaging, Targeted Therapy, and Theranostic Role of Radio-Copper

Molecular imaging allows for the quantification of functional parameters of an organ or process; moreover the interactions of a drug with its desired target can be analyzed, side effects can be determined, and the delivery, absorption, distribution, metabolism, and elimination in a living system can be precisely evaluated [25–27]. Among the molecular imaging techniques, positron emission tomography (PET) is most often used to tailor and deliver personalized treatment, as a result of receptor identification and mapping their density to a tissue or organ of interest, or by exploiting the imbalanced metabolism in different stages of pathological processes.

The positron-emitting radionuclide is customarily selected taking into account several factors, such as: the half-life of the radionuclide (this should match with the vector pharmacokinetics to allow optimal uptake), the energy of the positron emission (which determines the precision and image resolution), and the availability and cost of the production. Moreover, the specific/molar activity and carrier-free specifications, as quality parameters, become tremendously important when associated with molecular term (either imaging or therapy), together with radiobiological parameters, mainly the affinity, uptake, and retention profiles (radio)toxicity, blood clearance, and elimination route.

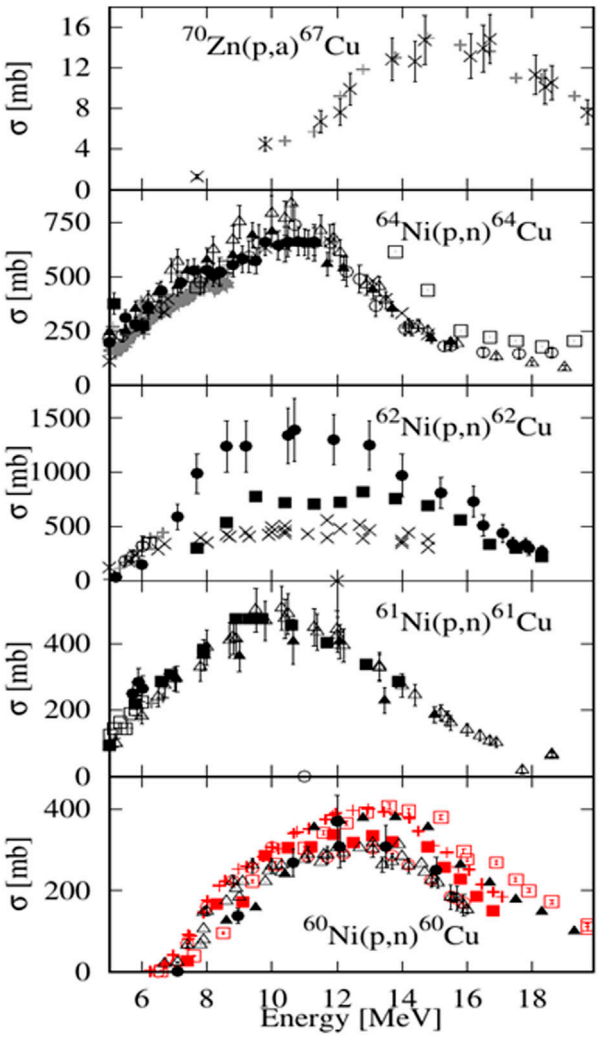
Five radioisotopes of copper (**Table 1**) can be produced at a cyclotron, with characteristics required for clinical use [28–31]. Based on their radioisotope emissions, ^{60}Cu, ^{61}Cu, ^{62}Cu, and ^{64}Cu are suitable for molecular imaging applications, while ^{64}Cu and ^{67}Cu are selected for targeted radionuclide therapy [30–32]. Due to their short half-lives, they are used in ionic form (as chlorides) or in combination with fast kinetic peptides. While the radiopharmaceuticals based on longer-lived radionuclides, such as ^{177}Lu, ^{89}Zr, or ^{90}Y enable the investigation of the biological processes over a number of hours, which is often demanded by the study or imposed by slow kinetics of the vector [33–35], copper-64 is a theranostic radionuclide of particular interest due to its simultaneous emission of both β^+ (17.52%) and β^- (38.48%) particles [36–44]. The positron emission allows for high resolution PET imaging, while low abundance gamma emissions do not affect the imaging process compared to other positron emitters [30, 45]. It decays also through electron capture (EC 43.53%), when high linear energy transfer Auger electrons are emitted. When this happens in the close vicinity of a cancerous cell nucleus, it may cause DNA damage, eventually triggering cell death and thus, achieving a therapeutic effect. Taking advantage of the positron emissions, real-time therapy follow-up can be performed by PET imaging, presumably at any time point during therapy.

Production of Medical Radioisotopes of Copper, with Particular Interest on ^{64}Cu

Researchers are investigating different routes to produce carrier-free and high specific activity copper radioisotopes [29]. Copper-64 can be produced in a reactor by (n,γ) and (n,p) reactions, on enriched targets [30], at thermal neutron fluxes ($6\text{-}7\cdot10^{12}$ $n^{\star}cm^{-2\star}s^{-1}$). The average specific activity of ^{64}Cu obtained was 2.4 TBq/g Cu, at the end of irradiation. Using this route, radionuclide impurities ^{65}Zn and ^{60}Co are co-produced and should be eliminated by radiochemical processing, using an anion exchange separator [31]. Higher specific activity can be achieved when fast neutron reactions are employed, but thermal neutron reactions also occur, leading to high amounts of long-lived radionuclide impurities, such as ^{65}Zn ($T_{1/2}$ = 245 days) [29].

The most common way to produce ^{64}Cu is by using a small/medium energy cyclotron [32, 36–49]. Several nuclear reactions can be triggered on nickel or zinc targets by proton beams: ^{64}Ni(p,n)^{64}Cu, ^{nat}Zn(p,xn)^{64}Cu or ^{68}Zn(p,αn) [49–57], but also deuterons induced reactions: ^{64}Ni(d,2n), ^{66}Zn(d,α), ^{64}Zn(d,2p)

TABLE 1 | Radioisotopes of copper produced in medium energy cyclotrons.

Radioisotope and half-life	Decay mode and energy	Most intense γ emissions	Nuclear reaction and cross-section data [74]
^{67}Cu 61.8 h	β$^-$ (100%) 121 keV (57%) 154 keV (22%) 189 keV (20%)	91.2 keV (7%) 93.3 keV (16.1%) 184.6 keV (48.7%)	
^{64}Cu 12.7 h	β$^-$ (38.5%) 191 keV (38.5%)	-	
	EC and β$^+$ (61.5%) 278.2 keV (17.6%)	1,345.77 keV (0.475%)	
^{62}Cu 9.7 min	EC and β$^+$ (100%) 1,321 keV (98)	875.7 keV (0.15%) 1,173 keV (0.342%)	
^{61}Cu 3.32 h	EC and β$^+$ (100%) 524 keV (51%)	282.9 keV (12.2%) 656.0 keV (10.8%) 1,185.2 keV (3.7%)	
^{60}Cu 23.7 min	EC and β$^+$ (100%) 872 keV (49%)	1,332.4 keV (88%) 1791.6 keV (45.4%)	

[52–54, 56]. Good yields of ^{64}Cu production, with low radionuclide impurities, were obtained using enriched ^{64}Ni or $^{64/66/68}$Zn targets [49]. The irradiation of ^{nat}Zn targets is a less expensive method to conveniently obtain lower activities of ^{64}Cu, while the use of deuteron beams on these targets requires energies above 20 MeV to obtain reasonable yields [56].

The ^{64}Ni(p,n)^{64}Cu reaction is used at large scale for the production of ^{64}Cu, although bearing the disadvantage of costly target material, this route is preferred for the high yields that can be achieved, even at small medical cyclotrons [54, 55, 58]. Using a 12 MeV cyclotron, specific activity of >87 × 10^4 GBq/g and an irradiation yield of >111 MBq/μAh were reported [42]. ^{64}Ni (99.5% enrichment) is electrodeposited from the ^{64}Ni(NO$_3$)$_2$ solution, resulting in a^{64}Ni solid target [46]. Alternatively, liquid targets consisting of solutions of ^{64}Ni(NO$_3$)$_2$ are conveniently used, with lower production yield [47]. The irradiation process parameters are tuned for best yields, according to the experimental set-up and needs of a site; optimal parameters: 2–4 h irradiation time, 40–50 mg ^{64}Ni on target, lead to 9.99–18.5 GBq of ^{64}Cu and high specific activity (11.47 × 10^6 GBq/g Cu). The production yield, on 15–55 mg of enriched ^{64}Ni targets, ranges from 82.9 to 185 MBq/μAh [48]. Separation of Cu from the Ni targets employs ion-exchange chromatography, using a cation exchanger column (AG1-X8). Enriched ^{64}Ni can be recovered up to 95% [42]. During proton irradiation of enriched ^{64}Ni, ^{61}Co is produced as a contaminant, which can be separated with 4M HCl as an eluent [49].

$^{64}CuCl_2$ as Radiopharmaceutical and/or Precursor

^{64}CuCl$_2$ is used either as a radiopharmaceutical or as a precursor for radiolabeling specific carriers, such as monoclonal antibodies,

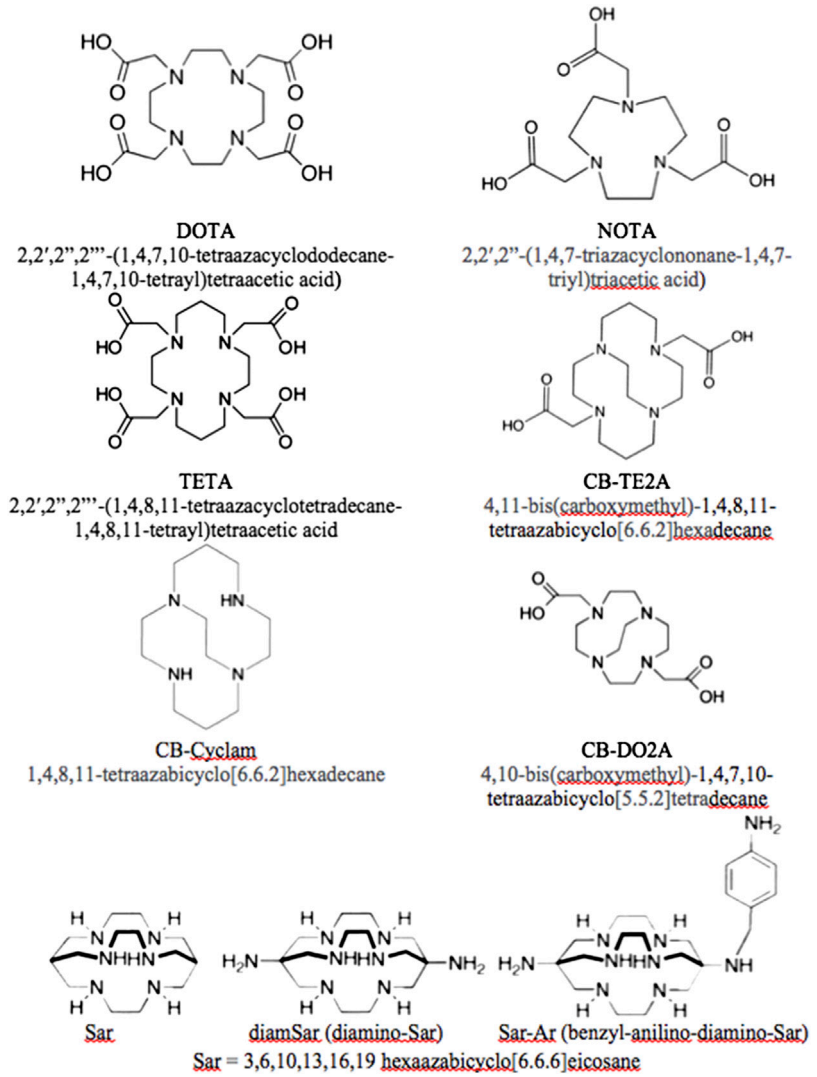


FIGURE 1 | Chelators used for binding radio-copper to biomolecules.

peptides, amino acids, hormones, nanoparticles, or small molecules, using chelating agents [58–60]. This is also the case for all the other copper radionuclides. Various cold copper complexes were studied and also used as anticancer agents [60].

After IV administration, $^{64}CuCl_2$ accumulates in the liver (uptake fraction 0.65), brain (uptake fraction 0.1), kidney (uptake fraction 0.01), and pancreas (uptake fraction 0.0002). Based on preclinical studies, the calculated effective dose (ED) is 70 mSv for the whole body of a 70 kg adult, after the intravenous injection of 925 MBq of $^{64}CuCl_2$ [61].

The chelators used for binding radio-copper to biomolecules (**Figure 1**) should have high thermodynamic stability; compact structures of macrocyclic or macro-bicyclic ligands with increased kinetic stability are preferred [62–71]. When dissociated from the complexes, Cu^{2+} is reduced to Cu^{+} and binds to SOD in high concentrations [63]. DOTA have been used for chelating ^{64}Cu, however, its ability to bind many different metal ions, *trans*-chelation to liver proteins, and its decreased stability compared to TETA/CB-TE2A make it less attractive [64, 65]. By comparison, NOTA and the hexaamino sarcophagine ligands demonstrate ease of conjugation, high radiolabeling yields, and *in vivo* stability [58, 66]. They also achieve better clearance from the blood, liver, and kidneys [65, 66]. The kinetic stability of copper (II) cross-bridged cyclam complexes is superior to those of the TETA and DOTA complexes [63], while ^{64}Cu-CB-TE2A proved to be the most stable, when compared to CB-cyclam, CB-DO2A, DOTA, and TETA, respectively [62].

Comparing the biodistribution, at 24 h p.i., of ^{64}Cu-CB-DO2A, ^{64}Cu-CB-TE2A, ^{64}Cu-DOTA, and ^{64}Cu-TETA, a larger amount of ^{64}Cu-labeled cross-bridged chelates was cleared form the blood, liver, and kidney than the non cross-bridged analogues; moreover, ^{64}Cu-CB-TE2A was the most resistant to *trans*-chelation in rat liver [65]. Hexaaza macrobicyclic

sarcophagines (Sar) are very compact structures, acting like a "cage" around Cu^{2+}, which increases the thermodynamic and kinetic stability, leading to low accumulation at non-targeted tissues. Evaluating the biodistribution data of ^{64}Cu-Sar, ^{64}Cu-diamSar, and ^{64}Cu-SarAr in balb/c mice, it was found that all three complexes had been cleared from the blood rapidly, while the uptake was low in bone, heart, stomach, spleen, muscle, lungs, and the gastrointestinal tract [66].

^{64}Cu as Radioisotope Contained in Theranostic Agents Intended for Different Tumors

$^{64}CuCl_2$ shows an increased and specific uptake in melanoma expressing high hCTR1: 12.7% ± 0.26 in B16F10 cells and 4.6% ± 0.04 in A375M cells, the tumor-to-muscle ratio was 4.11 ± 0.07 for B16F10 and 3.46 ± 1.25 for A375M. During $^{64}CuCl_2$ treatment, tumor growth in both melanoma models was slower than without treatment, suggesting that $^{64}CuCl_2$ radiotherapy is effective for hCTR1 high-expressing tumors [67].

In a xenograft model of glioblastoma multiforme (GBM) U87MG, the biodistribution of $^{64}CuCl_2$ indicated no brain uptake, while PET images showed an uptake in glioma cells; a decrease of the tumor volume with more than 68% was noticed, raising the survival rate of the treated mice [68]. SI113 inhibits SGK1, a protein with increased the expression of glioblastoma. The combination of SI113 and $^{64}CuCl_2$ has a synergistic effect and affects cell viability, triggering apoptosis, and necrosis. The inhibitory dose, tested in three cell lines in glioblastoma ((LI) PARI, ADF, and T98G) with different mutational status for p53, was 40 MBq [68].

In a study using the hypoxia-selective agent ^{64}Cu-ATSM on hamsters implanted with GW39 (human colorectal carcinoma), the inhibition of tumor growth was observed for a 220 MBq injected dose; the animals presented an increased rate of survival with no acute toxicity. After administration, PET scans revealed that ^{64}Cu-ATSM was localized in the GW39 tumor and PET imaging could be performed regularly [69].

Administration of 555 MBq of ^{64}Cu-TETA-Y3-TATE in a single dose to CA20948 rats, a model of somatostatin receptor-positive pancreatic cancer, decreased the tumor volume (29–73%) and inhibited its growth. The multiple dose radiotherapy study (3 × 370 MBq) decreased the tumor volume (36–81%) and provided a tolerable radiation exposure level over an extended period [70].

^{64}Cu-ATSM (^{64}Cu-diacetyl-bis(N-4-methylthiosemicarbazone) showed a high cytotoxic effect, decreasing the clonogenic survival of LL/2 cells (Mouse Lewis Lung carcinoma cells) in a dose dependent manner; the uptake of 1.50 Bq/cell of ^{64}Cu killed 99% of the cells. Under hypoxic conditions, ^{64}Cu was accumulated in the cells and produced DNA damage, detected by comet assay and Annexin V-FITC and propidium iodide staining methods [71].

DU-145 human prostate cancer xenografts were visualized by PET using $^{64}CuCl_2$, the cellular uptake was mediated by hCTR1, demonstrated by negative control PC-3 prostate cancer cells. Knockdown of hCTRl reflected the decreased cellular uptake and inhibition of tumor growth [72]. After $^{64}CuCl_2$ administration, a rapid uptake in the PCa lesions reached the maximum value in 1 h [73].

CONCLUSIONS

The biochemical pathways show copper metabolism in normal cells and highlight its increased activity in human cancer cells, at a higher metabolic rate. Its involvement in tumor progression and angiogenesis and its pivotal role in preserving the intracellular homeostasis are particular indicators used in functional imaging. Thus, specific processes are targeted by radio-copper chloride, but also specific vectors radiolabeled with copper radioisotopes are used. Moreover, the copper presence in intermitochondrial and intramitochondrial spaces, as constituents of cytochrome *c* oxidase, substantiates the selection of ^{64}Cu, a short range high LET emitter (Auger electrons), as a therapeutic agent, in a bioavailable chemical form, $^{64}CuCl_2$.

The uptake mechanism, kinetics, and metabolic parameters are very important findings for PET imaging using ^{60}Cu, ^{61}Cu, ^{62}Cu, or ^{64}Cu which are decisive when designing an individualized targeted therapy and, also, for dose calculations of high LET Auger electrons and β^- emissions of ^{64}Cu and ^{67}Cu. The concept of theranostic applications applies perfectly to copper radioisotopes, by matching pairs for diagnostics and therapy (e.g., ^{61}Cu and ^{67}Cu) or by taking advantage of the dual emissions of ^{64}Cu for both purposes. In this latter case, a real-time therapy follow-up brings important benefits for patients.

AUTHOR CONTRIBUTIONS

DN, RD, and RL reviewed the data regarding copper radioisotopes production and radiochemistry and edited the article. LC, RS, and DAN reviewed the data regarding biological assessment of ^{64}Cu-labelled biomolecules, CD, AN, ID, and DD reviewed pharmaceutical and pharmacological data.

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SUPPLEMENTARY MATERIAL

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