New Perspectives in Cancer Therapy: The Biotin-Antitumor Molecule Conjugates

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Abstract
Chemotherapy is still the first-line treatment of cancer, even if the drugs currently used in therapy generally possess high toxicity and poor selectivity. In the last two decades several efforts have been made to overcome these drawbacks by specifically carrying anticancer drugs to the tumors. Among the different approaches, the so called vitamin-mediated drug targeting has recently emerged as a novel and valuable strategy. Indeed, the linkage of cytotoxic drugs to selected vitamins, leading to vitamin-drug conjugates, would result in specifically delivering great amounts of the targeted drug at high doses to cancer cells. Among vitamins, biotin seems to be the most promising targeting agent. The aim of this review is to get an overview on recent success in the conjugation of biotin with molecules endowed with anticancer properties.

Keywords: Anticancer drugs; Biotin; Doxorubicin; Drug targeting; Gemcitabine; Taxol

Introduction
Chemotherapy is still the first-line treatment of cancer, even if the drugs currently used in therapy generally possess high toxicity and poor selectivity. Drugs commonly used in cancer therapy are characterized by different mechanisms of action i.e. i) toxicity to specific cancer cells, ii) anti-proliferative activity or iii) capability of modifying the cell cycle at specific phases [1-5]. As a direct consequence of the cellular activity of anti-cancer drugs, in particular with respect to rapidly proliferating and dividing cells, they show low selectivity towards not-cancerous cells like red blood cells, gut epithelia, bone marrow or hair follicles [6]. On the other side, due to the slow proliferation typical of certain tumors, several anticancer drugs used so far result ineffective against such tumors, thus leading to the need of high dose therapies to limit the cancer growing. These high doses could destroy part of the cancer cells but also result in a hard damage to the adjacent normal-proliferating cells. Due to these drawbacks, the cancer therapy has to be discontinued and sometimes stopped before the tumor mass has been reduced or eliminated [7,8]. Thus, reaching a real selectivity of anti-cancer drugs is a milestone and still a challenge in cancer research. To this aim, different approaches could be applied. One of the most common consists in providing the drug with a suitable carrier. The polymeric drug carriers emerged as the most effective ones because of their versatility of use as well as their straightforward chemical modification [9-17]. These systems usually bring to a passive drug targeting due to their ability to accumulate in a specific organ as a result of their route of elimination or for particular organotropism. A more specific drug targeting could be attained by providing the carrier with specific targeting agent, such as antibodies, vitamins, magnetic particles, hormones or peptides [18-24]. Another widely applied approach for drug targeting is the direct linking of the targeting molecule to the drug to form a new chemical entity pharmacologically active per se or a prodrug [7,25-27]. In this context, the so called vitamin-mediated drug targeting has recently emerged as a novel concept in specifically carrying anticancer drugs to the tumors [7,28-31]. As known, for their survival living cells need to consume vitamins during their life cycle. This is particularly true for those cells that rapidly divide, such as cancer cells. Indeed, the intense metabolic activity of cancer cell arises from their fast growth and is accompanied by a strong use of essential vitamins; consequently the receptors involved in vitamin internalization are overexpressed on the cell surface. This concept is essential from a therapeutic point of view. In fact, it has been argued that the linkage of cytotoxic drug to selected vitamins, leading to vitamin-drug conjugates, would result in specifically delivering great amounts of the targeted drug at high doses to cancer cells. Among vitamins, biotin seems to be the most promising targeting agent. In this context, biotin, folic acid, vitamin B12 and riboflavin, that are essential for the division of all cells and in particular for tumor cells, have been recently experimented as targeting agents [20,32-35]. Interestingly, among these vitamins, biotin seems to be the most promising targeting agent. It is well known that the uptake of biotin by mammalian cells is receptor-mediated [36]. Among the first insights on cell surface biotin receptors there are the studies from SM Grassl in 1992 and Janos Zempleni and Donald M. Mock in 1998. Studying the biotin uptake by human peripheral blood mononuclear cells (PBMC) Zempleni and Mock found that “…in the presence of ouabain, biotin uptake into PBMC is dependent on normal function of Na-K-ATPase. It seems likely that biotin is co-transported with sodium, because biotin uptake into PBMC was reduced when extracellular sodium was replaced by choline, lithium, or ammonium. Likewise, as observed by others, the biotin uptake into rat hepatocytes, rat intestinal cells, or Hep G2 cells was reduced by ouabain and by sodium-free media.” [37-39]. In a subsequent study, they also found that PBMC accumulate biotin by a transporter that is specific for biotin. These studies provided evidence that another, less specific transporter in mammalian cells may bind biotin, pantothenic acid, and similar compounds. However, the contribution of this transporter to biotin uptake into PBMC is quantitatively minor [38]. The main transporter for biotin has been found in the sodium-dependent multivitamin transporter (SMVT), which has been found to be overexpressed in several aggressive cancer lines such as leukemia (L1210FR), ovarian...
Among these agents, Paclitaxel is a natural highly oxygenated diterpenoid that was first isolated in 1971 from the stem bark of the western yew, Taxus brevifolia. It is widely used to treat a variety of solid tumors such as breast, ovarian, non-small cell lung, head and neck cancers [47,48]. Since Paclitaxel was discovered, its structure has been extensively studied and modified yielding the so called taxoids, a class of proven anticancer drugs which promote microtubule assembly and suppress microtubule dynamics, thus causing the block of mitotic activity and subsequent cellular apoptosis [3,5].

In 2010, Chen et al., applied what they called “an efficient mechanism-based tumor-targeting drug delivery system, based on tumor-specific vitamin-receptor mediated endocytosis...”. The Authors prepared a biotin conjugates with one of the new-generation taxoids, SBT-1214 [40]. The approach of this work is clear: to use the biotin- SBT-1214 conjugate 1 (Figure 1), which is characterized by an intracellularly labile disulfide linkage, to target the cancer cell by exploiting the overexpression of biotin receptor on the tumor cell surface.

In order to follow the entire process involved in the tumor targeting of the conjugate, the Authors synthesized three fluorescent biotin conjugates (Figure 2): i) the biotin-fluorescein conjugate 2, to observe the receptor-mediated endocytosis by tracking the fluorescent biotin in its route, ii) the biotin-coumarin conjugate 3, as a fluorogenic probe to evaluate the intracellular degradation of the disulfide linkage between the linker and coumarin, which becomes fluorescent only when it is released as a free molecule via disulfide cleavage of the spacer and iii) the biotin- SBT-1214 -fluorescein conjugate 4, to validate the whole internalization by receptor-mediated endocytosis and drug release processes, wherein the released fluorescent taxoid should bind to microtubules in the cancer cells.

In details, conjugates 2, 3 and 4 have been tested on L1210FR murine leukemia cell line, which overexpress receptors for biotin on their cell surface. Firstly, the Authors proved the internalization of the biotin-fluorescein conjugate 2 into the leukemia cells, by evaluating the intracellular fluorescence after incubation with the probe. Furthermore, they confirmed that the internalization was receptor-mediated by pre-incubation of the same cells with an excess of free biotin. They found that in these conditions the fluorescence of the biotin-fluorescein conjugate 2 decreased about 4.5 times, thus confirming that the biotin-fluorescein internalization is receptor-mediated. Secondly, the Authors confirmed the effectiveness of the fluorogenic biotin- coumarin conjugate 3 by incubating the chosen cell line with conjugate 3. Differently from
conjugate 2, the intracellular fluorescence for conjugate 3 could be only evidenced after reductive cleavage of the spacer. Due to the visualized intracellular fluorescence, this study clearly demonstrated that the intracellular release of coumarin via cleavage of the disulfide linkage by GSH followed by thiolactonization took place as designed. Successively, they tested the biotin-SBT-1214-fluorescein conjugate 4. These results confirmed the internalization of the conjugate and also clearly proved that the released fluorescent taxoid binds to the microtubules. A cross-confirmation of the previous data has been performed by testing conjugate 4 using two more cell lines, L1210 murine leukemia cell line and WI38 noncancerous human lung fibroblast cell line, which do not overexpress receptors for biotin on their cell surfaces. This experiment resulted in a much stronger (about 12-13 times) fluorescence in L1210FR cells (with biotin receptors overexpressed) as compared to that in L1210 cells or WI38 cells, so confirming the biotin-mediated drug targeting. Finally, the Authors evaluated the cytotoxicity of the conjugate 1 against L1210FR, L1210 and WI38 cell lines by using the MTT assays. The conjugate 1 resulted high cytotoxic against L1210FR (IC50 8.8 nM), while the cytotoxicity against L1210 (IC50 522 nM) and WI38 (IC50 570 nM) cell lines were 59 times and 65 times lower, respectively, as a further confirmation of the receptor mediated endocytosis. A fundamental consideration arises from the last results: the low toxicity that these conjugates showed against not-cancerous cells demonstrates that these systems could differentiate cancer cells from normal ones so reducing the side effects related to the use of cytotoxic drugs. These results are of paramount importance in the field of biotin targeted drugs because clearly demonstrated that: i) the biotin-drug conjugates are preferentially internalized into cancer cells, ii) the toxicity against normal cell is significantly reduced, and iii) the intracellular cleavage of the employed spacer allows a better systemic stability and a lower interaction with non-cancerous cells.

**Biotin conjugated to doxorubicin**

The antibiotic doxorubicin is extensively used against different human cancers, such as breast cancer, soft tissue sarcomas, and Hodgkin’s and non-Hodgkin’s lymphomas. The anticancer activity of doxorubicin has been addressed to several mechanisms of action: i) inhibition of DNA synthesis in the tumor cell, ii) generation of free radicals, iii) DNA adduct formation and DNA cross-linking, iv) interference with DNA strand separation and DNA helicase, v) interaction with cell membranes, vi) induction of DNA damage through interference with topoisomerase II, vii) induction of apoptosis and viii) growth arrest of tumor cells [49]. Nevertheless, the clinical use of doxorubicin is affected by several harmful side effects, among them, the cardiotoxicity is the most important one, leading to cardiomyopathy and congestive heart failure. Indeed, doxorubicin is known to cause cardiotoxicity through multiple routes, including the build-up of reactive oxygen species and disruption of the calcium homeostasis in cardiac myocytes. Several approaches have been proposed to limit its cardiotoxic effects, i.e. incorporation of the drug inside liposomes, formation of prodrugs proteolytically activated in the tumor cells, or the formation of polymeric prodrugs bearing specific drug-targeting moieties. Although these approaches are valuable, they are still not resolving the drawbacks connected to doxorubicin side effects [50]. Aiming at limiting the adverse side effects of doxorubicin, in 2010 Ibsen S. et al. designed and synthesized the biotin-doxorubicin conjugate 5 (Figure 3), in which the amine group of doxorubicin was derivatized with a photocleavable biotinylated spacer [51]. They demonstrated that the active drug is released only after the exposure to UV at 350 nm, thus minimizing the cytotoxic effects on not-cancerous cells.
Biotin conjugated to gemcitabine

Gemcitabine is an anti-metabolite used to control non-small cell lung, pancreatic, metastatic breast and current ovarian cancers [54,55]. After cellular uptake, gemcitabine is phosphorylated to gemcitabine monophosphate (dFdCMP) which, in turn, is converted to gemcitabine di- and triphosphate (dFdCDP and dFdCTP, respectively) that are the active drug metabolites. The triphosphate analogue of gemcitabine acts i) replacing one cytidine during DNA replication and ii) inhibiting the DNA polymerase [56]. Some characteristic of gemcitabine, i.e. its short plasma half-life (9-13 min in human) due to its rapid renal clearance, and its myelosuppression side effect, contribute to decrease the gemcitabine chemotherapeutic index [57].

In their efforts to overcome the side effects of gemcitabine, in two recent papers Maiti et al. and Bhuniya et al. synthesized two new theranostic anticancer targeted prodrugs of gemcitabine (conjugates 6 and 7, Figure 4), which are characterized by a disulfide cleavable linker containing a fluorescent probe (coumarin for 6 and IR fluorescent BODIPY fluorophore for 7) [55,58]. A theranostic is a special type of Drug Delivery System (DDS) useful for therapeutic and diagnostic applications, which is able to provide not only a specific cellular drug release, but also a real time monitoring of the drug released in tissue [59].

The Authors studied the behavior of 6 and 7 in the presence of glutathione (GSH), which is the most abundant thiol in cells, or dithiothreitol (DTT) and demonstrated that gemcitabine release is due to cleavage of the sulfide bond upon GSH or DTT treatment,
followed by an intramolecular nucleophilic substitution of the thiol at the carbamate moiety. This mechanism of gemcitabine release is supported by further studies carried out in the presence of different biologically relevant analyte containing or not thiol groups, such as cysteine, homocysteine, non-thiol amino acids or metal ions. Results clearly evidenced that conjugates 6 and 7 undergo only a thiol-mediate cleavage, with no significant interference from other molecules present in biological environment. To investigate whether the biotin moiety can guide 6 and 7 to biotin receptor-positive or biotin receptor-negative tumor cells, Authors evaluated the fluorescence by confocal microscopy in A549 (biotin receptor-positive) and WT38 (biotin receptor-negative) cells pre-incubated with 6 and 7. A strong fluorescence intensity was only observed in the A549 cells, thus confirming that biotin acts as targeting agent to cancer cells. Additionally, to investigate the intracellular location of gemcitabine release, Authors performed co-localization experiments using fluorescent endoplasmic reticulum (ER) and lysosome-selective markers. They found that biotin-gemcitabine conjugate 7, containing the near IR BODIPY fluorophore, localized to the ER, while biotin-gemcitabine conjugate 6, containing coumarin, localized to the lysosome, probably by receptor mediated endocytosis. The subsequent thiol-induced disulfide cleavage released gemcitabine which, in turn, diffused to the cell nucleus. Finally, MTT assay performed on A549 cells clearly evidenced a stronger anticancer effect for the biotin conjugates 6 and 7 with respect to their analogues without the biotin moiety, further confirming the biotin-mediated targeting to tumor cells. In summary, also in these studies the conjugation of anticancer drugs with biotin emerged as a successful approach to spare cancer and non-cancer cells, thus increasing the cell specificity of the drug.

**Biotinylaton of Potential Anti-tumor Drug Candidates**

**Biotin conjugated to Annonaceous Acetogenis derivatives**

Squamocin (compound 8, Figure 5) and bullatacin (compound 9, Figure 5) are annonaceous acetogenins (ACGs) belonging to adjacent bis-THF type of acetogenins [41,60,61]. ACGs are secondary metabolites occurring in some plants of Annonaceae family. They are derivatives of long-chain fatty acid (C32 or C34) bearing a terminal γ-lactone ring and have been reported to potentially inhibit the activity of NADH-ubiquinone oxidoreductase (respiratory complex I). These long-chain fatty acid derivatives display impressive cytotoxicity against various tumor cell lines (IC_{50} value ranging from 10^{-6} M to 10^{-14} M) as well as in vivo antitumor effect [41,62-64].

In a just-published research paper, Shi et al. report on the synthesis of nineteen biotinylated derivatives of 8 and 9 as well as on their cytotoxicity against three cancer cell lines: 4T1 (breast cancer), P815 (mastocytoma) and L1210 (leukemia). The first two cell lines overexpress the receptor for biotin, while the third one is not overexpressing the receptor [41]. Generally, they found that the cytotoxic activity of the biotin-ACG conjugates against L1210 cell line is almost the same as that of the parent 8 and 9. This datum is fundamental to assess that biotinylation of 8 and 9 does not adversely affect their cytotoxic potential. On the other side, the biotin-ACG conjugates showed significantly higher cytotoxicity than 8 and 9 against 4T1 and P815 cell lines. In particular, the biotin-squamocin conjugate 10 (Figure 6) resulted ten and twenty six times more active than 8 against 4T1 and P815 cells, respectively. Similarly, the biotin-bullatacin conjugate 11 (Figure 6), resulted three and eight times more potent than 9 against 4T1 and P815 cells, respectively. Taken together, the results clearly demonstrate that biotin targeted derivatives of 8 and 9 are still active against different cancer cell lines and that cytotoxicity is significantly increased by biotin conjugation.

Concerning the influence of the number of biotinyl residues on the cytotoxic activity, the Authors found that the addition of a second biotinyl residue does not generally result in further improvement of the potency of the biotin-ACG conjugate, with the only exception of the already mentioned biotin-squamocin conjugate 10. Indeed, this conjugate, which is characterized by two biotin moieties, resulted up to three times more potent and two times more selective than the corresponding mono-biotinylated derivative 12 (Figure 6). Interestingly, the Authors also noticed that the optimal site of attachment of biotin strongly depend on whether or not the 6-amino-caproic acid residue is present as a linker between the ACG scaffold and the biotinyl residue. Particularly, concerning biotin-squamocin conjugates, the preferred site of attachment of biotin is the C-28 hydroxyl group when biotin is directly attached to the scaffold, and become the C-15 hydroxyl group when the linker between biotin and squamocin is present. In the case of biotin-bullatacin conjugates, the optimal site for biotinyl derivatization is the C-15 hydroxyl group in the absence of the linker, which changes to the C-4 and C-24 hydroxyl groups in the presence of the linker. Moreover, regarding the effect of the linker on the biological activity, Authors showed that biotin-ACG conjugates bearing the linker are generally more potent than the analogues which lack such linker, thus proving that the presence of the linking spacer positively affect the anticancer potential of the conjugates. Indeed, the biotin-squamocin conjugate 12 (Figure 6), biotinylated at C-15 and bearing the linker between squamocin and biotin, is almost four and ten times more potent against 4T1 cells and P815 cells, respectively, than the analogue without the linker. Similarly, conjugates 11 and 13 (Figure 6), biotinylated at C-24 and C-4, respectively, and both bearing the linker between bullatacin and biotin, are twelve to forty nine times more active against 4T1 cells and P815 cells, respectively, than the corresponding analogues without the linker. In summary, in this study a broad investigation of the effect of biotin conjugation on the anticancer activity of two ACGs has been described. The results clearly proved that biotin conjugation significantly increases the anticancer potential ACGs, which, in turn, is significantly affected by i) the number of biotinyl residues included in the conjugates, ii) the point of attachment of the biotinyl residue, as well as iii) the presence or not of a spacer between ACG and biotin.
Biotinylation of Protein with Antitumor Effect

Biotin conjugated to p53 protein

p53 is a transcription factor involved in various cellular functions, such as cell-cycle regulation, initiation of apoptotic cell death and DNA repair. Mutated or otherwise deactivated p53 is observed in the majority of human cancers [65]. Several mechanisms, i.e. mutations in p53 DNA binding domain and enhanced proteosomal degradation upon ubiquitination, are responsible for p53 inactivation. Due to its vital functions, p53 has attracted a great attention, and several approaches have been pursued to deliver p53 into tumor cells [66]. In this context, in 2013 Fahrer et al. demonstrated that biotinylation of p53 represents a successful strategy to target and deliver the protein to cancer cells [67]. In this study the Authors take advantage of the already developed C2-streptavidin transporter [68,69] to introduce the biotin conjugation to p53 could be used for wide number of labile proteins or peptides of pharmaceutical interest.

Conclusions

This review gives a highlight on recent progresses on the so-called biotin-mediated drug targeting of molecules endowed with anticancer properties. Particularly, the review has been organized in three different sections in which we have examined recent success in the conjugation of biotin with i) anticancer drugs commonly used in cancer therapy (toxoids, doxorubicin and gencitabine) or their derivatives, as well as ii) anticancer drug candidates or iii) protein with antitumor effect, furnishing biotin conjugates able to preferentially deliver the anticancer molecules to cancer cells.

In summary, from the analysis of the literature on this topic clearly emerged that the conjugation of biotin with anticancer molecules represents an attractive and highly innovative approach to improve efficiency and efficacy and reduce cytoxicity of anti-cancer therapy.
Although only few targeted drugs have been developed so far, this area is in constant growth and we strongly believe that this approach could give an added value to cancer therapy.

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